Validation of *Fusarium graminearum*-induced wheat candidate genes with virus-induced gene silencing (VIGS)

Masterarbeit

eingereicht von

Daniel Fink

Wien, November 2014

Betreuer: Univ.Prof. Dipl.-Ing. Dr.nat.techn. Hermann Bürstmayr

Ausführender Betreuer: Dipl.-Ing. Dr. Wolfgang Schweiger

Department IFA-Tulln

Institut für Biotechnologie in der Pflanzenproduktion

Danksagung

Ich möchte folgenden Personen meinen aufrichtigen Dank für ihre Betreuung und ihre wissenschaftliche und praktische Unterstützung aussprechen:

Univ.Prof. Dipl.-Ing. Dr.nat.techn. Hermann Bürstmayr

Dipl.-Ing. Dr. Wolfgang Schweiger

Dipl.-Ing. Dr. Barbara Steiner

Bsc. Simone Zimmerl

Ao.Univ.Prof. Dipl.-Ing. Dr.nat.techn. Marc Lemmens

Bsc. Imer Maloku

Dipl.-Ing. Andreas Teutschl

Ing. Matthias Fidesser

Lisa-Maria Schmidt

Außerdem gilt mein besonderer Dank meiner Freundin, meiner Familie und meinen Freunden für ihre Unterstützung.

Abstract

Bread wheat (*Triticum aestivum*) belongs to the most important food crops worldwide. One of its most prevalent and devastating pathogens is *Fusarium graminearum* (teleomorph: *Gibberella zeae*) which infects wheat and other small grain cereals throughout temperate regions all over the world. The related disease Fusarium head blight (FHB) leads to severe losses in grain yield and quality due to mycotoxin contamination (Desjardins 2006). Due to their impact on animal and human health, mycotoxin contents in food and feed are strictly limited and regulated in several countries including the EU (van Egmond 2004). Crop rotation, tillage regime modification and treatment with fungicides provide only a minor contribution to FHB control. Therefore developing a functional understanding of the underlying biology and deployment of resistance genes in elite breeding material is the most promising approach (Buerstmayr et al. 2009). Fusarium resistance is a complex quantitatively regulated mechanism governed by many quantitative trait loci (QTL), of which *Fhb1* on chromosome 3B is a prominent major contributor of type II resistance (spread within plant) (Buerstmayr et al. 2003). As the sequence of this genomic region is yet unknown, several efforts were made to obtain sequence information with the goal to identify resistance candidate genes. Prior to this work a BAC library (Bacterial artificial chromosomes) of CM-82036, a wheat genotype harbouring *Fhb1* was screened with genetic markers to reconstruct the genomic sequence of this important QTL. The genes found in this region were then used to map RNAseq- reads of Fusarium- and mock- inoculated plants. Based on this data we found several genes differentially expressed in resistant cultivars and identified them as a terpene synthase, F-box protein, calcium sensor protein and a putative GDSL lipase-like protein. Each of these could contribute differently to FHB resistance. While terpenes act as toxins (Wittstock & Gershenzon 2002), F-box proteins are a key element in protein recognition and degradation (Skaar et al. 2013) and calcium sensors play an important role in eliciting biotic and abiotic stress response (Reddy et al. 2011). Moreover it is known that two genes coding for GDSL lipases in *Arabidopsis thaliana* elicit local and systemic resistance to pathogens (Gottwald et al. 2012). Once the candidates were identified we isolated and cloned them into a viral plasmid DNA in order to achieve a knock-down effect on plant transcripts during following *Fusarium* infection of a resistant cultivar. Plants with silenced target genes should show severe symptoms of FHB, if the candidate contributes strongly to resistance.

The goal behind this approach is to find the main resistance gene for FHB, which could be a key element for successful resistance breeding and ensurance of food security.

Kurzzusammenfassung

Brotweizen (*Triticum aestivum*) gehört zu den weltweit wichtigsten Nahrungspflanzen. Ein weit verbreiteter und verheerender Krankheitserreger ist der Pilz *Fusarium graminearum* (teleomorph: *Gibberella zeae*), der neben Weizen auch andere Getreidearten in gemäßigten Zonen befällt und global auftritt. Ährenfusariosen (Fusarium head blight; FHB) sind das Resultat eines erfolgreichen Befalls und führen neben hohen Ertrags- und Qualitätsverlusten auch zur Mykotoxinbelastung des Ernteguts (Desjardins 2006). Aufgrund ihrer toxischen Wirkung auf Mensch und Tier gibt es Grenzwerte in der EU und anderen Ländern, die deren Gehalt in Lebens- und Futtermitteln beschränken. Da Fruchtfolgen, Bodenbearbeitungsmaßnahmen und Fungizidbehandlungen den Befall nur geringfügig beeinflussen können, ist das Verstehen von Resistenzmechanismen und das Einkreuzen von Resistenzgenen ein besserer Ansatz (Buerstmayr et al. 2009). Fusariumresistenz ist ein komplexer quantitativ regulierter Mechanismus, wobei *Fhb1* auf Chromosom 3B als wichtigster QTL (Quantitative Trait Locus) für Typ II-Resistenz gilt (Ausbreitung in der Pflanze) (Buerstmayr et al. 2003). Da aber die genomische Sequenz dieses Lokus nicht bekannt ist, wurde intensiv auf das Entschlüsseln dieser Sequenzinformation hingearbeitet. Der vorliegenden Arbeit ist die Rekonstruktion der genomischen Sequenz um *Fhb1* aus der Linie CM-82036 vorausgegangen. Anhand von sequenzierter RNA aus Fusarium- und Wasser-inokulierten Pflanzen wurden vier Gene gefunden, die in resistenten Pflanzen hochreguliert waren. Dabei handelte es sich um codierende Gene für eine Terpensynthase, ein F-box Protein, ein Calcium Sensorprotein und eine GDSL-Lipase. Jedes dieser Gene kann dabei anders zur Resistenz beitragen. Während Terpene als Toxine wirken (Wittstock & Gershenzon 2002) sind F-box Proteine wichtig für das Erkennen und Abbauen von Proteinen (Skaar et al. 2013). Calcium Sensorproteine spielen eine wichtige Rolle bei der Reaktion auf biotischen und abiotischen Stress (Reddy et al. 2011) und von zwei codierenden Genen für GDSL Lipasen in *Arabidopsis thaliana* weiß man, dass sie lokale und systemische Resistenz gegen Pathogene auslösen (Gottwald et al. 2012). Sobald die Kandidatengene identifiziert waren, wurden Fragmente von ihnen isoliert und in eine virale Plasmid-DNA kloniert. Die Virusinfektion einer resistenten Pflanze soll infolge einer Fusarium-inokulation die Zahl der funktionierenden Transkripte verringern und die Translation von Resistenzproteinen verhindern. Pflanzen mit geringerer Transkriptzahl eines Kandidatengens sollten daher starke FHB Symptome zeigen, wenn es sich tatsächlich um ein Resistenzgen handelt.

Das Ziel dieser Methode ist es, das wesentliche Resistenzgen gegen FHB zu finden. Dies könnte ein entscheidender Beitrag zur erfolgreichen Resistenzzüchtung und zur Gewährleistung der Nahrungsmittelsicherheit sein.

Table of contents

1. Introduction

1.1. Wheat – economic importance

Wheat is a substantial part of human and animal nutrition. As a staple crop for 35% of the human population it supplies approximately 20% of the worldwide calorie demand (http://www.cymmt. org/; in Scofield et al. 2005). According to acreage, wheat is the foremost crop plant in the world, followed by maize and rice. The overall production of wheat in 2012 adds up to about 660 million tons (FAO, 2014). Due to the maintenance of a continuous wheat production for global food supply, crop improvement, regarding yield as well as resistance to pathogens, is of major importance.

1.2. *Fusarium* head blight (FHB) or scab

Fusarium head blight as one of the most prevalent diseases in wheat (*Triticum aestivum*) and other small grain cereals is mainly caused by the fungus *Fusarium graminearum* (teleomorph *Gibberella zeae*. The fungus overwinters on plant debris and reaches flowering wheat heads via splash water, where the spores germinate and infect the most susceptible floral tissue. FHB as a result occurs worldwide and most frequently in regions providing sufficient warmth and humidity for fungal penetration causing severe losses in grain yield and quality (Kugler et al. 2013).

FHB infection and the contamination of grains with mycotoxins produced by the fungus pose a huge threat to human and animal health. Especially the mycotoxin family of trichothecenes acts immunosuppressive due to its inhibition of protein, DNA and RNA synthesis, inhibition of mitochondrial function and effects on cell division and membrane function (Rocha et al. 2005). Therefore mycotoxin contents in cereals are strictly regulated in many countries including the European Union (van Egmond 2004).

1.3. *Fusarium* resistance strategies

Measurements to contain the disease in agronomic practice are only partially successful. The understanding of FHB resistance on a molecular level and the deployment of resistance genes in wheat varieties seem to be more promising strategies.

Two major components of host plant resistance have been widely accepted by most authors actively contributing to *Fusarium* research. Type I resistance counteracts initial infection and type II resistance operates against the spread of the pathogen within the host (Schroeder & Christensen 1963). During pathogen attack and plant resistance reactions an enormous range of attacking enzymes and counteracting inhibitors is held by the apoplast (Misas-Villamil & van der Hoorn 2008), since fungal penetration induces the activity of numerous genes in response. Studies on differential gene expression patterns due to pathogenic activity reveal the triggering of an oxidative burst as part of the hypersensitive response (HR) initiating the production of ROS in attacked cells (Zhou et al. 2005). Induced locally acting signalling cascades can also be activated systemically by synthesis and trans-cellular transport of hormones like jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) to protect distant tissues in advance of the spreading pathogen (systemic acquired resistance, SAR; type II resistance) (Grant & Lamb 2006).

Although resistant cultivars are available, there are still three factors limiting the success of breeding approaches: (1) resistant cultivars are lacking in agronomic performance, (2) the inheritance of resistance is oligogenetic to polygenetic and (3) the screening for FHB resistance is environmentally biased and costly (Buerstmayr et al. 2002).

Despite those obstacles, the implementation of functional breeding systems is a crucial approach for the development of resistant cultivars.

1.4. *Fhb1* – main resistance QTL (quantitative trait locus)

Resistance to FHB is a quantitative trait, which means that there are several genes involved in host resistance reactions. For the research on FHB resistance, a large number of potential candidate genes, even minor contributors, have to be taken into account. A recent applicable tool for resistance selection in conventional breeding is the use of DNA-based markers, which are either closely linked or directly attached to genes of interest. This marker assisted selection approach in combination with QTL mapping revealed several strong QTL from different varieties, although especially Chinese Spring wheat cultivars Sumai 3 and its descendent CM-

82036 deliver the most promising ones (Buerstmayr et al. 2009) - *Fhb1* on the short arm of chromosome 3B and *Qfhs.ifa.5A* on the short arm of chromosome 5A (Anderson et al. 2001). The identification of those main resistance QTLs lead to the development of near isogenic lines (NILs) with differing susceptibility to *Fusarium graminearum* due to the origin of the harboured QTL (Kugler et al. 2013).

RNA sequencing of Fusarium inoculated NILs revealed line specific transcriptomic responses to the fungus. In order to identify those linked to *Fhb1*, work at the Institute for Biotechnology in Plant Production (IFA-Tulln) has focussed on sequencing the genomic region of CM-82036 which encodes for *Fhb1*. Annotated genes on this locus have been linked to the expression data and promising candidate genes have been selected. Namely these are a terpene synthase, a calcium binding protein, a GDSL lipase and an F-box protein (Table 1).

Table 1: Gene expression data derived from infection trials of susceptible (C4) and resistant (CM) cultivars 30 and 50 hours after mock- (M) and Fusarium (F)-inoculation. The gene transcript levels are represented by the respective numbers. Red squares indicate low and green squares indicate high expression levels of the respective genes.

C4 F30			C4 F50			C4 M30			C4 M50			CM F30			CM F50			CM M30			CM M50			putative function
0	0	1	0	0	0	0	0	1	0	0	0	24	25	27	19	17	24	16	16	9	19	15	16	Terpene synthase
82	68	89	73	49	80	69	67	87	62	51	100	134	112	228	148	105	135	139	136	98	101	94	108	Ca binding protein
0	0	0	0	0	0	2	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	transcription factor IIE
0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	2	1	0	2	0	0	0	poreforming toxin like
0	1	3	0	0	0	1	1	1	0	0	1	17	16	26	2	5	4	29	20	10	2	3	17	WSX1
29	19	28	37	27	53	14	8	12	3	4	10	46	27	33	147	91	63	4	7	8	10	3	9	GDSL lipase
64	51	46	141	89	142	13	9	9	5	3	7	77	58	85	205	124	105	10	3	6	2	3	10	? GDSL
2	0	0	2	0	1	2	0	0	0	0	0	1	0	3	1	3	0	1	0	0	0	0	1	Cystatin Hv-CPI6
3	4	5	5	2	9	4	2	2	4	2	3	162	141	241	170	121	149	200	178	137	187	154	210	F-box

1.5. F-box proteins

Ubiquitination of proteins is a central mechanism regulating several different cellular processes, including signalling pathways related to hormones, light, sucrose, developmental coefficients and pathogens. The bio-chemical background is a collaboration of E1 (ubiquitin-activating), E2 (ub-conjugation) and E3 (ub-ligation) enzymes triggering mono- and polyubiquitination for protein degradation by the 26S proteasome (Devoto et al. 2003; Hua & Vierstra 2011).

F-box protein families belong to the functional group of E3 ubiquitin ligating enzymes. They recruit substrate proteins by highly specific substrate-protein interfaces and are tightly

regulated to prevent unspecific protein degradation (Skaar et al. 2013). In case of hormone signalling, F-box proteins are main contributors to ethylene synthesis and auxin, strigolactone and jasmonate related pathways by ubiquitination of the repressing transcription factors, which is reviewed in detail by Hua & Vierstra (2011) and Somers & Fujiwara (2009).

Besides ethylene, jasmonic acid induced gene expression is suspected to be amongst the essential mechanisms of *Fusarium* head blight resistance (Li & Yen 2008), which justifies the consideration of F-box proteins to be resistance contributors.

1.6. Terpene synthases

Terpene synthases play a key role in the synthesis of terpenes and are most likely regulated by transcription factors (Cheng et al. 2007). Their products are the structurally most diverse group amongst plant natural metabolites and are involved in plant interaction with insects, pathogens and other plants (Dudareva et al. 2004; Paschold et al. 2006). Additionally they regulate plant growth, development and environmental interactions (Tholl 2006) as well as direct and indirect defence response (Cheng et al. 2007). In case of indirect defence responses involving herbivores, the volatile nature and signalling function of terpenoids warns neighbour plants about attacking insects and also attracts herbivore predators. Direct response to pathogen attack by plant terpenoids is strongly linked to the production of phytoalexins, exhibiting anti-microbial properties, as it is reviewed in detail by Cheng et al. (2007).

Although the knowledge of structural and mechanistic properties increased due to the characterization of numerous terpene synthases, their exact role in physiological and ecological interactions remain unclear (Tholl 2006). Nonetheless they must not be disregarded in the contribution to FHB resistance.

1.7. Calcium sensors

Ca^{2+} is known as an important secondary messenger during stress reactions in plant cells. Stress exposure causes rapid rise of cellular Ca^{2+} levels, which is sensed by a number of Ca^{2+} and Ca^{2+}/CaM binding proteins regulating downstream signalling and appropriate physiological responses. Thereby Ca^{2+} sensors can directly bind to *cis*-elements on promoters or to DNA binding proteins and activate or inactivate them, both resulting in induction or repression of

specific gene expression. Furthermore CDPKs and phosphatases, regulated by cellular Ca^{2+}, are activated and phosphorylate/dephosphorylate DNA binding proteins to regulate gene expression. Ca^{2+} mediated reactions can either occur due to abiotic (drought, salt, cold, heat, mechanical stress) or biotic stresses elucidated by herbivore or fungal attack. Biotic stress responses are induced systemically by trans-membrane ion fluxes, involving Ca^{2+} signalling in plant microbe interactions for gene expression of SA regulators. Besides this general reaction against biotic stress, plants also synthesize transcription factors as response to fungal chitin reception, indicating their specific involvement in fungal defence (reviewed in Reddy et al. 2011).

Due to the strong overexpression of a specific Ca^{2+} binding protein in resistant plants (Table 1), its involvement in *Fusarium*-related reactions is hypothesized and therefore has to be examined.

1.8. GDSL lipases

GDSL motif enzymes are a very diverse family of recently discovered lipases with many undescribed characteristics (Brick et al. 1995; Upton & Buckley 1995). Current descriptions of GDSL esterases/lipases indicate their involvement in plant morphogenesis, development, synthesis of secondary metabolites and defence responses (Zhang et al. 2006; Agee et al. 2010; Oh et al. 2005).

Response to biotic or abiotic stresses often requires the enhancement of a signal by feedback loops. Recent studies in *Arabidopsis thaliana* revealed, that GDSL lipase 1 (GLIP1) expression requires ethylene signalling and vice versa regulates systemic immunity by feedback regulation of ethylene signalling. This reaction occurs in response to necrotrophic pathogens (Kim et al. 2013). Also expression of another GDSL lipase gene (GLIP2) in *Arabidopsis* indicates enhanced resistance to fungal pathogens (Lee et al. 2009). Both genes are known to play important roles in plant resistance against necrotrophic and hemibiotrophic pathogens by eliciting local and systemic response (Gottwald et al. 2012).

Several studies revealed up-regulation of GDSL motifs during the infection of resistant wheat heads with *Fusarium* spores, controversial to GDSL expression profiles in susceptible cultivars (Muhovski et al. 2012; Li & Yen 2008; Wang et al. 2005).

Although there is no evidence of wheat homologs to *Arabidopsis* GLIP1 and GLIP2 genes, the involvement of expressed wheat GDSL motifs in resistance reactions towards *Fusarium graminearum* is strongly hypothesised nonetheless.

1.9. Post transcriptional gene silencing (PTGS)

Transcriptomic regulation is assumed important as a defence mechanism against unwanted nucleic acids originating from viruses or the own genome. While RNA interference protects many organisms from transposon activation and resulting genome instability, plant PTGS is tightly linked to genome defence against RNA viruses (reviewed in Hammond et al. 2001).

The functional background is the recognition and degradation of dsRNA and subsequent targeting of the organism's own mRNA with sufficient homology. In this process, incoming dsRNA is cleaved into fragments of 21-23 nucleotides by a DICER enzyme (Xie et al. 2004). The resulting small interfering or guide RNA strand is incorporated by the plant RISC-complex (RNA-induced silencing complex) (Hammond et al. 2000; Zamore et al. 2000) and subsequently binds to highly homologous mRNA, which is degraded by catalytic RISC components (Hammond, Boettcher, et al. 2001; Liu et al. 2004; Song et al. 2004).

Triggering this plant immune system regulates mRNA translation at a post-transcriptional level. Therefore gene expression can occur, but translation into functional proteins is extensively inhibited by mRNA degradation, protecting the genome from detrimental nucleic acids.

1.10. Virus-induced gene silencing (VIGS)

VIGS has become a widely applied reverse genetic method for the determination of gene function over recent years. Many viruses have been discovered and modified for the application on dicots, but only two remained applicable on monocot plants. Especially barley stripe mosaic virus (BSMV) has emerged as the most practicable candidate for VIGS experiments on wheat (Holzberg et al. 2002; Lacomme et al. 2003; Scofield et al. 2005).

Holzberg et al. (2002) initiated the first successful application of VIGS in monocots by silencing a phyteone desaturase gene (PDS) using BSMV as viral vector. PDS is part of the carotenoid synthetic pathway and protects chlorophylls from photooxidation (Bartley & Scolnik 1995). The result of the VIGS experiment were phenotypes displaying bleached leaves, proving the fundamental practicability of the VIGS concept (Holzberg et al. 2002).

The cornerstones for the successful implementation of a VIGS system are the design of an appropriate vector carrying target gene information, and the host plant infection triggering plant response. The method relies on the functional concept of PTGS cleaving dsRNA for viral defence (Figure 1). VIGS induces degradation of viral RNA and targets host mRNA, causing local

(siRNAs) and systemic (RdRP) spread of silencing throughout the plant. The systemic spread occurs regardless of viral spread (Becker & Lange 2010).

BSMV VIGS is very advantageous regarding experimental costs and is easy to apply. Additionally VIGS is very suitable for high throughput studies to explore gene function (Yuan et al. 2011).
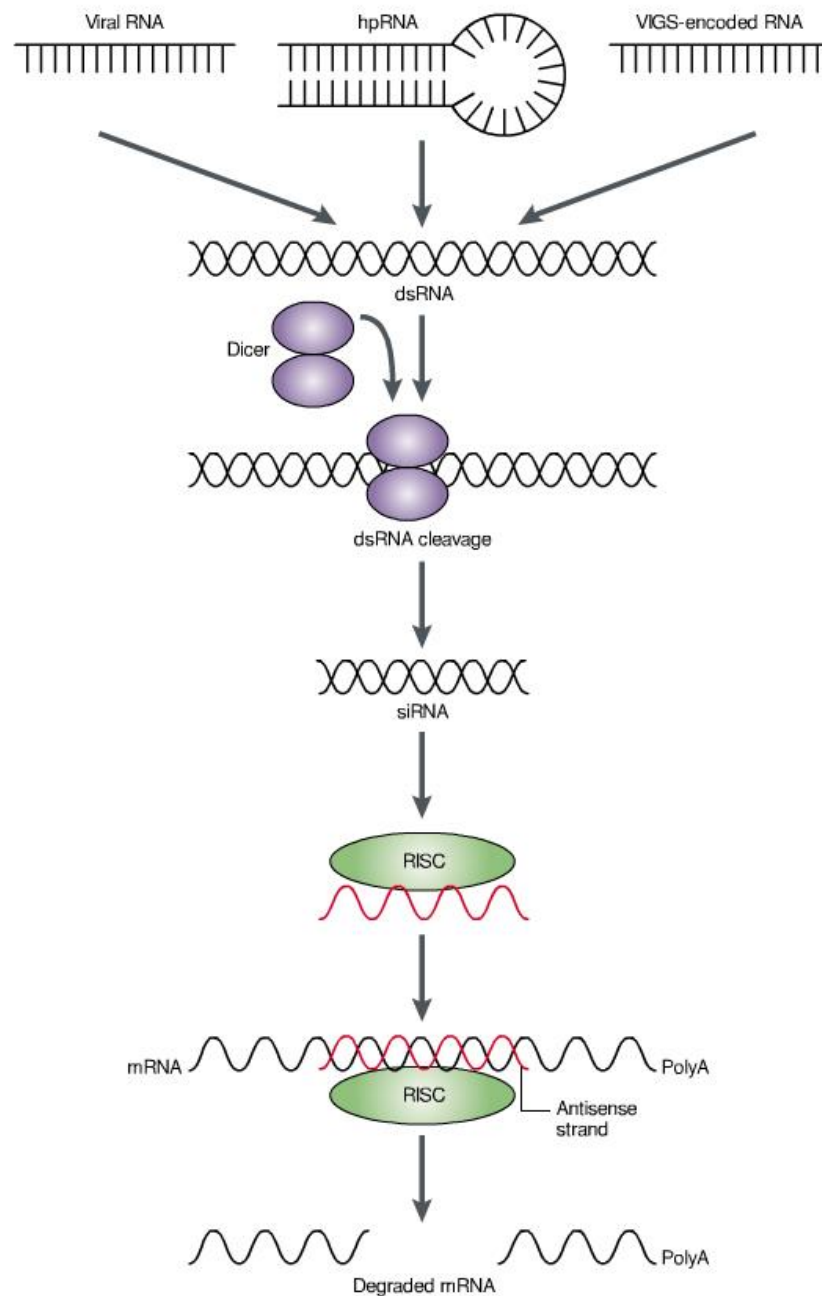
Figure 1: Schematic mechanism of RNA induced gene silencing (Waterhouse & Helliwell 2003). VIGS-encoded RNA becomes double stranded during viral replication and is recognized by DICER molecules triggering the activation of the gene silencing pathway.

2. Materials and methods

2.1. Plant and infection materials

2.1.1. CM-82036

CM-82036 is a spring wheat derivative of the Chinese cultivar Sumai-3 and the Brazilian cultivar Thornbird. It possesses a high resistance to FHB as it hosts the two most prominent resistance QTL *"Fhb1"* and *"Qfhs.ifa-5A"* besides many smaller ones for example on chromosome 6B.

2.1.2. NIL 34

NIL 34 is a susceptible wheat near isogenic line derived from CM-82036 x Remus backcrosses (BC5F2). NIL34 harbors the susceptible *Fhb1* allele in the genetic background of CM-82036 and is used as positive control for *Fusarium* infection trials.

2.1.3. Growth conditions

The plants were sown in multipots and kept in the greenhouse until germination followed by vernalization until the two leaf- stage. At this time they were potted and transferred to the climate chamber with controlled conditions of 14°C, 50% humidity and 12 hours of daylight. As soon as they had developed 3-4 tillers the plants were treated against pests and temperature and illumination were raised step by step to 24°C at 16 hours of daylight until viral infection.

2.1.4. Plant nutrition, aphid and mildew prevention

Before shoot growth, plants were nourished with "Blaukorn" fertilizer. At this time point plants were also treated with JuwelTop (1 mL/L; Nufarm, Linz, Austria) and Biscaya (0,3 mL/L; Bayer, Wien, Austria).

2.1.5. *Fusarium graminearum* conidia suspension

A suspension of *Fusarium graminearum* macroconidia was used for plant inoculation. All suspension stocks were provided at the beginning of inoculation trials. For the actual inoculation the suspension had to be diluted to a concentration of 10.000 conidia per milliliter.

2.1.6. Barley Stripe Mosaic Virus (BSMV)

BSMV is a tripartite virus infesting barley and sometimes wheat, producing mild effects of chlorotic discolorations and growth impairment. Usually it is spread by seed, natural vectors are still unknown. Its genome consists of α-, β-, and γ- RNA. RNAα encodes the methyltransferase/helicase subunit of the RNA-dependent RNA polymerase (RdRp). RNAβ defines the coat protein and triple gene block (TGB) proteins for viral cell-to-cell movement. RNAγ finally encodes the polymerase subunit of the RdRp and the γb protein, the major factor in viral pathogenesis, long distance movement and the suppression of host RNA silencing defenses (Yuan et al., 2011). For using the BSMV as a VIGS vector, a fragment of the gene of interest can be inserted either after the stop codon or at the beginning of the γb gene which prevents γb expression (Bruun-Rasmussen et al. 2007).

2.2. Primer design and candidate gene selection

The selection of candidate genes was based on available RNAseq data. *Triticum aestivum* cultivars CM-82036 and Remus were mock- and *Fusarium*-inoculated and investigated for differential gene expression 30 and 50 hai (hours after infection) (Kugler et al. 2013). Based on these data 4 candidate genes (Table 1, introduction) were chosen for further testing. The gene sequences are given below and contain the whole Open Reading Frame (ORF) of the candidate genes. Start codons are given in green (ATG) and stop codons in red letters (TGA, TAG, TAA).

Gene sequences (Open reading frames)

Terpene synthase (~1550 nt)

ATGCAGAGGTCCGAGGAATGGATGAGGGAGAGGGTGGAGGAGCTCAAGGGGCGAGTGCGCACGAT
GTTCTCCAACGACAGTGTGGCCGAGGCGGTGACATTGATGGACACACTTGAGCATCTCGGCGTGGATG
GCCACTTCCGCGAAGAGATTGACTCGGCCATAAGCCGGATCGTTCACCCGGATGAGTCTGCTGGTTCTG
ATGACCTTCATGTTGTCGCGAGTCGGTTTCGGTTGCTTCGGCAGCATGGGATATGGGTGTCCACAGATG
CATTGGACAAGTTTAGAGACGGCACGGGCAACTTCAAGGCAAGCCTGAGCAGTGACCCAAGGGCTCTA
CTAAGCTTGTACAACGCAGCTCACATGGCAGTACCGGGTGATGGCCCGGCCCTCGACGATGTCATCGA
CTTCACACGGCACCAGCTCGAGGCCATTGCAGCGAAAGGTGAGCTTCGGTCACCGTTGGCGGAGCAGG
TCGCCCGCGCCCTCGACCATCCTCTCCCACGGTTCACCAAGCTGCTAGAGACCATGTATTACGTCGGTG
AGTATGCGCAGGAGGAGACACACGACAATACGCTGCTAGAGCTCGCTAGGCTCAACTCTCACCTCATG
AGGTCTCTTCACCTCAGGGAGCTAAAGGCACTGTCCTTGTGGTGGAGGGATCTTTACGACACAGTGAAT

CTACCGTACACTCGGGACCGTATGGTCGAGGTCTACTTTTGGAGTTGCGGAATGATCCCTGAGGAGGA
ACAGTCACGCGCACGATTGATGTTCGCCAAGACGTTTGGGTTGGTGACTTTTCTTGATGATACCTATGA
TGTCCATGCCACCTTAGAGGAATGTCATAGTTTCACCGAAGCCATGCAAAGATGGGATGATAGCGCGG
TTTCTATTCTACCTGAATACCTTGGCATGCTCTACATCAAAATGCTAAGCAACTTCAAAGATTTTGAGAA
TATGTTAAAACCGCACGAGAAGTACCGCATGTCTTATGCAAAAAAAACGTACCAACTACAGGCGGAAT
ACTATATGCAAGAGGCCCAATGGTCCAACGACAAGCACCAGCCGACCTTCAAGGAACACGAGGAGCTG
TCGGTCATGTCTTCGGGCTTGCCGATGCTTAACCTCGTGGCCCTCATGGGCTACGGCGCCATAGCAACC
AAGAAGGTATTCGAGTGGACATGCGCCGTCCCCGACGTAGTCCGTGCCGGTGCACAGATCGGCCGCTT
CCTCAACGACATCTCCTCTTACAAGTTGGGGAAGAACAAGAAGGACGTGGCTAGCGTCGTGGAGTGCT
ATATGGTGGAGAAGGGCACGACAGGGGAGGAGGCAGTGGCGGCAATTGCCGCCATGACGGAGAATA
GTTGGAGGACGATGAACCAGGCATGCATGGTGATGGACCGTGCGTTATTGCCAGCAGCGCAACTGGT
GGTGAACATAGCAAGGTCAGACGAGGTCATCTACCTCCGCGGTAGGGATGGTTACACCTTCGGTAGCC
ACGTCAAGGACCTTGTCACCATGCTCTTCCTCGCCCCCATCCCCCTTTGA

F-box protein (1215 nt)

ATGGAGGAACCGACGGCGGCCGGCCCCGACTGGTCAAAGCTGCCGCCGGACGTCCTCACCACCGTCCT
CGGCGACCTCGAGTTCCCGGATCTATTCCGCGCCGCCGACGTCTGCACCGCCTGGCGGGCCACCGCCC
GCGCCCTCCGCCGCCTCGGGATCTACAGCCGCCCCCAAACCCCCTGCCTCCTCTACACCAGCGCCGCCG
CCGGCCCCCGCGCCGCCGAGCTCTTCAGCCTCGCCGACAAGAAGGCCTACAGGGCGCGCCTCCCGGAC
CCTCCCATCGGGGAGCGCAACATCATCGGCTCCTCGTACGGGTGGCTCGTCACCGCCGACGCCCGCTCC
GAGCTCCACCTCCTCAACCCCGCCACCGGCGAGCAGGTCGCGCTCCCCTCCGTCGCCACCATCGAGCAG
GTAAGCCCCATCCTCGACCGCCATGGCAACCTTGAAAGGTACCATCTTTCTCTCCATGGGGATGATCCG
CAGCCCTATGGGGTGGACGAGCTCCGCGGAGTCCTCTATCTCAAGGCCGTGCTGTCCTGCGATCCCGCA
TTGGGGGATTGCACGGTCGTGCTGATTCACAATCCCTATAGGGATCTCTCGTTTGCGAGGGTCGGCGAT
GACAAGTGGCACTGGATACCTTCGGCACCTCGTGAACCGCCGCGGTACTCAGACTGCATATTTGGCGAT
GATGGTGCGCTCTACGCCATGGATCTCCTCGGCGGGATGTATCGCTATGCCATCCAAGGTTCTTGTGCC
ACCCGGGATATGATTTTCAAGGAAACTTCGCCATTCGTGGCATATAATGGATACCTATCCAAGACATCA
TGTGGCAGTGTGCTGCAAATATGGAGGGTCAAGAGGAAAACAAGGGGGGAGCAAGAAGAGATGCAT
ACAGTTGATATTGAGGTGTACATGACTGACCTTGACAAGCAGCAGATAGTTCGTGTGCGGACGTTGGG
GGATTACGCGTTGTTCATCGGGCACAACTATACTTGTTGCCTTTCCACACAGGACTACCCAGGGCTTCTG
CGGAACCATGTCTATTTTACGGACGATGACGAGTATTGGCTGATAGATTCGAAAGACAATCGTCGGGA
TGTTGGAATACTAGATTTGGAGGATTTAAGTGCCACTGATGTTGTATCTCCTCAACCATGGTTGAATTG
GCCAATTCCCGTATGGATCACTCCAAGTTTTAATAAGATCCATAAATAG

Ca²⁺-binding protein (825 nt)

ATGTGTATATTTGGACAACCATGTAGACAAGCTGTTGGACGTCTGTTTTGGGCTCACGTCGTGCAAATG
GTCGAGGACAAGAAGAAGAGACTCCTTGAGAAGAAGGAAGCTCCACTGAAATGGCAGCAGAAACTGG
AAGGGGCAATTAAGGCCACTGAAGAAAAGGAGAAGAAGCTCAAGTCGAAAAAGCACAGGAGGCGAA
GCTATTCTTCCTCAGAATCCGACAGTGAATCCGAGAGCGACAGTGATCGGAAACGCAGGAAGAGGAA
GGACCGCAAAAGGCACAAAAAACATGGCCACTCTGACTCTGATGGTGCCAGGAGGCGCAAGCACAGG
TCAAAGAGGAGGAGCTCGGACTCTAGCGATGAGAGCGACAGTGATGAATATGATAGCGAATCTGAAG
AGGATCGCCGAAGGAAGAAGCACTCGCACAGGAGGAAGCATCGCCGGCACTCTTCAAGGTCAGAGTC
TGATGCTTCAGATTACAGCAGCGATGATGATGAGCGGAGATCAACCAGGAAGGACCACACTAGGAGC
CGCAGGCGTCGCCACCGATCCTCAGACGATGAATCTGAGGAGAAGATCAGGTTGAGGCATAGGAAGC
GTCACAGATCAAGTGACGAGGACAAGCCGTCAGATTCTGACAACCATAAGCGTCACAGGAGCCGCTCT
ATGTCCTTGGATGACGGTGCTGCTGGCGAGCCAGACAAGATGAATGATGGCAAGGGGTCTCACAAAA
GCCGGCACCACCGCCGCCACCACCATCACCATCATGATCATCGTGCGAACTCTGCTGAACCCAGTGACG
GGAAGCAACTCGTGTAA

GDSL (1170 nt)

ATGGCTTCAACTCGCCGTGGCACCACCATGGCGACCAACAAACTCGTCATGTTCGTCGTGTTGCTGGTG
GTACTAGAGAGGGTGCGTTCCGACGATTCTCCATGCGGCTTCCCGGCGATCTTCAACTTCGGCGACTCC
TACTCGGACACCGGAGCCTTCCCGGCCCTCTTCCCGGCGGTGCAGCCGCCCTACGGCCGGACCTTCTTC
AGCATGCCGGCCGGGCGGCAAAGCGACGGCCGCCTCACCATCGACTTCATGGCTCAAAGCCTGGGGCT
GCGTTACCTGAATGCGTATCTGGATTCACTGGGGAGCAACTTCACTCAGGGAGCCAATTTCGCGAGCG
CCGCCGGAACCATCAGACGGGTGAACGGGAGCCTGTGGACCTCCGGGTACAGCCCCATTTCGCTGGAC
GTGCAGATCTGGCAATTCCAGCAGTTCATCAACAGGAGCCAGTTTGTCTACAACAACATAGGTGGAATC
TACCGCGAGATCCTGCCGAAACCCGAGCACTTGGTCTCCAAGGCGCTTTACACCTTGGACATCGGCGCC
AACGACCTCGCCATGGGCTATGTAGCCAACATGACGACAGAGCAAGTCGAGGCCTACGTCCCGGATCT
GATGGAGAGGCTTGCCTCGGCGATCCAGACGGTGTATGACCTCGGTGGGAGGTACTTCTGGGTGCACA
ACACGGGGACGCTCGGGTGCCTGCCGTACCGCCCGGACCTCGCCGCGGAGAAGGACGGCGCCGGCTG
CTCCATCGCGCTCAACGCCGGCCCCCGGTTCTTCAACGCGCGGCTCAAGGAGACCGTGGCCAGGCTCA
GGGTGGCTCTCCCCGAGGCCGCCTTCACCTACGTCGACGTGTACACGGCCCTGTACAGGCTGATGAGC
GAGGCCAAGAAGATCGGGTTTGCGGACCCTCTACATGTGTGTTGCGGGTATGGCGGTGGCGAGTACA
ACTTCGACAAGGACATCCGGTGCGGCGTTAAGGTGGAGGTGAACGGCAGGCTCCGGGAAGGGAAGTC
GTGCGAGGACCCATCCAAGAGCGTGAGCTGGGATGGTGTGCACTTGACCGAGGCGGCTTACAAGTTA
ATCTTCGACCAGATTGTGGACGGCGCGCTCTCCGACCCGCCGGTGCCTCTGCGGCGGGACTGCCAGGG
AAAAGGAAAATGA

To validate the effect of genes on *Fusarium* resistance, we used two constructs for each candidate gene. Primers were designed based on the template sequence and discriminating all other wheat homologous sequences that were identified by a previous BLAST search. Table 2 gives the sequences of all primer pairs used in the experiments. The primer stocks can be found in the box called "Oligo Stocks 4" in drawer 3 of freezer 5.

Table 2: Primers for candidate gene amplification

Candidate gene	Construct name	Lab ID	Primer sequence 5' → 3'	Product length (bp)	Annealing temperature (°C)
Terpene synthase	TS1	VF 51	GTACGGATCCATCCCTGAGGAGGAACAG	128	56.3
		VR 51	GTACCCCGGGATGGCTTCGGTGAAACTATGA		57.5
	TS2	VF 52	GTACGGATCCTGCCAGCAGCGCAACTG	128	57.3
		VR 52	GTACCCCGGGGATGGGGGCGAGGAAGA		57.3
F-box protein	FB1	VF 54	GTACGGATCCCCATCCTCGACCGCCAT	214	57.3
		VR 54	GTACCCCGGGCAGTGCCACTTGTCATCG		56.3
	FB2	VF 55	GTACGGATCCCCTTTCCACACAGGACTAC	133	57.5
		VR 55	GTACCCCGGGGGCACTTAAATCCTCCAAATC		57.5
Ca-sensor	CS1	VF 57	GTACGGATCCAGCTATTCTTCCTCAGAATCC	144	57.5
		VR 57	GTACCCCGGGTGTGCTTGCGCCTCCTG		57.3
	CS2	VF 56	GTACGGATCCACAACCATGTAGACAAGCTG	133	56.4
		VR 56	GTACCCCGGGTCAGTGGCCTTAATTGCCC		57.5
GDSL	HL1	VF 60	GTACGGATCCGCCGCGGAGAAGGACG	180	58.4
		VR 60	GTACCCCGGGCCCGATCTTCTTGGCCTC		58.4
	HL2	VF 61	GTACGGATCCGAGGTGAACGGCAGGCT	123	57.3
		VR 61	GTACCCCGGGGCCGTCCACAATCTGGTC		58.4

The primers for PCR amplification were designed following the guidelines of the PREMIER Biosoft website (PREMIER Biosoft, Palo Alto CA, USA; http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html). As wheat is a hexaploid organism, its genetic material consists of the three genomes A, B and D. Every genome carries members of the same gene family which differ only by single bases along their nucleotide sequence (single nucleotide polymorphisms, SNPs). In order to design genome specific primers for VIGS constructs it is mandatory to ensure 3'-ends fitting to SNPs differing from the other chromosomes.

Additionally the according inserts were checked for the formation of small interfering RNAs which are synthesised by plants in PTGS response. For this purpose we used the online tool pssRNAit (Samuel Roberts Noble Foundation, 2013; http://plantgrn.noble.org/pssRNAit/). After finding proper binding sites, restriction sites for BamHI (GGATCC) and Cfr9I (XmaI)

(CCCGGG) had to be added to the 5'-end of each primer in order to clone the insert in reverse direction for stable constructs. An elongation of restriction sites by the bases GTAC at the 5' end was mandatory to ensure sufficient restriction efficiency. All primers were designed on available *Fhb1* contig data using the Geneious software.

2.3. PCR amplification of candidate genes

Once the primers were received and diluted to 2,5 µmol, test PCRs could be carried out to check for primer specificity. For this purpose genomic DNA and cDNA of CM-82036 that has been collected at 30, 50 and 72 hours after *Fusarium* inoculation (hai) was used. Optimal annealing temperatures were determined individually for every primer by PCR with temperature gradients and reached from 56 to 59 °C. The conditions for the reactions were as follows (Tables 3 & 4).

Table 3: Protocol for PCR reactions. Annealing temperatures depend on primer combinations.

Step Nr.	Temperature (°C)	Process	Duration
1	95	Denaturation	2 minutes
2	95	Denaturation	30 seconds
3	56-59	Primer annealing	30 seconds
4	72	Elongation	30 seconds
5	72	Elongation	5 minutes
6	4	Cool down	∞

Table 4: Reagents in the PCR mix

PCR mix protocol

5x Green Go Taq reaction buffer

1 mM $MgCl_2$ (25 mM)

0,2 mM dNTPs (2 mM)

500 nM Forward primer

500 nM Reverse primer

100 ng template DNA

0,25 u GoTaq G2 DNA polymerase

The PCR product was checked for accurate length on a 2% agarose gel (amount of agarose in % of final volume with 1xTAE buffer) in volumes of 5 μL. Once the accurate primer/temperature/DNA combination was identified, 80 μl PCR reactions could be prepared for each silencing construct. As the reaction can be inhibited by large volumes, it was better to split the reactions into 40 μl volumes. Once the reaction was done, the PCR product was loaded on a 2% agarose gel for 45 minutes at 80 V. The matching bands were cut out under UV light and purified using the GeneJET Gel Extraction kit (Thermo Fisher Scientific, Waltham MA, USA) with abidance of the enclosed protocol.

2.4. Agarose Gel Electrophoresis

Primer specificity, propriety of restricted inserts and cloning success was checked on agarose gels. Gel concentration depended on fragment size and reached from 1% to 2%. Also runtime and voltage were adapted according to fragment size. 1x TAE buffer was used for every agarose gel.

2.5. Restriction digestion of the PCR product and γ-plasmid

For subsequent cloning into the viral BSMV vector, the inserts as well as the plasmid DNA had to be prepared by restriction digestion with the according enzymes (Thermo Fisher Scientific, Waltham MA, USA).

Table 5: Protocol for restriction digestion

Reagent	Plasmid restriction	Insert restriction
10x Reaction buffer Cfr9I	1x	1x
BamHI	30 u	20 u
Cfr9I(XmaI)	15 u	10 u
DNA	~5000 ng	~500 ng
H_2O (to final volume)	to 60 μL	to 40 μL

The restriction reactions were incubated at 37 °C for two hours on an Eppendorf thermoshaker (Eppendorf AG, Hamburg, Germany).

2.6. Ligation of PCR products into the viral vector

For the ligation of inserts and plasmids it was necessary to estimate the plasmid/insert ratio for sufficient ligation efficiency according to the following equation (UCLA; https://www.mcdb.ucla.edu/Research/Arispe/Protocols/Vector_Insert_Ratio.pdf):

$$\text{DNA amount (ng)} = \frac{ng\ of\ vector * kb\ size\ of\ insert}{kb\ size\ of\ vector} * molar\ ratio\ of\ \frac{insert}{vector}$$

As ligation efficiency could be very dissimilar, insert/vector ratios of 6:1 and 3:1 were used. The DNA amounts of insert and vector were measured using a NanoDrop spectrometer. Once the ligation reactions were mixed, they were incubated at room temperature for 1-2 hours and at 4°C overnight.

2.7. *E. coli* transformation

The obtained constructs were subsequently transformed into *E. coli* cells by heat shock transformation. Bacterial cells are stored at -80 °C and were already available in according aliquots at the time of the experiment. For transformation, 100 μL for each construct were thawed on ice to avoid damage of bacterial cells. For every transformation we took 10 μL the construct and mixed it with 100 μL of *E. coli* cells. After incubation on ice for 30 minutes the transformation batches were heat shocked for one minute at 42 °C on an Eppendorf thermoshaker and then put on ice again for 15 minutes. In order to enhance transformation success, 1 mL of LB media was added to each tube followed by 1,5 h incubation at 37 °C. It was crucial to include positive (empty plasmid) and negative controls (empty bacterial cell) to provide comparability for transformation validation.

2.8. Preparation of LB/Agar plates with ampicillin

LB/Agar plates were prepared by mixture of Luria Broth medium (25 g/L) and Agar (20 g/L) with filtered water. For sterilization the medium had to be autoclaved for 50 minutes at 1 bar. The autoclaved medium was cooled down to 50 °C before adding ampicillin to the medium (500 µL/500 mL). The whole volume was distributed on petridishes. The work was carried out on a laminar flow work bench to avoid any contamination with any unwanted bacteria or fungi.

2.9. Propagation of silencing constructs

The transformed *E. coli* cells were spread using a glass spatula on LB/Agar plates containing ampicillin as selective antibiotic. The application volume for each transformation batch was 200 µL per plate. After dispersion with a disinfected spatula the plates were incubated over night at 37°C until bacterial colonies were visible. Medium sized colonies were picked with an autoclaved toothpick and transferred to glass tubes filled with 3 mL liquid LB/Amp medium. Incubation at 37 °C at 170 rpm overnight lead to the duplication of selected colonies and their subsequent purification according to the STETL protocol. The presence of the insert was double−checked by PCR with flanking plasmid sequencing primers and via double digestion using BamHI and Cfr9I restriction enzymes both loaded on 1,5% agarose gels. Clones carrying plasmids with correct insert size were propagated again and subsequently isolated using the Promega PureYield Plasmid Miniprep System (Promega Corporation, Madison WI, USA) protocol.

2.10. Storage of clones in glycerol stocks

1 mL of each selected bacterial clone from Miniprep purification was stored in 0,5 mL of 70% glycerol until the sequencing results arrived. Table 6 lists the clones for every candidate gene. They can be found in a box named "Daniel HL VIGS Glycerolstocks" in the -80 °C freezer.

Table 6: Selected bacterial clones stored in glycerolstocks at -80 °C.

Candidate	Clone	Primer
Terpene synthase	TS1 C TS1 D TS1 E	VF/VR 51
	TS2 F TS2 G TS2 I	VF/VR 52
F-box protein	FB1 A FB1 D FB1 H	VF/VR 54
	FB2 C FB2 E FB2 I	VF/VR 55
Ca binding protein	CS1 B CS1 C CS1 F	VF/VR 57
	CS2 C CS2 D CS2 E	VF/VR 56
GDSL lipase	HL1 A HL1 C HL1 G	VF/VR 60
	HL2 B HL2 D HL2 E	VF/VR 61
F-box protein	FB1 STET	VF/VR 54

2.12. Sequencing of silencing constructs and MIDI purification

Plasmids descendent from PureYield Plasmid Miniprep purification were adjusted to a concentration of 100 ng/μL. 10 μL of every plasmid were mixed with 25 μmol of a sequencing primer in forward direction and sent to LGC genomics, Berlin, for sequencing. The results were compared to the original amplified sequence in Geneious and matching clones were used for further duplication in 50 mL LB/Agar/Amp medium out of glycerol stocks. The 50 mL cultures were incubated at 37°C at 170 rpm overnight again and subsequently purified according to the "QIAGEN plasmid midi kit" protocol (Qiagen, Venlo, Netherlands).

2.13. *In vitro* transcription of viral RNAs

Purified plasmids were linearized using MluI and BcuI restriction enzymes (Promega, Madison, WI, USA). 1 μg of every plasmid (α, β, γ) were transcribed into viral mRNA using the "Cellscript amplicap – max t7 high yield message maker kit", following the manufacturer's instructions (Cellscript, Madison, Wyoming, USA).

2.14. Rub-inoculation of plants

Plants were inoculated with the viral solution by rub- inoculation of the fully developed flag leaves. The viral inoculum contains 0,7 μL of every viral α, β and modified γ BSMV mRNA were mixed with 23 μL of FES buffer in the S2 laboratory. In total 25 μL of infectious buffer mixture were applied per adult plant. The infection solution was distributed between thumb and index finger and rubbed onto the flag leaves from tip to base. The inoculation was finalized by moving the fingers from base to tip twice while pressing the leaf firmly producing a squeaking sound.

FES buffer, 1% (store at 4°C)

GP buffer	100 mL
$Na_4P_2O_7 * H_2O$	5g
Bentonite	5g
Celite	5g
ddH_2O	Up to 500 mL

GP buffer (store at room temperature)

Glycine	18,77 g
K_2HPO_4 dibasic	26,13 g
ddH_2O	Up to 500 mL

2.15. Inoculation with *Fusarium graminearum*

Plants were inoculated with *Fusarium graminearum* conidia (10.000/mL) at the stage of anthesis. Two florets on two opposing spikelets at the upper third of the wheat head were inoculated with 10 µL conidia suspension. The heads were moistened with water and wrapped in a plastic bag for 24 hours in order to support fungal spread.

2.16. Scoring for head blight symptoms and RNA sampling of infected spikelets

Scoring for FHB symptoms occurred 2, 4, 6, 8, 10, 12, 14, 18 and 21 days after inoculation (dai). Additional to disease scoring on spikelets, development of fungal spread was also determined on the rachis. Every brownish discoloration on spikelets and rachis was counted as infection and entered into an according scoring list. At 4 dai infected spikelets were cut off and frozen in liquid nitrogen to provide RNA samples for silencing validation. The samples used for RNA extraction were homogenized in 15 mL tubes using a pre-chilled mortar.

2.17. RNA extraction and reverse transcription into cDNA

RNA extractions were performed using the peqGOLD RNAPure™ extraction protocol (Peqlab, Erlangen, Germany). The samples were mixed with 1 mL extraction buffer and incubated at room temperature for 5 minutes. The addition of 0.2 mL chloroform, the subsequent incubation on ice and centrifugation at 5100 rpm for 30 minutes yielded in the formation of three liquid phases. The topmost aqueous phase was transferred to new 1.5 mL tubes and precipitated with isopropanol followed by two washing steps with 1 mL 75% RNAse-free Ethanol and the eluation in 50 µL DEPC water. RNA-concentrations were adjusted to 200 ng/µL prior to the quality check on a 1.5% agarose gel. Obtained high quality RNA was transcribed to cDNA using the RevertAid First Strand cDNA Synthesis Kit protocol (Thermo Fisher Scientific). The cDNA was diluted 1:100 and stored at -21°C.

2.18. Validation of silencing

The plants displaying strong symptoms were tested for the silencing success. For that purpose, PCRs were performed with transcribed plant cDNA using opposing primers. So for plants carrying the TS1 silencing construct, the transcripts were searched for by using the TS2 primer pairs in a 35 cycled PCR. For comparison purposes in unsilenced plants, WT control plants were tested with the same primer pairs. The PCR reactions were checked on an 1,5% agarose gel for band intensity.

2.19. Validation of consistent cDNA concentrations

Plant cDNA was also checked for the presence of GAPDH as constitutively expressed gene. Strong and indifferent gene expression of GADPH in silenced and control plants indicate same cDNA concentration in all samples. Silencing results are therefore not influenced by concentration differences.

Primer Name	Sequence	Temp °C
GAPDH1_F	CCAACCTCCCATCTCCGTCTC	58.3
GAPDH1_R	AATCGGCACCAGCCTCACCC	57.9

2.20. Validation of systemic viral spread

Systemic spread of BSMV was determined by PCR, using primers targeting the BSMV α RNA sequence in plant cDNA. The sequences of this primer pair is given in the table below.

Primer Name	Sequence	Temp °C
BSMV Alpha Fwd	GTACGGCGCAACATCTCCTC	62.5
BSMV Alpha Rev	CACCCGCATTCACCTCACCTG	65.3

3. Results

3.1. VIGS experimental framework

In this VIGS experiment four candidate genes were used, based on RNAseq data derived from *Fusarium* inoculated CM-82036 plants. Namely, these candidate genes encode a terpene synthase, an F-box protein, a calcium binding protein and a GDSL lipase. They are all located at a section called *Fhb1* on the short arm of chromosome 3B, which is strongly associated with FHB resistance. The candidate sequences were searched for on NCBI database to obtain information about other homologous genes and possible off-targets. The following chapters present the results starting with the fundamental information provided by NCBI BLAST followed by the preparation of silencing constructs, the sequencing results, phenotypic disease scoring and the validation of silencing.

3.2. NCBI BLAST results

The NCBI database was searched to confirm sequence information and gene identities. As some genes are not yet to be found on the targeted 3B genome, information from highly similar 3D sequences was adduced (Table 7). The e-value should be very low and provides information about sequence specificity and whether the same sequence is likely to be found in another genome. Short sequences usually deliver high e-values, as they are more likely to be found in other genomes as well. Identity is given by the amount of SNPs along the alignment, another indicator for target specificity. Annotations give a hint about the protein function, and best BLAST hit informs about organisms carrying most similar sequences. Once gene identities were verified, they were carried on to the practical part of the experiment, starting with the preparation of silencing constructs.

Table 7: Data for silencing sequences derived from BLAST database.

	Length (bp)	E value 3B	E value 3D	Ident % 3B	Ident % 3D	Annotation	Best BLAST hit
TS1	128	4E-51	2E-54	96	98	terpene syntase, putative	*Oryza sativa*
TS2	128	3E-42	2E-44	92	93	terpene syntase, putative	*Oryza sativa*
FB1	214	-	6E-97	-	98	f-box, putative	*Brachypodium distachion*
FB2	133	3E-62	4E-57	100	98	eukaryotic translation initiation factor,putative, expressed	*Oryza sativa*
CS1	144	-	2E-35	-	96	sarcoplasmic reticulum histidine-rich calcium-binding protein precursor, putative, expressed	similar to *Brachypodium distachion*
CS2	133	2E-55	8E-54	97	96	sarcoplasmic reticulum histidine-rich calcium-binding protein precursor, putative, expressed	*Brachypodium distachion*
HL1	180	-	4E-68	-	93	SGNH_plant_lipase_like	similar to *Brachypodium distachion*
HL2	123	-	3E-38	-	91	SGNH_plant_lipase_like	similar to *Brachypodium distachion*

3.3. Preparation of the silencing constructs

Primer pairs with the highest specificity according to test PCRs were used for the amplification of the silencing sequences. As a higher amount of the PCR products was needed for successful cloning, 80 µL PCRs were performed and their products were checked on an agarose gel followed by the excision of the according bands.
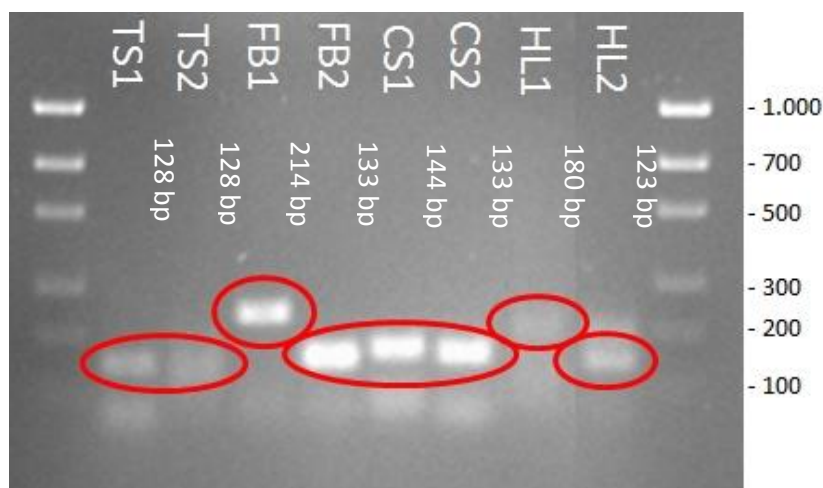
Figure 2: 3 µL of PCR products taken from 80 µL PCRs applied on an agarose gel. The residual volumes of 77 µL per reaction were applied on a separate agarose gel for subsequent excision of correct bands under UV light.

Some bands in Figure 2 are unspecific. In this case, only PCR products matching the calculated length were excised and carried on to the construction of the VIGS vector. The comprised DNA was extracted and purified for further cloning.

Following the restriction digestion by BamHI and Cfr9I (XmaI) restriction endonucleases, the fragments were cloned into the γ-plasmid for subsequent transformation of competent XL-1 *E. coli* strain. The transformed bacteria were plated on LB media supplemented with ampicillin as selective antibiotic. Medium-sized colonies were harvested and propagated in 3 mL of liquid LB media containing ampicillin, until the bacterial density was high enough for STETL-miniprep purification of positive clones. The clones carrying the correct silencing fragment sizes were propagated again in a greater volume in order to perform isolation steps for maximum plasmid purity. Figure 3 shows agarose gels used to confirm the correct inserts prior to sequencing and high-quality isolations.
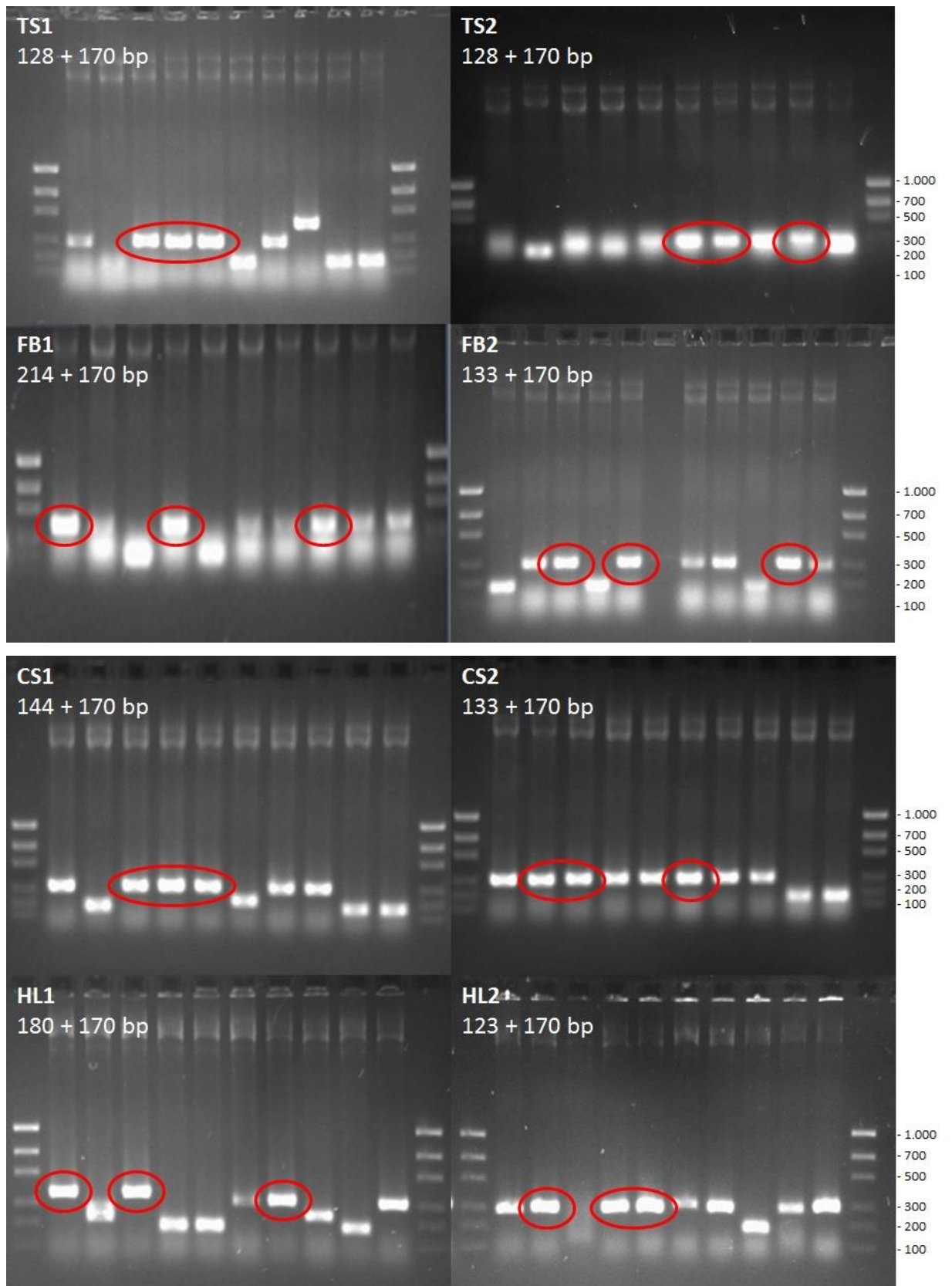
Figure 3: Agarose gels to check for presence of the inserts. PCR was carried out with primers flanking the inserts, which is why 170 bp had to be added to the respective insert size. Encircled bands represent clones carrying plasmids with the correct insert sizes, which were transferred to further verification steps.

Most transformants displayed correct fragment sizes. Nonetheless some clones seemed to carry only a part of the target sequence. This would indicate secondary restriction sites within the fragments, but they were not detectable with the bioinformatics software. One clone of TS1 even seemed to carry a longer fragment than all the others, which could possibly be derived from inverted double-ligation. The selection of clones for further experimental steps was based on their insert sizes and the band intensities. In case of multiple positive clones, those with the strongest band intensity were selected for further confirmation steps.

Additionally to PCR insert confirmation, isolated plasmids from selected clones have also undergone double digestion with BamHI and Cfr9I (XmaI) restriction enzymes for further insert verification. The results of these reactions are presented in Figure 4.
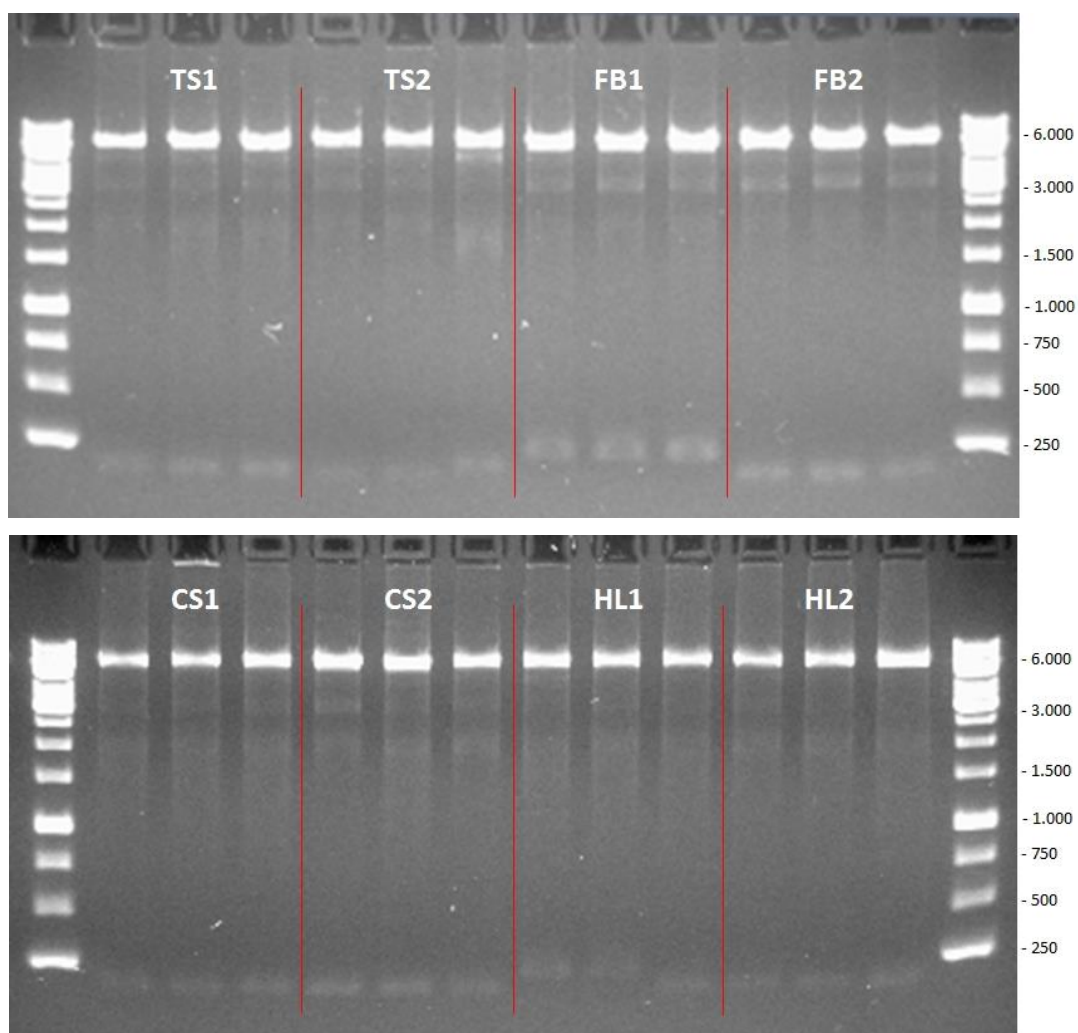
Figure 4: Agarose gels after BamHI and Cfr9I (XmaI) double digest of STETL purified plasmids. The strong bands at 5.500 bp represent the plasmid backbone, the weak bands below 250 bp represent the transferred plant gene fragment.

As BamHI and Cfr9I restriction sites on the BSMV γ plasmid are flanking the cloned insert left and right, the restriction reaction with those enzymes cut out the insert again. Gel electrophoresis separated the plasmid backbone and the insert, giving information about insert size and therefore successful cloning of the correct gene fragment. Figure 4 clearly shows the successful ligation of the inserts into the plasmids. The plasmid backbones are represented by the strong bands at the top of the gel. The inserts can be found at the bottom as weak bands. The difference in band intensity was caused by the higher amount of plasmid DNA compared to insert DNA, as the plasmid was much longer than the insert.

Almost every construct harboured the correct insert except for HL1 and TS2 transformants seemed to carry differential fragments. For further validation of correct fragment ligation the clones in Figure 4 were propagated again, followed by high quality isolation according to the "Promega PureYield Plasmid Miniprep System" protocol for subsequent sequencing and construct validation. Sequencing results can be found in chapter 4.4.

As soon as the correct insert was confirmed, one candidate per construct was isolated following the "Qiagen Plasmid Midi Kit" protocol for maximum purity, and then linearized prior to mRNA transcription.

The linearization of silencing- and wild-type γ constructs was performed using MluI, and α- and β-plasmids were linearized by SpeI restriction endonucleases, following the according protocol. All linearized constructs are shown in Figure 5 below.
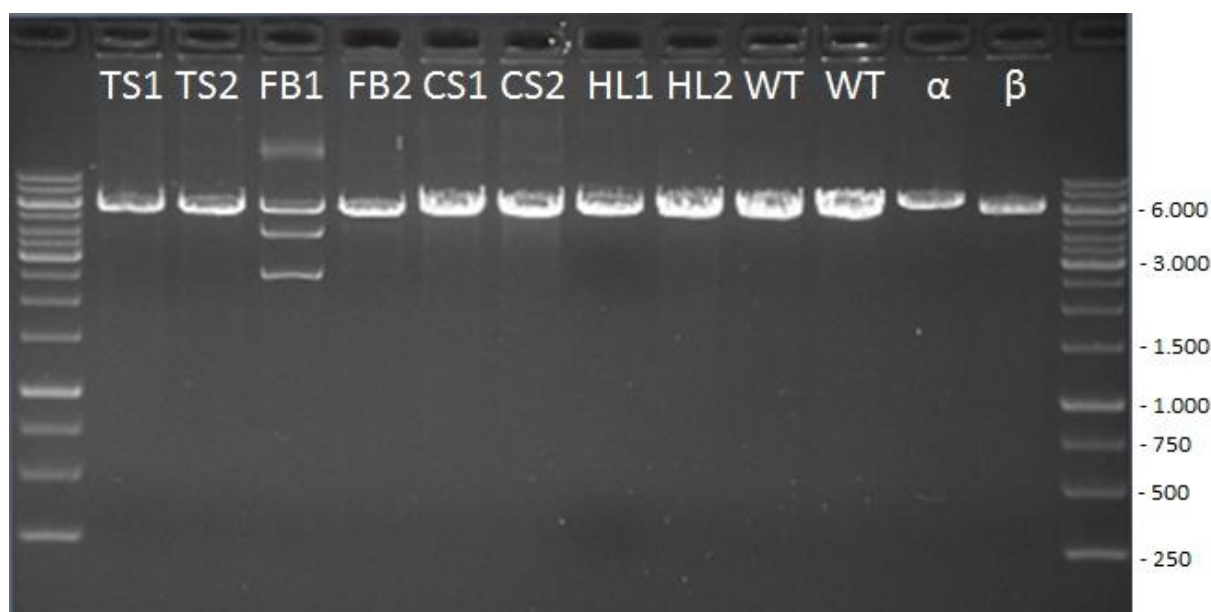
Figure 5: Linearized constructs after digestion with MluI (transformed and WT-γ) and SpeI (α, β) restriction enzymes.

While the vast majority of silencing constructs showed the expected length, FB1 displayed multiple unexpected bands. This indicated either only partial digestion or the restriction enzyme didn't bind at all, which would have left the plasmid undigested. In order to investigate the source of this error, the selected FB1 clone was propagated again from glycerol stocks. As the bacterial colonies all derived from the positive clone, only one colony was transferred to liquid medium and isolated again following the STETL miniprep protocol.

The STETL and Qiagen MIDI-kit purified FB1 plasmids were double-digested again with BamHI and Cfr9I enzymes for insert verification (Figure 6).
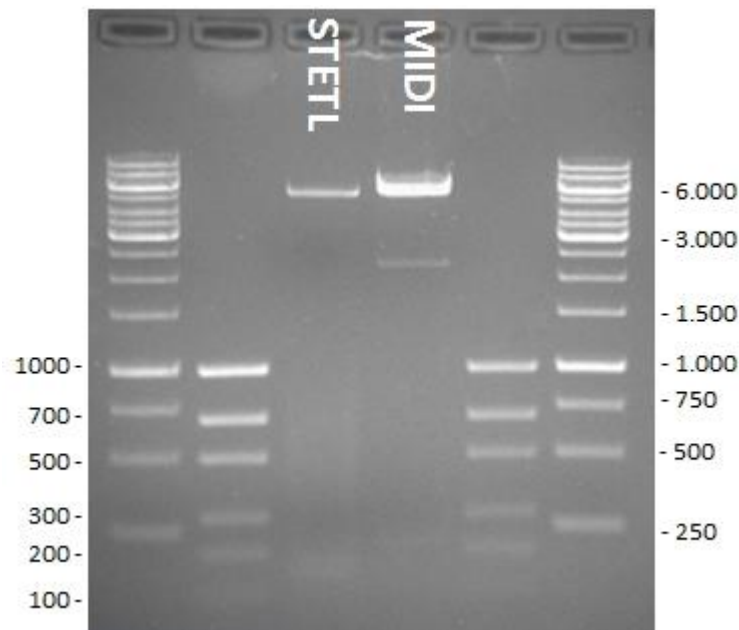
Figure 6: Restriction control of STETL and MIDI-purified plasmids. STETL plasmids display either a smaller fragment at 100-200 bp or an entire loss of the gene fragment, while MIDI-preps display one correct band at 200 bp and an incorrect one at ~2.500 bp.

This figure shows that plasmids isolated with the STETL-miniprep protocol carried either the wrong insert (very weak band between 100 and 200 bp) or have lost the insert entirely, whereas plasmids isolated with the high quality Qiagen MIDI-kit showed the correct band at 200 bp but also displayed a second fragment between 2.000 and 2.500 basepairs in length. The only possible explanation would be the presence of secondary binding sites within the plasmid backbone, which could not be confirmed after bioinformatics research and regarding the fact, that all other constructs were also incorporated in the very same γ-plasmid and represented correct linearization products this seemed unlikely.

For further investigation, the STETL-purified FB1 construct was transformed again into *E. coli*, following the exact same procedure like before, prior to linearization. Interestingly, the control

gel after double digestion with BamHI and Cfr9I (Figure 7) showed plasmids with correctly embedded inserts as well as false ones similar to constructs derived from MIDI-kit isolation in Figure 6.
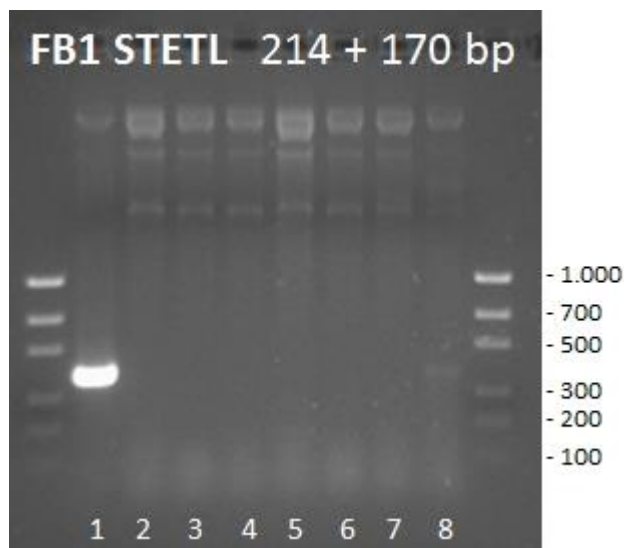
Figure 7: Repeated purification of FB1 silencing constructs with subsequent double digestion. Lane 1 contains the correct insert size and is used for sequencing along with the FB1 construct derived from MIDI preps. Lanes 2-8 display wrong fragment sizes.

The only positive candidate (carrying the correct insert) on lane 1 was sequenced along with the FB1 plasmid derived from MIDI-kit purification. The STETL-purified plasmid didn't show sufficient homology, while the insert of the MIDI-purified FB1 construct was 100% identical to the original sequence (see Chapter 3.2; Sequencing results). After discussing whether this construct should be excluded from further experimental steps, it was included in the infection trials nonetheless. So every construct visible in Figure 5 was transcribed into mRNA afterwards.

The transcription of linearized plasmids into viral mRNA was carried out after sequence confirmation. Prior to the preparation of the viral inoculum for wheat infection, the transcripts were checked on a gel for mRNA quality determination (Figure 8).
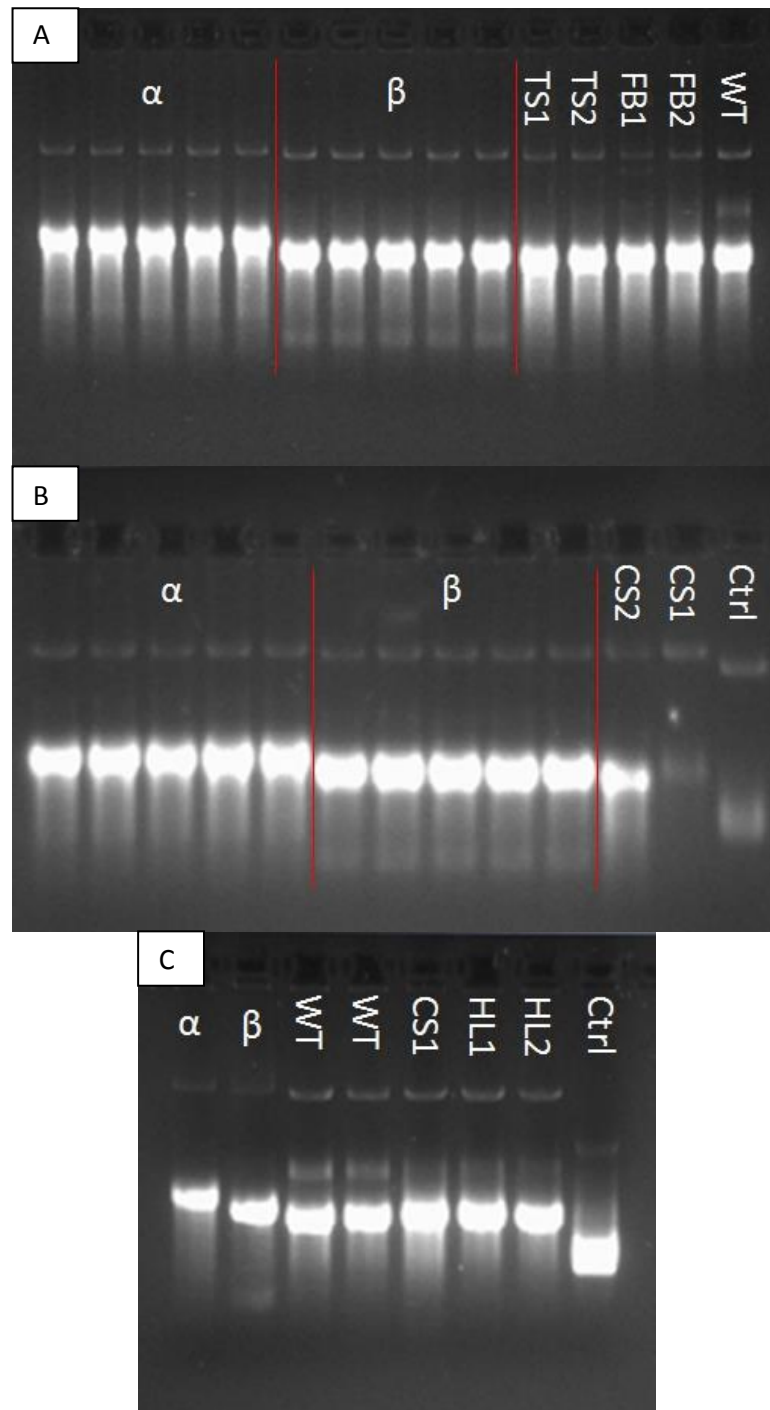
Figure 8: mRNA quality check on agarose gels. Strong bands indicate high mRNA quality, weak bands at the topside of the gels represent the residual plasmid backbone. Pictures A and B display sufficient mRNA yields of all transcripts except for CS1, which was repeatedly and successfully transcribed in sufficient concentration along with HL1 and HL2 constructs (C).

The figure above represents the check of transcribed viral mRNA on an agarose gel. Due to sample number limitations, transcriptions were performed in three steps (A, B and C). Weak bands at the topside of the gels represented plasmid backbone DNA. Strong bands indicated

good mRNA quantity and quality. All mRNA transcriptions were successful, except for CS1, which was transcribed repeatedly along with WT and HL constructs.

3.4. Sequencing results

Three plasmids of every silencing candidate were sequenced in order to identify accurate constructs and therefore raise silencing efficiency. The following pictures show chromatograms aligned with the template exon sequence originated from sequencing results of every silencing candidate. Only totally identical clones should be transcribed into viral mRNA. If there was no 100% sequence identity, candidates with highest sequence homology were also used for mRNA transcription. The best sequencing results are displayed in Figures 9 and 10 below.
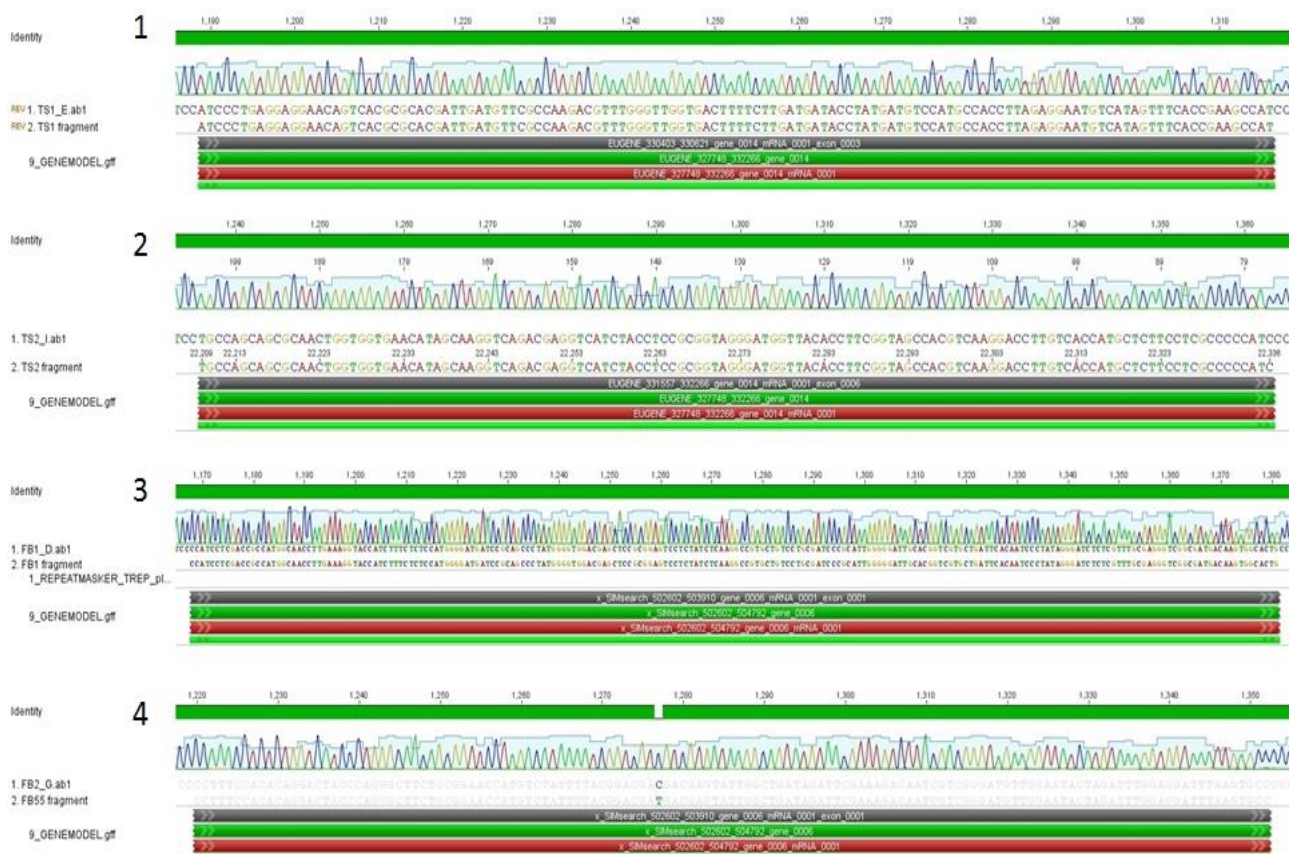
Figure 9: Alignments of chosen silencing candidates and template exon sequence. While the chosen constructs in alignment 1, 2 and 3 show 100% homology, alignment 4 harbors one SNP in the sequence central region.
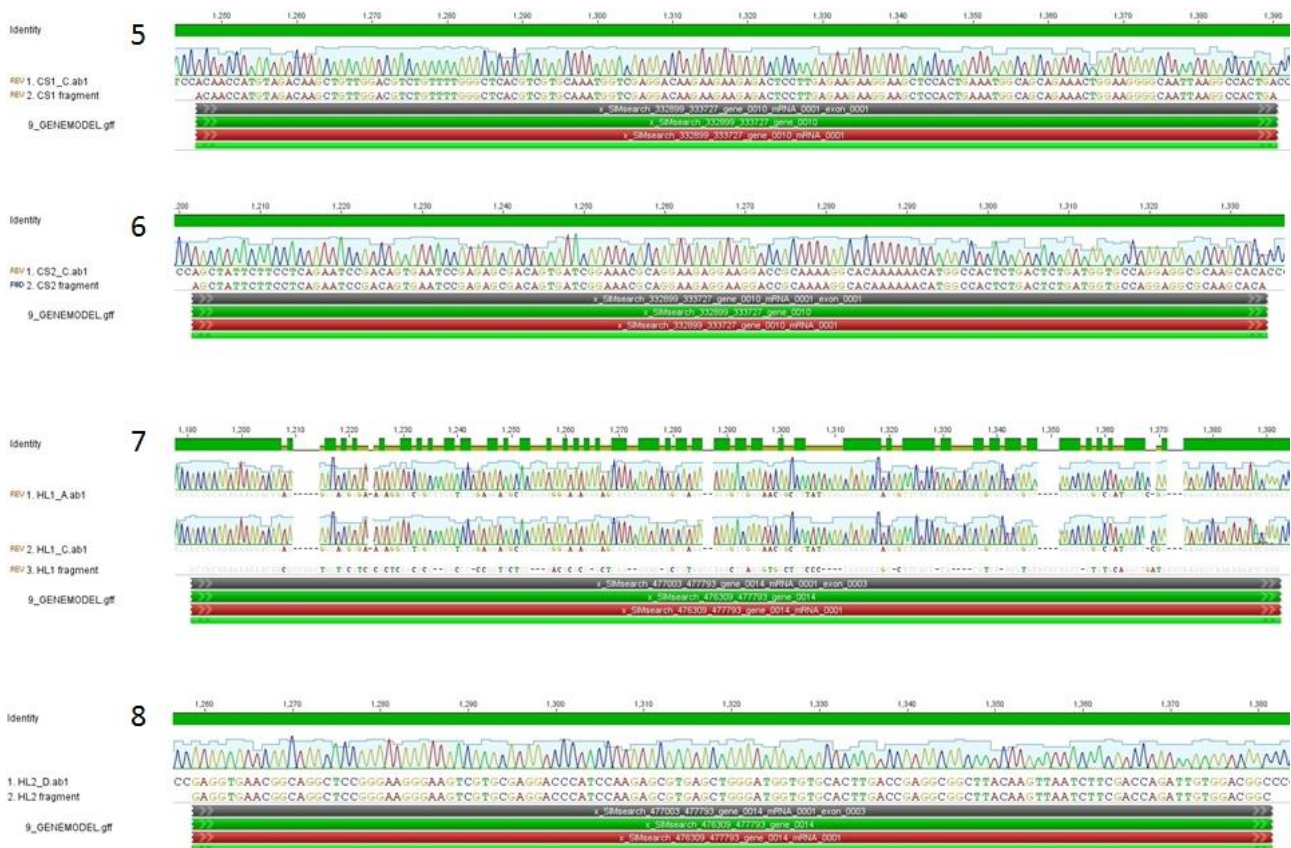
Figure 10: Alignments of chosen silencing candidates and template exon sequence. While the chosen constructs in alignment 5, 6 and 8 show 100% identity, candidate 7 displays gaps compared to its template exon. The fact that both plasmids carry the exact same insert indicates amplification of a homeologous sequence during the isolation of the candidate gene fragment due to insufficient primer specificity.

Chromatogram quality in sequencing results above was very high, which can be read out of single high peaks on every sequenced base. Low quality would be displayed by low peaks for more than one base at a certain position along the sequence.

Except for the constructs FB2 (alignment 4) and HL1 (alignment 7) the transformants showed 100% sequence identity to the template exons of the candidate genes. FB2 displayed one polymorphism in the central region of its sequence. This construct showed a C/T SNP, the other possible candidate harboured an A/G SNP a few bases away, which would not affect the silencing outcome, as siRNA formation occurs randomly along the silencing sequence. HL1 constructs were 100% identical to each other but an alignment to their template sequence of the target gene showed a lot of gaps and SNPs, which indicated the amplification of a homeologous off-target gene fragment caused by insufficient primer specificity. Nonetheless the candidate was tested in the VIGS experiment out of curiosity regarding gene function analysis of its unknown gene.

As already mentioned in the previous chapter, plasmids derived from low quality (STETL) and high quality (MIDI) isolations of construct FB1 were sequenced due to controversial outcome during linearization. The sequencing data are shown in Figure 11.

Figure 11: Sequencing results derived from MIDI (9) and STETL (10) purification after controversial results during plasmid linearization. While sequence 9 shows high sequencing quality and 100% identity to the target gene fragment, sequence 10 displays a sequence with very low quality and confidence.

It is clearly visible that MIDI-kit purified plasmids harboured an insert with 100% identity to the target gene fragment, while the STETL-kit purified plasmids showed no homology to the template exon and additionally displayed very poor sequence confidence. The reason for this could be simply the loss of the insert, as it was already assumed in Chapter 4.3.

3.5. Phenotypic disease scoring

Prior to viral infection, an infestation by mildew was noticed on plants during shoot growth and was treated accordingly by fungicides. A second infestation by mildew occurred during FHB disease scoring and was treated by down-regulation of humidity to 40%. Thereby plants were prevented from eventual physiological effects caused by fungicide treatment.

Once the flag leaf was fully developed, resistant CM-82036 plants were infected with the viral inoculum. 25 µL of infection solution were applied on fully developed flag leaves by rub-inoculation. Every viral construct, including the WT virus, was tested on 30 resistant plants.

Point inoculations with *Fusarium graminearum* macroconidia were carried out 9-10 days after viral infection on four florets of two opposite spikelets. This time span should allow systemic spread of the transformed virus within the plant. Figure 12 shows a wheat leaf not inoculated

with viral inoculum displaying clear symptoms of BSMV infection recognizable by yellow dots. This was accounted for phenotypic evaluation of systemic viral spread.

Figure 12: Non-inoculated leaf displaying yellow dots indicating systemic viral spread.

Visual scoring for FHB symptoms on spikelets and rachis started from 2 dai until 14 dai with a 2-day interval followed by two terminal scoring time points at 18 and 21 dai. Every spikelet showing brownish discolorations was counted as successfully infested with FHB.

Regarding rachis scoring, an initial infection was counted 0,5, referring to the discoloration of the transition between spikelet and rachis. Once the fungal spread has progressed and overcome the distance between one spikelet to another, the symptom was quantified as one infested rachis section. Additional infested rachis tissue was added up.

RNA samples were taken from inoculated spikelets at 4 dai for subsequent silencing validation (Chapter 4.6). Data from spikelet and rachis scoring were collected and entered in an excel sheet resulting in heat maps (Appendix, Chapter 7.3) and diagrams showing disease

development over the scoring intervals. Due to limited space, the infection trials were performed in parallel in two different climate chambers with identical environmental conditions.

Figure 13: resistant CM (A) and susceptible NIL (B) cultivars after FHB infection. While resistant plants only show initial infection symptoms on inoculated spikelets (negative control), susceptible plants display maximal symptoms along the whole wheat head. Rachis infection already affects the stem.

Each trial set included positive and negative control plants to compare disease spread between susceptible (NIL) and resistant (WT) cultivars. Figure 13 shows *Fusarium* inoculated wheat heads of highly resistant (A) and non-resistant (B) cultivars. Resistant plants infected with the WT virus were expected to develop only minor disease symptoms, as they harbour the resistant *Fhb1* locus and the unmodified virus doesn't interfere with responsive resistance gene expression. Highly susceptible NILs should display a maximum of disease severity due to their susceptible *Fhb1* allele. As already mentioned in the introduction (Chapter 1.4), resistance against FHB is a quantitative trait and therefore influenced by more than one gene with different contribution. Since silencing of one up-regulated gene cannot undo the effect of other resistance genes, symptom intensities on plants carrying the modified virus should lie between those of WT and NIL phenotypes.

The results derived from the phenotypic scoring on silenced and control plants are represented in the figures below, illustrated as the percentage of plants with the same amount of infected tissue. All plants were randomized in order to inhibit errors derived from differential environmental conditions due to wind and light exposure within the climate chambers (Appendix, Chapter 7.1).

TS, FB and WT2 trials were carried out in climate chamber 1, while CS, HL and WT1 plants were treated and scored in climate chamber 3. Progress of spikelet infection is given on the left and progress of rachis infection is given on the right side.
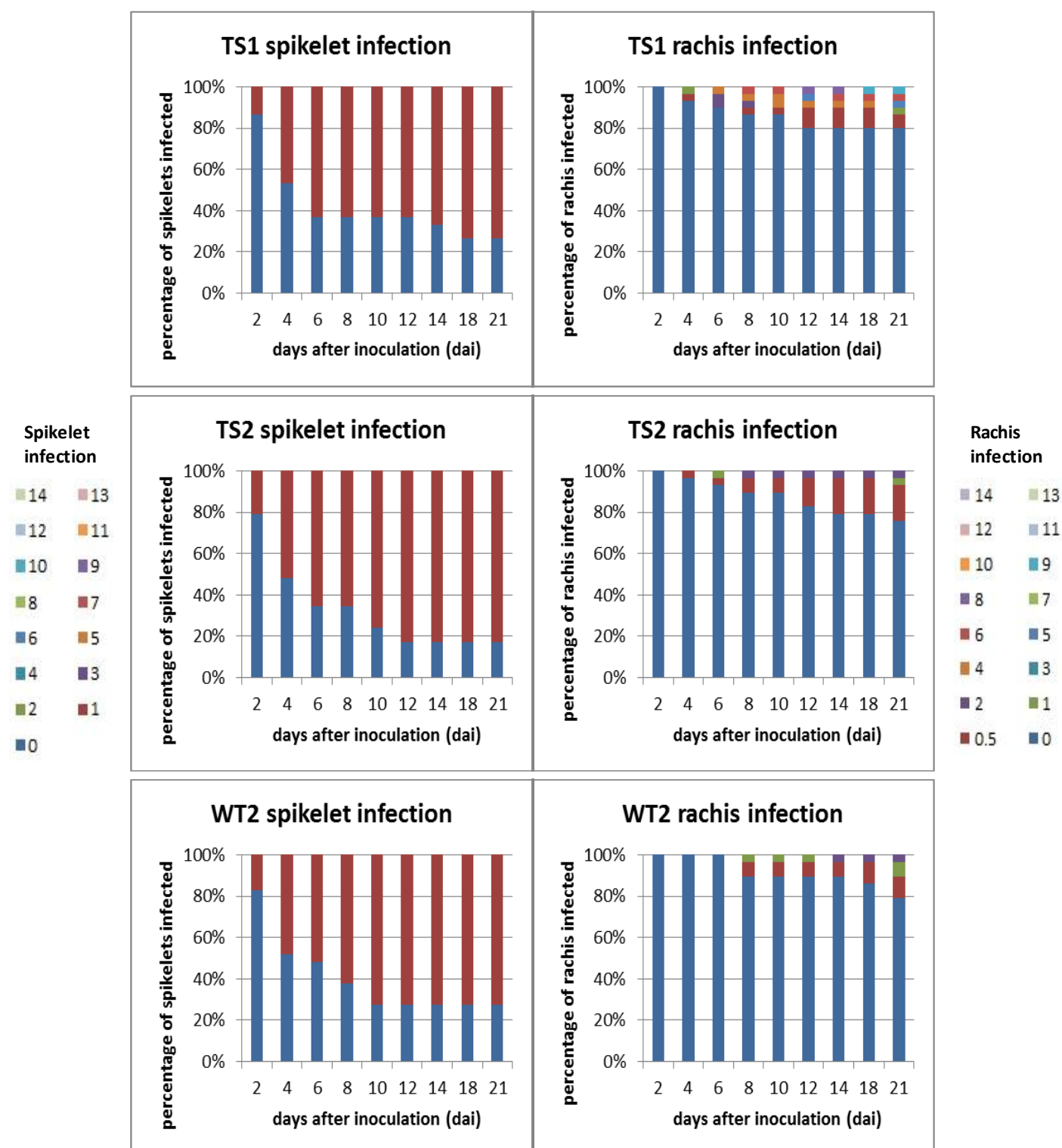
Figure 14: relative FHB infection patterns on spikelets (left) and rachis (right) on TS and WT plants. Each plot represents the percentage of 30 plants displaying symptoms on spikelets or rachis. The blue bars indicate no infection of the respective tissue, brown bars indicate initial infection. The interpretation keys for each tissue are given on the left and right side of the figure.

Figure 14 gives the percentage of tissue (either spikelets or rachis) infected by FHB. It describes disease development on spikelets (left) and rachis (right) of plants carrying the TS silencing construct compared with the tissue of WT control plants. It is clearly visible, that disease development on spikelets as well as on rachis was very weak.

Spikelet infection didn't exceed 1 infected spikelet on all plants. Additionally to slow disease development over time, approximately 20-30% of silenced plants failed to develop any disease symptoms even 21 dai. The expectation for silenced as well as for WT plants would be an infection of the two inoculated spikelets at least.

Rachis infection also developed similarly on TS and WT2 plants. About 80% of all plants didn't display any FHB symptoms along the rachis 21 dai. The remaining 20% did develop FHB symptoms along the rachis, but only single plants were affected by severe infection.

Based on these phenotypic data, terpene synthase could either be excluded from the set of genes most interesting for FHB resistance breeding, or a more effective TS construct is needed for gene silencing.
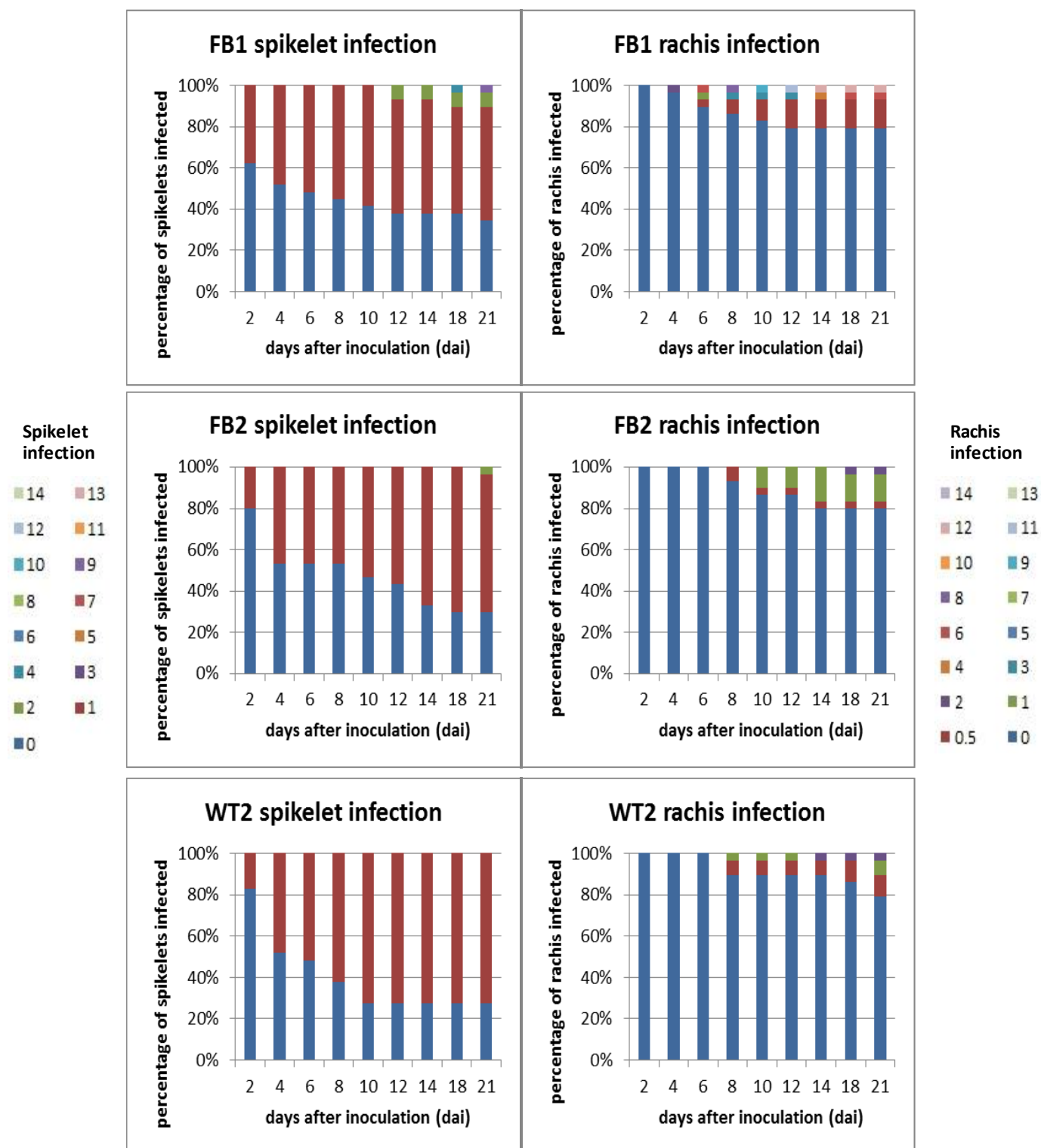
Figure 15: relative FHB infection patterns on spikelets (left) and rachis (right) on FB and WT plants. Each plot represents the percentage of 30 plants displaying symptoms on spikelets or rachis. The blue bars indicate no infection of the respective tissue, brown bars indicate initial infection. The interpretation keys for each tissue are given on the left and right side of the figure.

This figure gives the infection pattern of FB silenced plants compared to WT control plants.

Regarding spikelet infection, FB1 plants developed symptoms very soon. At 2 dai about 40% of

all FB1 plants already showed one infected spikelet. At 12 dai single plants started to display

FHB symptoms at 2, and later on 4 and 9 spikelets. However, 1-2 plants out of 30 could not be accounted as proof for increased susceptibility, as their amount reached only about 5% of all FB1 plants.

FB2 plants seemed to develop FHB symptoms slower than plants carrying the FB1 construct. At 2 dai only about 20% of the test group displayed one infected spikelet. This percentage rose to 70% over time until 21 dai, when single plants developed symptoms on a second spikelet as well. Despite slight differences in infection speed and intensity, FB plants did not display fundamental differences in spikelet infection compared to WT2 plants.

Fungal spread on rachis also developed quicker on FB1 than on FB2 and WT plants. On single plants the fungus nearly reached the stem with several infested rachis sections, but as this only affected 1-2 plants again, increased FHB severity could not be determined.

In conclusion, silencing of the F-box protein didn't affect fungal spread within wheat heads.
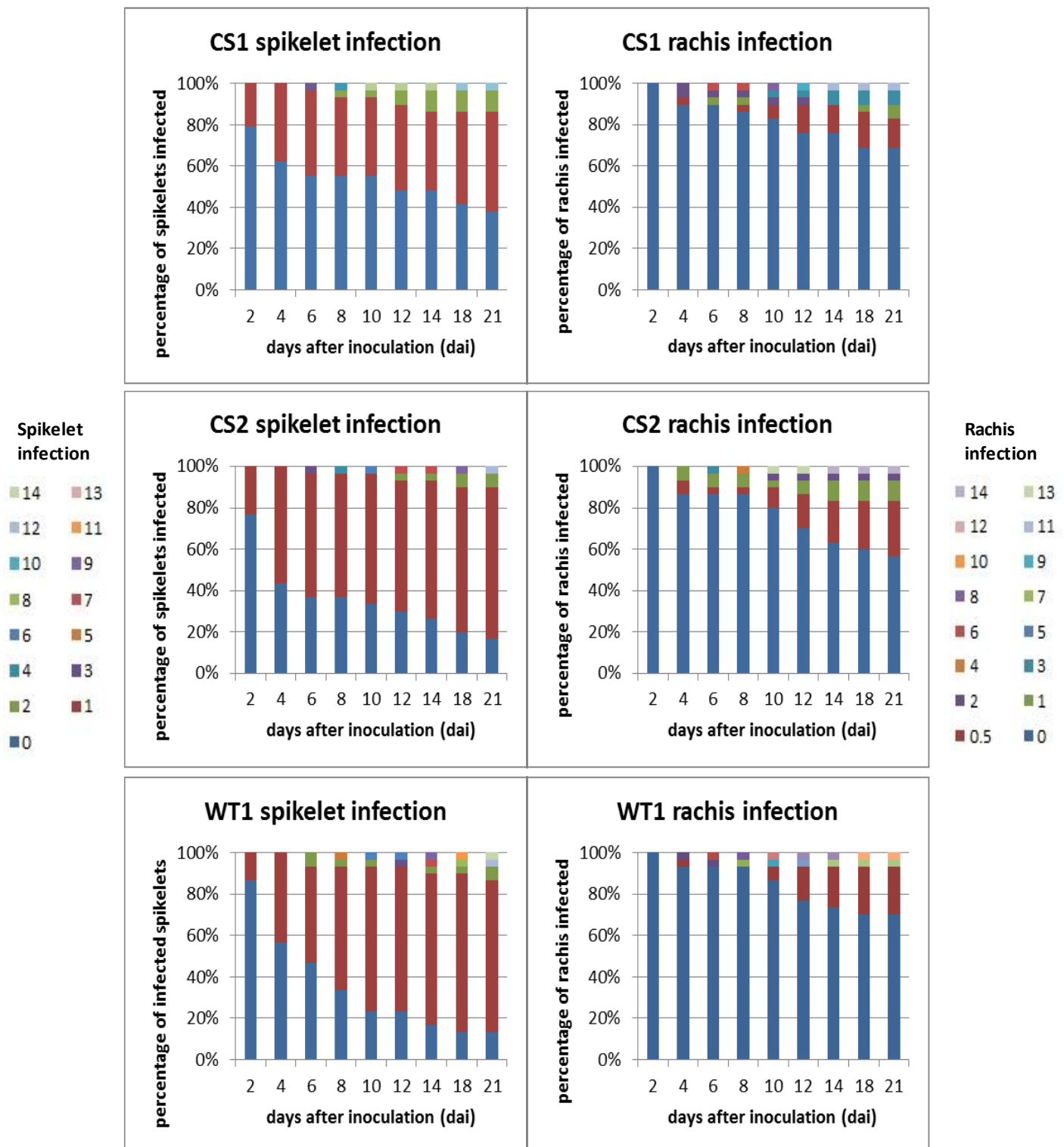
Figure 16: relative FHB infection patterns on spikelets (left) and rachis (right) on CS and WT plants. Each plot represents the percentage of 30 plants displaying symptoms on spikelets or rachis. The blue bars indicate no infection of the respective tissue, brown bars indicate initial infection. The interpretation keys for each tissue are given on the left and right side of the figure.

Here (Figure 16) the infection patterns of spikelets and rachis on CS and WT plants are illustrated. As visible, infection of CS1 spikelets occurred slowly, as nearly 40% of the plants showed no FHB symptoms at all 21 dai and only 15% displayed 2 or more infected spikelets.

42

Plants harbouring the CS2 silencing construct displayed a more rapid progress of FHB on spikelets, but still the results didn't differ from WT1 infection patterns. Even the high number of spikelets infected on single CS plants was outreached by WT1 controls.

The same picture can be taken from rachis infection. Plants started to display symptoms already at 2 dai, but the progress of FHB along the rachis was slow. About 60-70% of all inoculated plants didn't show any symptoms of FHB infection along the rachis at 21 dai. Only few CS plants were infested heavily, but they were also exceeded by FHB infestation on WT rachis.

Silencing of the calcium binding protein therefore seemed to have no effect on FHB susceptibility.
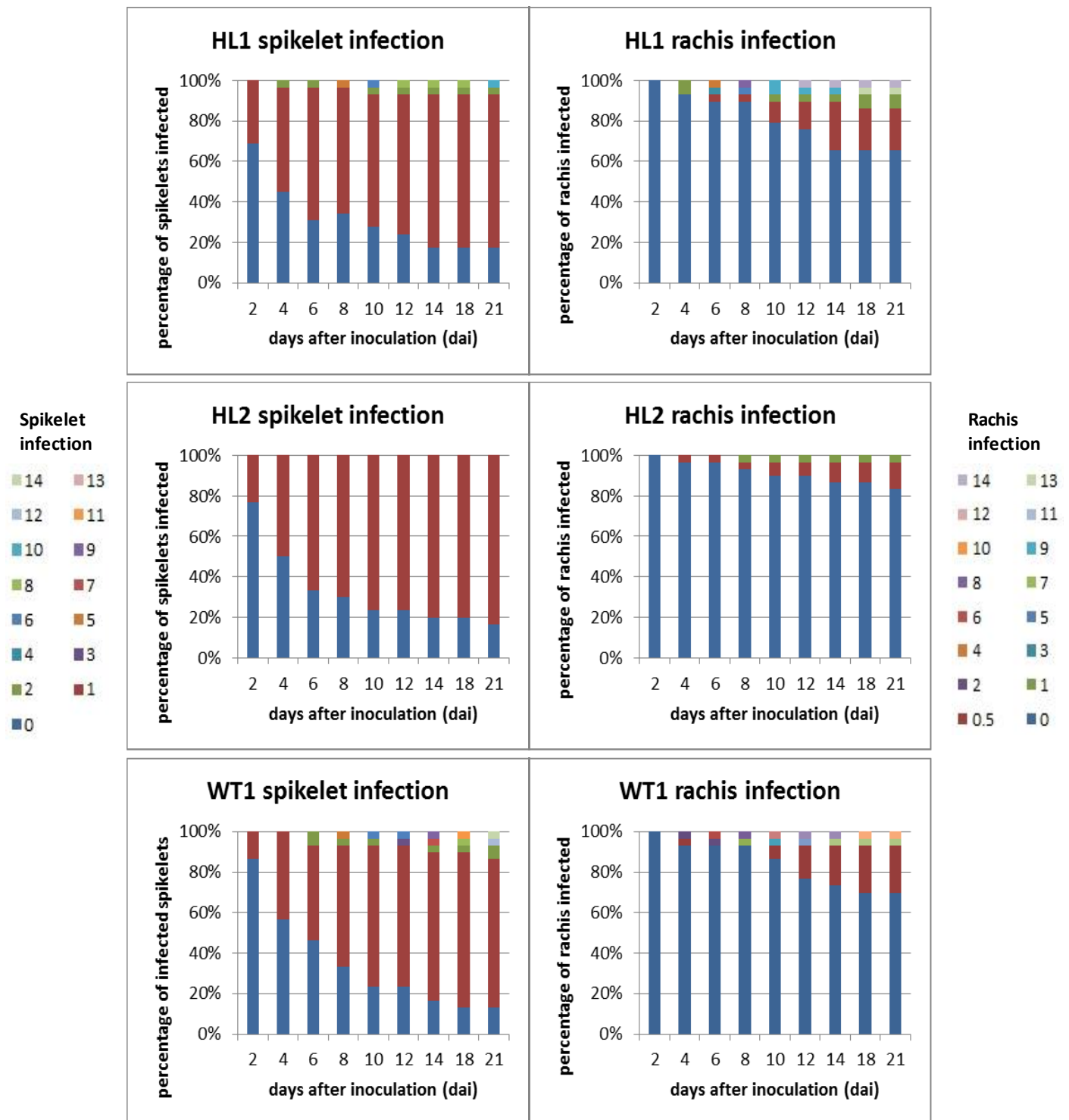
Figure 17: relative FHB infection patterns on spikelets (left) and rachis (right) on HL and WT plants. Each plot represents the percentage of 30 plants displaying symptoms on spikelets or rachis. The blue bars indicate no infection of the respective tissue, brown bars indicate initial infection. The interpretation keys for each tissue are given on the left and right side of the figure.

Figure 17 gives the infection patterns for silenced HL plants compared to the wild type control (WT). Spikelet infection on HL1 started with 30% showing one infected spikelet at 2 dai. At 21 dai over 80% developed FHB symptoms at least at one inoculated spikelet. Only single individuals exceeded this initial infection stage.

HL2 spikelet infection started with about 25% of wheat heads showing FHB symptoms on one spikelet and reached about 85% at 21 dai.

Rachis scoring delivered a very similar pattern. Fungal spread on rachis occurred very slowly and didn't overcome the stage of initial infection on most HL individuals.

WT individuals exceed HL infection in the development of spikelet and rachis symptoms. Comparison of HL and WT infection patterns therefore lead to the conclusion, that silencing of the HL gene did not affect FHB resistance reactions.

Relying on the collected phenotypic data, the test plants carrying the silencing constructs displayed no increased susceptibility after *Fusarium* inoculation. Comparison of silenced and resistant WT control plant infection patterns displayed very high similarity in spikelet and rachis infection. In single cases, FHB symptoms on WT plants even exceed those on silenced plants.

Figure 18 below gives the FHB infection patterns on spikelets and rachis of susceptible NIL plants only inoculated with *Fusarium graminearum* conidia. They were kept as control plants in both climate chambers and exposed to the same environmental conditions. It is clearly visible that infection and spread on both tissues occurred very rapidly and very severely. Not only almost all plants developed symptoms for initial FHB infection, but also even 50-70% displayed multiple infected spikelets and severe rachis infection.
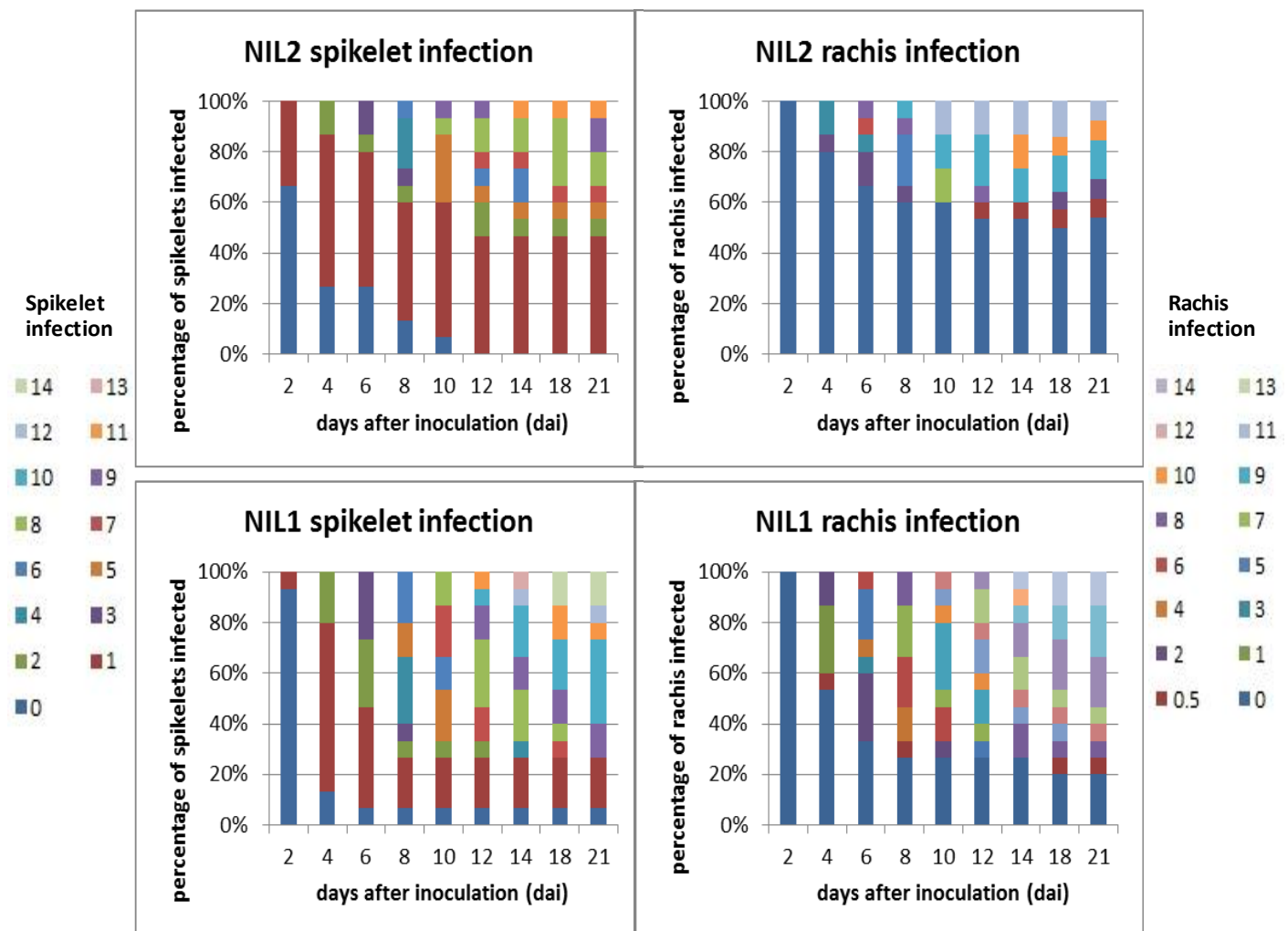
Figure 18: FHB infection patterns on spikelets and rachis of susceptible NIL plants lacking the resistant *Fhb1* allele. NIL2 plants were exposed to the same conditions as TS, FB and WT2 plants, while NIL1 were kept with CS, HL and WT1 plants. NILs were not treated with any viral construct and only inoculated with *Fusarium* conidia. Most of them showed maximum symptom intensity.

As already mentioned above, NILs don't harbour the resistant *Fhb1* allele and are therefore susceptible to FHB infection. Successful silencing of a *Fusarium* responsive gene in CM plants carrying the resistant *Fhb1* allele should produce a phenotype similar to susceptible NILs. Instead, silenced plants displayed similarly high resistance to FHB like their negative (WT) controls. So either none of the candidate genes is involved in resistance reactions, controversial to their up-regulated state during FHB infection, or the VIGS experiment failed, which would have left the plant transcripts totally unaffected by the silencing constructs. The determination of this case is described in the next chapter.

3.6. Validation of silencing

Successful silencing needed to be verified in order to substantiate the data derived from phenotypic scoring. Silenced and WT control plants were tested by performing PCR and subsequent check on agarose gels. Strong bands in Figure 19 indicate strong presence of the candidate genes, weak bands do the opposite.
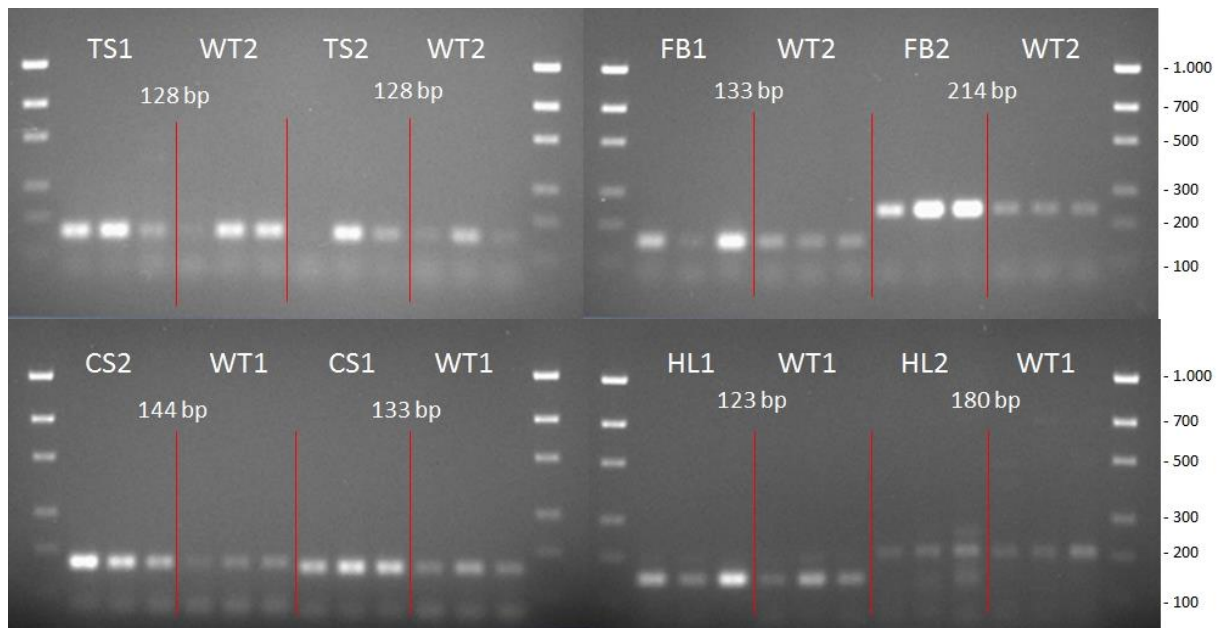
Figure 19: Validation of silencing for each construct including wild type control plants (WT). Transcripts were verified using opposing primers in silenced plants and their WT control. Strong bands indicate high transcript abundance, weak bands indicate low abundance of candidate gene transcripts.

Figure 19 above shows the validation of gene silencing by PCR with opposing primers for every transcript. The silencing of terpene synthase for example was verified using TS2 primers for TS1 silenced plants and vice versa. Using TS1 primers for TS1 silenced plants would only have confirmed strong presence of the virus, as a high amount of viral replicates carrying this insert could be expected. The verification was performed the same way for each construct in silenced and WT control plants.

The bands for the according gene were strong in silenced plants, similar to those in WT controls. This indicated still high transcript abundance, although a silencing of those expressed genes was expected. The conclusion is the failure of the VIGS experiment, as the successful silencing of candidate genes could not be assumed. Nevertheless the strong difference in transcript abundance even between individuals treated with the same construct seemed unusual and induced another control PCR targeting the constitutively expressed GAPDH gene,

which should not be influenced by silencing. The purpose of this was the screening for possible cDNA concentration differences between tested individuals due to different yields from RNA extraction (Figure 20).
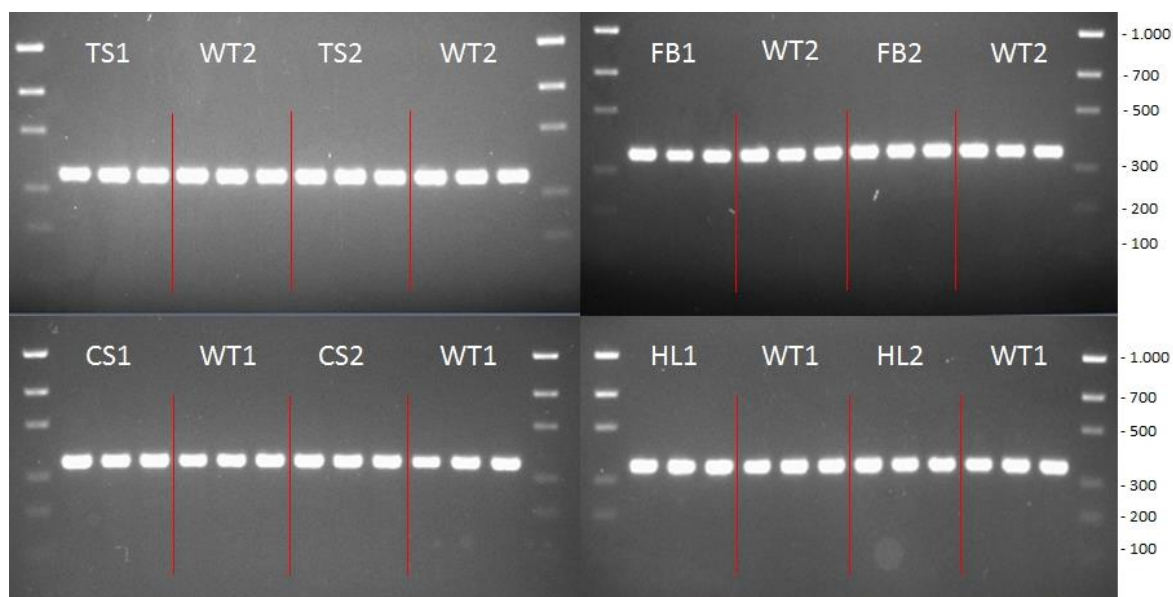
Figure 20: Check for the constitutive expression of GAPDH in silenced and WT control plants. The consistent band strength indicates the same cDNA concentration of all samples.

The GAPDH control gel showed the same band intensity in silenced plants and their WT controls. Therefore differences in cDNA concentration were not the reason for discrepancies in silencing validation. Incomplete systemic spread of BSMV could have been another cause for these differential silencing results.

To prove systemic viral spread within inoculated plants, another PCR targeting the BSMV α genome was performed with according primers and also checked on an agarose gel (Figure 21).
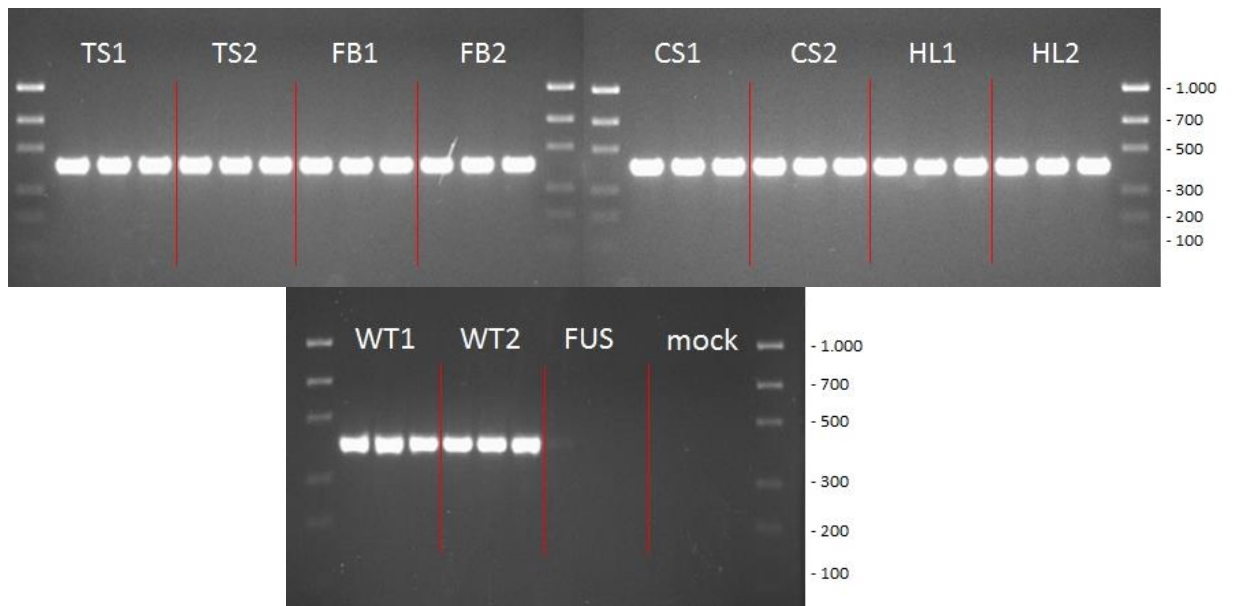
Figure 21: Check for systemic spread of the virus by PCR. All plants infected with the virus show clear strong bands at the expected length of ~380 bp. FUS and mock inoculated plant cDNA was taken from another experiment and was not infected with BSMV. This proves the systemic spread and the presence of BSMV at 4 dai.

The gels showed clear strong bands in consistent distribution for the BSMV α fragment in every plant treated with the virus, indicating systemic spread. For comparison, a set of virus-free plant cDNA from *Fusarium*- (FUS) and mock-inoculation trials was used to prove the absence of BSMV α in non-virus inoculated plants.

Given the fact, that there was no difference in cDNA concentration or limited viral spread, the failure of this VIGS experiment seemed clear.

4. Discussion

The aim of this master thesis was to post-transcriptionally silence four possible FHB resistance genes to determine their influence on resistance reactions. These genes, encoding a terpene synthase, F-box protein, Ca-binding protein and a GDSL lipase, are located on the resistance locus *Fhb1* and were up-regulated in resistant plants, compared to a susceptible cultivar. While three candidate genes were constitutively expressed, the gene encoding a GDSL lipase was differentially up-regulated after inoculation of spikelets with *Fusarium graminearum* conidia suspension. The applied method was VIGS, which transiently silences genes in plants by triggering post transcriptional gene silencing of plant transcripts. VIGS is a versatile tool for functional genomics, but is also used for molecular breeding and crop improvement (Senthil-Kumar & Mysore 2011).

The experiment was conducted with highly resistant plants (CM-82036) harbouring both resistance alleles (*Qfhs.ifa-5A, Fhb1*) and plants derived from a line near isogenic to CM-82036 (NIL34), which harbour the susceptible allele of *Fhb1*. Silencing constructs were tested on 30 CM-82036 plants each. As every candidate gene was tested with two constructs, we had to allocate the plants in two climate chambers with the same temperature and humidity regime due to limited space. 30 CM-82036 plants infected with the unmodified virus (WT) and 15 non-virus infected NIL34 plants were carried along as negative and positive controls in each climate chamber. After *Fusarium* inoculations we expected silenced plants to develop a phenotype similar to highly susceptible NIL34 plants. Scoring for FHB symptoms was performed on spikelet and rachis tissue every second day starting 2 dai until 14 dai, followed by two terminal scoring dates on 18 and 21 dai. One inoculated spikelet per plant was taken as RNA sample 4 dai for validation of silencing. The phenotyping results looked very similar in all silenced plants. Most of them only developed slight symptoms for FHB infection not comparable to severe symptoms in positive controls. Of course there were outliers, but as their phenotype only reflected a small minority they didn't give proof for increased susceptibility. In fact their phenotype was not different to unsilenced resistant plants, controversial to the expected outcome.

Silencing validation was carried out with RNA-derived cDNA of three randomly selected plants per silencing construct and showed contrasting results. We did not observe silencing in any of the tested plants. Some silenced plants even displayed higher transcript abundance than their negative controls. To confirm equal cDNA concentrations we checked every individual for constitutive GAPDH expression as photometric analysis was not feasible due to enzymatic

residues caused by cDNA transcription. Additionally the individuals were checked for BSMV presence in the wheat ears.

Consistent cDNA concentrations and systemic BSMV spread showed that in this experiment no silencing was achieved with VIGS. Therefore the tested candidate genes cannot be excluded from the list of potential resistance contributors. Possible reasons for the failure and alternative approaches are outlined in the following chapters.

4.1. Silencing validation is limited by primer specificity

For every target gene we designed two constructs on different exons or ORF segments. This was done to obtain construct stability, as this is influenced by the sequence itself (Chapter 5.2). Validation of silencing was performed using PCR with primers for fragment isolation. Of course primers designed for construct 1 would in this case bind to the target transcript as well as to the virus carrying the silencing sequence. As this would only confirm viral presence in the respective tissue, we used construct 2 primers to validate silencing by construct 1. The sequence incorporated in the virus cannot be amplified and therefore this method should specifically hit the target transcript. Unfortunately this method didn't meet the expectations, as there is obviously no sign of consistent silencing in our plant material, at least when using the chosen primers. It could be possible though, that primer specificity is too low and another homoeologous sequence of same fragment size is amplified.

4.2. Silencing is influenced by sequence length and the silencing sequence itself

According to published studies, silencing success is often correlated with insert sequence length. While some sources indicate upper range limits to 500 nt (Lu 2003), Bruun-Rasmussen et al. (2007) indicate, that sequences longer than 130 nt are more likely to get lost and negatively affect virus accumulation. Therefore, small sequences lead to more longer lasting silencing effects (Bruun-Rasmussen et al. 2007).

In this experiment FB1 (214 nt), CS2 (144 nt) and HL1 (180 nt) constructs exceed this proclaimed sequence length of 130 nt. Although it seems unlikely, it is still possible that the

virus lost the silencing sequence during replication within the plant and therefore plants didn't develop an FHB susceptible phenotype.

Besides the demands regarding insert length, the sequence itself can also influence construct stability. Ding et al. (2006) compared actin and PDS genes in a silencing experiment. The actin sequence was more stable than the insert from the PDS gene, although the actin sequence was longer (398 bases versus 240 bases; Ding et al. 2006, in Scofield & Nelson 2009). The failure of this VIGS experiment could also be linked to improper sequences of candidate genes. Maybe choosing other exons or fragments within the ORF would have delivered better results.

4.3. Formation of siRNA leads to silencing of off-targets

During PTGS, formation of siRNA occurs from viral dsRNA. Silencing efficiency is correlated with the formation of sequence-specific siRNA out of the target sequence carried by the virus (Khvorova et al. 2003; Schwarz et al. 2003; Amarzguioui & Prydz 2004; Reynolds et al. 2004; Ui-Tei et al. 2004). The location of siRNA sequence in the target (Birmingham et al. 2006) and target accessibility (Luo & Chang 2004; Pancoska et al. 2004; Brown et al. 2005) are also influencing silencing efficiency. Additionally the structure of siRNA itself may also have an effect on its efficiency to induce gene silencing (Xu et al. 2006).

In the initial phase of this experiment (primer design for silencing sequence amplification) we used the online tool pssRNAit (Samuel Roberts Noble Foundation, Inc.; http://plantgrn.noble.org/pssRNAit/) to minimize the risk of off-target siRNA formation. Based on these data the selection of appropriate insert sequences occurred. Of course this is the best way to deal with potential off-target silencing prior to experimental implementation. Nonetheless prediction of off-target siRNA formation is still limited, as there is no complete genome sequence available for wheat (Scofield & Nelson 2009). So after all it is still possible, that the formation of siRNA lead to additional off-target silencing of yet unknown genes.

4.4. Environmental and physiological influences for carrying out a VIGS pipeline

At the time this VIGS experiment was carried out, there has been a problem with mildew and lice infestation at the institute. Therefore it was nearly impossible to protect our plants from

insect and pathogen attack. Although they were treated with an insecticide/fungicide mixture preventively at an early stage, symptoms of mildew infestation were visible on some plants about 4 days after *Fusarium* inoculation in the climate chambers. As we didn't know about potential interference with silencing by fungicidal modes of action, reduction of humidity from 50% to 40% was preferred as counteracting measurement to impair mildew spread. Initial *Fusarium* attack has already occurred and should therefore not be affected. Although there is no information about interference of mildew response, an impact on silencing should not be considered, as systemic spread of siRNA should already have occurred at this point. Certainly it would be possible though that resistance reaction against mildew masked *Fusarium* susceptibility on some plants.

Temperature plays also a big role for viral replication and silencing. Cakir & Tör (2010) investigated silencing effectiveness in dependence of temperature and revealed, that growth conditions between 18 and 22°C delivered better systemic silencing results while the virus wasn't able to spread the same way at 26°C. Another study showed that silencing was more effective at 22°C and even slight temperature changes can affect movement and/or replication of the virus (Jiang et al. 2008). Fu et al. (2006) further revealed that high temperatures (22-28°C) combined with high humidity (60-70%) inhibit VIGS symptoms in tomato. Low temperatures (15-18°C) and humidity (30-40%) on the other hand contributed positively to silencing effectiveness. Looking at the results of their study, high temperature seems to influence the rapidity of silencing while low humidity contributes to long lasting effects. Although 22°C were exceeded in this experiment (24°C), PCR control of systemic viral spread delivered satisfying results. Humidity was set to 50% at the beginning and was down-regulated to 40% due to the attempt to impair mildew spread. Additionally to this change of environmental conditions, regulation of climate chamber 1 was malfunctioning for a few days during the last week of the experiment and caused variation in humidity from 40-52%. Whether these dynamics in environmental conditions influenced the outcome can only be speculated. As already mentioned, there is information about the influence of sequence length and the sequence itself on silencing. The question is, if there could be a connection between insert loss affinity and the given environmental conditions, which maybe were not optimal. Another factor in carrying out a VIGS experiment is correct timing. Tournier et al. (2006) gave proof to the hypothesis, that spread of silencing is driven by source-sink relationships and silencing signals are transported via the phloem to developing tissue. We performed BSMV inoculations at the stage of late shoot growth. The aim was to inoculate the fully developed flag leaf to trigger viral spread towards wheat heads, following the principle of source-sink

relationships. For that purpose plants in the same developmental stage were inoculated on one day to decrease the number of infection time points for efficient inoculum use. As a trade-off we therefore had to inoculate the last developed leaf on some plants, as the flag leaf was not sufficiently developed. Although this time point may not be perfect, I doubt its influence on the experimental outcome. Scofield et al. (2005) silenced PDS in wheat and barley and observed most severe photobleaching in 3rd and 4th leaves when 1st and 2nd leaves were inoculated. This simply underlines the importance of source-sink relationships in silencing experiments. BSMV inoculation in our trials should already have hit the spike as main sink at the chosen time point.

4.5. Is VIGS not the right assessment for functional validation of *Fusarium* resistance genes?

Resistance against *Fusarium graminearum* is a quantitative trait which means polygenetic inheritance and multiple genes contributing to resistance reactions. More than 100 QTL have been described in association with FHB resistance (Buerstmayr et al. 2003). The most promising QTL in this regard is *Fhb1* on chromosome 3B. But even this knowledge doesn't make searching for the genetic background of FHB resistance much easier as there are still multiple genes involved and the main resistance factor is still unknown. Silencing one of these up-regulated genes could possibly be compensated by the action of other responsive genes. This would mask a susceptible phenotype.

VIGS is a tool for transient silencing of candidate genes by mRNA degradation. This means that a number of transcripts is still available for the synthesis of functional defence protein. So there is no chance to achieve a loss-of-function phenotype by the application of VIGS. Only 75-90% downregulation of target gene expression can be accomplished by silencing (Pflieger et al. 2008; Orzaez et al. 2006). A big question is, if resistance is really correlating with gene transcript abundance. An experiment on monogenically to oligogenically inherited *Fusarium oxysporum* resistance in *Arabidopsis thaliana* shows resistance correlating with resistance gene transcript abundance (Chen et al. 2014). Nonetheless there is no threshold value known for transcript amount and resistance reactions. The remaining gene expression could theoretically still be enough to produce functional protein and, in case of resistance genes, a resistant phenotype (Unver & Budak 2009).

Loss-of-function phenotypes at this point can only be achieved by the application of TILLING (Targeting Induced Local Lesions in Genomes). In this approach plants carrying a randomly

mutated genome (by application of ethyl methanesulfonate; EMS) are tested for gene function. A mutation in the gene of interest results in total loss of function and the according protein cannot be synthesized any longer. Compared to VIGS this method is more time consuming, but still it delivers results with higher confidence.

Another possible approach for resistance gene validation would be virus mediated overexpression. It follows the same mechanics and is basically limited by the same factors as VIGS, but operates the opposite direction (Lee, Hammond-Kosack, and Kanyuka 2012). Fusarium responsive genes could thereby be overexpressed in susceptible plants and validated for their function in resistance reactions.

4.6. Focussing on DON-related resistance mechanisms - a more promising approach?

DON (Deoxynivalenol) belongs to the trichothecene mycotoxin family, its impact on plants and mammals has been reviewed by Rocha et al. (2005). It is known that trichothecenes suppress protein synthesis. Therefore molecular mechanisms contributing to trichothecene resistance such as active metabolization might be an important factor for FHB resistance (Lemmens et al. 2005). In former studies it was shown that pathogens with mutations in genes linked with the synthesis of toxic compounds were often nonvirulent (Graniti, 1991) or at least didn't cause same symptom severity, which suggests trichothecenes to be pathogenesis factors for *Fusarium graminearum* (Hohn & Desjardins 1992; Proctor et al. 1997). DON seems to be necessary for the pathogen to progress from spikelet to rachis and throughout the wheat head (Jansen et al. 2005). DON non-producing *Fusarium* strands are not able to inpair host cell thickening and therefore their movement is hindered (Bai et al. 2002; Jansen et al. 2005; Maier et al. 2006). Furthermore susceptible cultivars accumulate more DON than resistant ones (Goswami & Kistler 2005; Wilde & Miedaner 2006). This indicates that DON isn't substantially needed for FHB infection but strongly contributes to disease levels, which is why DON resistance can be strongly considered in resistance breeding approaches. In 2010 a barley uridine diphosphate-glycosyltransferase (UGT) was discovered to be capable of detoxifying DON by transformation to non-toxic DON-3-glycoside (Gardiner et al. 2010; Schweiger et al. 2010). *Arabidopsis thaliana* transformed with this barley-derived UDP-glycosyltransferase already delivered very promising results in increased DON resistance. It still needs to be tested if the same result could be observed in wheat and reduction of fungal spread and DON accumulation could be achieved (Shin et al. 2012).

The *Fhb1* QTL is linked not only to resistance against spread of the fungus but also provides resistance against DON alone. Lemmens et al. (2005) assumed that *Fhb1* harbours a gene either encoding for an UDP-glucosyltransferase (UGT) or a gene involved in regulating UGT activity. Successful silencing of *Fhb1* candidate genes and subsequent DON inoculation trials could therefore be used to identify putative UGT regulators.

5. References

Agee AE, Surpin M, Sohn EJ, Girke T, Rosado A, Kram BW, Carter C, Wentzell AM, Kliebenstein DJ, Jin HC, et al (2010) MODIFIED VACUOLE PHENOTYPE1 is an Arabidopsis myrosinase-associated protein involved in endomembrane protein trafficking. Plant Physiol **152**: 120–32

Amarzguioui M, Prydz H (2004) An algorithm for selection of functional siRNA sequences. Biochem Biophys Res Commun **316**: 1050–8

Anderson J a., Stack RW, Liu S, Waldron BL, Fjeld a. D, Coyne C, Moreno-Sevilla B, Fetch JM, Song QJ, Cregan PB, et al (2001) DNA markers for Fusarium head blight resistance QTLs in two wheat populations. TAG Theor Appl Genet **102**: 1164–1168

Bai G-H, Desjardins AE, Plattner RD (2002) Deoxynivalenol-nonproducing Fusarium graminearum Causes Initial Infection, but does not Cause DiseaseSpread in Wheat Spikes. Mycopathologia **153**: 91–98

Bartley GE, Scolnik PA (1995) Plant Carotenoids : Pigments for Photoprotection , Visual Attraction , and Human Health. **7**: 1027–1038

Becker A, Lange M (2010) VIGS--genomics goes functional. Trends Plant Sci **15**: 1–4

Birmingham A, Anderson EM, Reynolds A, Ilsley-Tyree D, Leake D, Fedorov Y, Baskerville S, Maksimova E, Robinson K, Karpilow J, et al (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Methods **3**: 199–204

Brick DJ, Brumlik MJ, Buckley JT, Cao JX, Davies PC, Misra S, Tranbarger TJ, Upton C (1995) A new family of lipolytic plant enzymes with members in rice, arabidopsis and maize. FEBS Lett **377**: 475–80

Brown KM, Chu C, Rana TM (2005) Target accessibility dictates the potency of human RISC. Nat Struct Mol Biol **12**: 469–470

Bruun-Rasmussen M, Madsen CT, Jessing S, Albrechtsen M (2007) Stability of Barley stripe mosaic virus-induced gene silencing in barley. Mol Plant Microbe Interact **20**: 1323–31

Buerstmayr H, Ban T, Anderson JA (2009) Review QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat : a review. Plant Breed **128**: 1–26

Buerstmayr H, Lemmens M, Hartl L, Doldi L, Steiner B, Stierschneider M, Ruckenbauer P (2002) Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. I. Resistance to fungal spread (Type II resistance). Theor Appl Genet **104**: 84–91

Buerstmayr H, Steiner B, Hartl L, Griesser M, Angerer N, Lengauer D, Miedaner T, Schneider B, Lemmens M (2003) Molecular mapping of QTLs for Fusarium head blight resistance in spring wheat. II. Resistance to fungal penetration and spread. Theor Appl Genet **107**: 503–8

Cakir C, Tör M (2010) Factors influencing Barley Stripe Mosaic Virus-mediated gene silencing in wheat. Physiol Mol Plant Pathol **74**: 246–253

Chen YC, Wong CL, Muzzi F, Vlaardingerbroek I, Kidd BN, Schenk PM (2014) Root defense analysis against Fusarium oxysporum reveals new regulators to confer resistance. Sci Rep **4**: 5584

Cheng A, Lou Y, Mao Y, Lu S, Wang L, Chen X (2007) Plant Terpenoids : Biosynthesis and Ecological Functions. **49**: 179–186

Desjardins AE (2006) Fusarium Mycotoxins: Chemistry, Genetics, and Biology. APS Press, St. Paul, MN

Devoto A, Muskett PR, Shirasu K (2003) Role of ubiquitination in the regulation of plant defence against pathogens. Curr Opin Plant Biol **6**: 307–311

Ding XS, Schneider WL, Chaluvadi SR, Mian M a R, Nelson RS (2006) Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant Microbe Interact **19**: 1229–39

Dudareva N, Pichersky E, Gershenzon J (2004) Biochemistry of Plant Volatiles 1. **135**: 1893–1902

Van Egmond HP (2004) Natural toxins: risks, regulations and the analytical situation in Europe. Anal Bioanal Chem **378**: 1152–60

Fu D, Zhu B, Zhu H, Zhang H, Xie Y, Jiang W (2006) Molecules and Enhancement of Virus-induced Gene Silencing in Tomato by Low Temperature and Low Humidity. **21**: 153–160

Gardiner S a, Boddu J, Berthiller F, Hametner C, Stupar RM, Adam G, Muehlbauer GJ (2010) Transcriptome analysis of the barley-deoxynivalenol interaction: evidence for a role of glutathione in deoxynivalenol detoxification. Mol Plant Microbe Interact **23**: 962–76

Goswami RS, Kistler HC (2005) Pathogenicity and In Planta Mycotoxin Accumulation Among Members of the Fusarium graminearum Species Complex on Wheat and Rice. **95**: 1397–1404

Gottwald S, Samans B, Lück S, Friedt W (2012) Jasmonate and ethylene dependent defence gene expression and suppression of fungal virulence factors: Two essential mechanisms of Fusarium head blight resistance in wheat? BMC Genomics **13**: 369

Graniti A (1991) Phytotoxins and their involvement in plant diseases. Introduction. Experientia **47**: 751–755

Grant M, Lamb C (2006) Systemic immunity. Curr Opin Plant Biol **9**: 414–20

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature **404**: 293–296

Hammond SM, Boettcher S, Caudy a a, Kobayashi R, Hannon GJ (2001a) Argonaute2, a link between genetic and biochemical analyses of RNAi. Science **293**: 1146–50

Hammond SM, Caudy a a, Hannon GJ (2001b) Post-transcriptional gene silencing by double-stranded RNA. Nat Rev Genet **2**: 110–9

Hohn TM, Desjardins AE (1992) Isolation and Gene Disruption of the Tox5 Gene Encoding Trichodiene Synthase in Gibberella pulicaris. **5**: 249–256

Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J **30**: 315–27

Hua Z, Vierstra RD (2011) The cullin-RING ubiquitin-protein ligases. Annu Rev Plant Biol **62**: 299–334

Jansen C, von Wettstein D, Schäfer W, Kogel K-H, Felk A, Maier FJ (2005) Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proc Natl Acad Sci U S A **102**: 16892–7

Jiang C-Z, Lu F, Imsabai W, Meir S, Reid MS (2008) Silencing polygalacturonase expression inhibits tomato petiole abscission. J Exp Bot **59**: 973–9

Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs Exhibit Strand Bias. Cell **115**: 505

Kim HG, Kwon SJ, Jang YJ, Nam MH, Chung JH, Na Y-C, Guo H, Park OK (2013) GDSL LIPASE1 modulates plant immunity through feedback regulation of ethylene signaling. Plant Physiol **163**: 1776–91

Kugler KG, Siegwart G, Nussbaumer T, Ametz C, Spannagl M, Steiner B, Lemmens M, Mayer KF, Buerstmayr H, Schweiger W (2013) Quantitative trait loci-dependent analysis of a gene co-expression network associated with Fusarium head blight resistance in bread wheat (Triticum aestivum L.). BMC Genomics **14**: 728

Lacomme C, Hrubikova K, Hein I (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. 543–553

Lee DS, Kim BK, Kwon SJ, Jin HC, Park OK (2009) Arabidopsis GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. Biochem Biophys Res Commun **379**: 1038–42

Lee W-S, Hammond-Kosack KE, Kanyuka K (2012) Barley Stripe Mosaic Virus-Mediated Tools for Investigating Gene Function in Cereal Plants and Their Pathogens: Virus-Induced Gene Silencing, Host-Mediated Gene Silencing, and Virus-Mediated Overexpression of Heterologous Protein. Plant Physiol **160**: 582–590

Lemmens M, Scholz U, Berthiller F, Dall'Asta C, Koutnik A, Schuhmacher R, Adam G, Buerstmayr H, Mesterházy A, Krska R, et al (2005) The ability to detoxify the mycotoxin deoxynivalenol colocalizes with a major quantitative trait locus for Fusarium head blight resistance in wheat. Mol Plant Microbe Interact **18**: 1318–24

Li G, Yen Y (2008) Jasmonate and ethylene signaling pathway may mediate Fusarium head blight resistance in wheat. Crop Sci **48**: 1888–1896

Liu J, Carmell M a, Rivas F V, Marsden CG, Thomson JM, Song J-J, Hammond SM, Joshua-Tor L, Hannon GJ (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science **305**: 1437–41

Lu R (2003) Virus-induced gene silencing in plants. Methods **30**: 296–303

Luo KQ, Chang DC (2004) The gene-silencing efficiency of siRNA is strongly dependent on the local structure of mRNA at the targeted region. Biochem Biophys Res Commun **318**: 303–10

Maier FJ, Miedaner T, Hadeler B, Felk A, Salomon S, Lemmens M, Kassner H, Schäfer W (2006) Involvement of trichothecenes in fusarioses of wheat, barley and maize evaluated by gene disruption of the trichodiene synthase (Tri5) gene in three field isolates of different chemotype and virulence. Mol Plant Pathol **7**: 449–61

Misas-Villamil JC, van der Hoorn R Al (2008) Enzyme–inhibitor interactions at the plant–pathogen interface. Curr Opin Plant Biol **11**: 380–388

Muhovski Y, Batoko H, Jacquemin J-M (2012) Identification, characterization and mapping of differentially expressed genes in a winter wheat cultivar (Centenaire) resistant to Fusarium graminearum infection. Mol Biol Rep. doi: 10.1007/s11033-012-1823-5

Oh IS, Park R, Bae S, Kwon J, Kim S, Lee E, Kang Y (2005) Secretome Analysis Reveals an Arabidopsis Lipase Involved in Defense against Alternaria brassicicola. **17**: 2832–2847

Orzaez D, Mirabel S, Wieland WH, Granell A (2006) Agroinjection of Tomato Fruits . A Tool for Rapid Functional Analysis of Transgenes Directly in Fruit 1. **140**: 3–11

Pancoska P, Moravek Z, Moll UM (2004) Efficient RNA interference depends on global context of the target sequence: quantitative analysis of silencing efficiency using Eulerian graph representation of siRNA. Nucleic Acids Res **32**: 1469–79

Paschold A, Halitschke R, Baldwin IT (2006) Using "mute" plants to translate volatile signals. Plant J **45**: 275–91

Pflieger S, Blanchet S, Camborde L, Drugeon G, Rousseau A, Noizet M, Planchais S, Jupin I (2008) Efficient virus-induced gene silencing in Arabidopsis using a "one-step" TYMV-derived vector. Plant J **56**: 678–90

Proctor RH, Hohn TM, McCormick SP (1997) Restoration of wild-type virulence to Tri5 disruption mutants of Gibberella zeae via gene reversion and mutant complementation. Microbiology **143**: 2583–2591

Reddy ASN, Ali GS, Celesnik H, Day IS (2011) Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. Plant Cell **23**: 2010–32

Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A (2004) Rational siRNA design for RNA interference. Nat Biotechnol **22**: 326–30

Rocha O, Ansari K, Doohan FM (2005) Effects of trichothecene mycotoxins on eukaryotic cells: a review. Food Addit Contam **22**: 369–78

Schroeder H, Christensen J (1963) Factors affecting resistance of wheat to scab caused by Gibberella zeae. Phytopathology 831–838

Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the Assembly of the RNAi Enzyme Complex. Cell **115**: 199–208

Schweiger W, Boddu J, Shin S, Poppenberger B, Berthiller F, Lemmens M, Muehlbauer GJ, Adam G (2010) Validation of a candidate deoxynivalenol-inactivating UDP-glucosyltransferase from barley by heterologous expression in yeast. Mol Plant Microbe Interact **23**: 977–86

Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a Virus-Induced Gene-Silencing System for Hexaploid Wheat and Its Use in Functional Analysis of the Lr21 - Mediated Leaf Rust Resistance Pathway 1. **138**: 2165–2173

Scofield SR, Nelson RS (2009) Resources for virus-induced gene silencing in the grasses. Plant Physiol **149**: 152–7

Senthil-Kumar M, Mysore KS (2011) New dimensions for VIGS in plant functional genomics. Trends Plant Sci **16**: 656–65

Shin S, Torres-Acosta JA, Heinen SJ, McCormick S, Lemmens M, Kovalsky Paris MP, Berthiller F, Adam G, J. MG (2012) Transgenic Arabidopsis thaliana expressing a barley methylation and chromatin patterning UDP-glucosyltransferase exhibit resistance to the mycotoxin deoxynivalenol. J Exp Bot **63**: 695–709

Skaar JR, Pagan JK, Pagano M (2013) Mechanisms and function of substrate recruitment by F-box proteins. Nat Rev Mol Cell Biol **14**: 369–81

Somers DE, Fujiwara S (2009) Thinking outside the F-box: novel ligands for novel receptors. Trends Plant Sci **14**: 206–213

Song J-J, Smith SK, Hannon GJ, Joshua-Tor L (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. Science **305**: 1434–7

Tholl D (2006) Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. Curr Opin Plant Biol **9**: 297–304

Tournier B, Tabler M, Kalantidis K (2006) Phloem flow strongly influences the systemic spread of silencing in GFP Nicotiana benthamiana plants. Plant J **47**: 383–94

Ui-Tei K, Naito Y, Takahashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R, Saigo K (2004) Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acids Res **32**: 936–48

Unver T, Budak H (2009) Virus-induced gene silencing, a post transcriptional gene silencing method. Int J Plant Genomics **2009**: 198680

Upton C, Buckley JT (1995) A new family of lipolytic enzymes? Trends Biochem Sci **20**: 178–179

Wang Y, Yang L, Xu H, Li Q, Ma Z, Chu C (2005) Differential proteomic analysis of proteins in wheat spikes induced by Fusarium graminearum. Proteomics **5**: 4496–503

Waterhouse PM, Helliwell C a (2003) Exploring plant genomes by RNA-induced gene silencing. Nat Rev Genet **4**: 29–38

Wilde F, Miedaner T (2006) Selection for Fusarium head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat. **98**: 2002–2004

Wittstock U, Gershenzon J (2002) Constitutive plant toxins and their role in defense against herbivores and pathogens. Curr Opin Plant Biol **5**: 300–7

Xie Z, Johansen LK, Gustafson AM, Kasschau KD, Lellis AD, Zilberman D, Jacobsen SE, Carrington JC (2004) Genetic and functional diversification of small RNA pathways in plants. PLoS Biol **2**: E104

Xu P, Zhang Y, Kang L, Roossinck MJ, Mysore KS (2006) Computational estimation and experimental verification of off-target silencing during posttranscriptional gene silencing in plants. Plant Physiol **142**: 429–40

Yuan C, Li C, Yan L, Jackson AO, Liu Z, Han C, Yu J, Li D (2011) A high throughput barley stripe mosaic virus vector for virus induced gene silencing in monocots and dicots. PLoS One **6**: e26468

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell **101**: 25–33

Zhang Z, Ober JA, Kliebenstein DJ (2006) The Gene Controlling the Quantitative Trait Locus EPITHIOSPECIFIER MODIFIER1 Alters Glucosinolate Hydrolysis and Insect Resistance in Arabidopsis. **18**: 1524–1536

Zhou W, Kolb FL, Riechers DE (2005) Identification of proteins induced or upregulated by Fusarium head blight infection in the spikes of hexaploid wheat (Triticum aestivum). Genome **48**: 770–780

6. Appendix

6.1. Plant distribution in climate chambers

Plants were randomized in each climate chamber due to eventually diverse climatic conditions. They were distributed in a way that the same amount of plants carrying each construct is exposed to each climatic condition. This should prevent diverse physiological development to minimize errors. The distribution of plants in climate chambers is given in Tables 8 and 9.

Table 8: Plant distribution in climate chamber 1

Distribution of plants chamber 1

Legend: HL1 (yellow), HL2 (blue), CS1 (orange), CS2 (red), WT (white), NIL (light grey)

Left block:

1	2	3	4	5
1	2	3	4	5
1	2	3	4	5
10	9	8	7	6
		5	4	
10	9	8	7	6
15	14	13	12	11
15	14	13	12	11
15	14	13	12	11
20	19	18	17	16
	10	9	8	
20	19	18	17	16
25	24	23	22	21
25	24	23	22	21
25	24	23	22	21
30	29	28	27	26
		15	14	
30	29	28	27	26

Right block:

1	2	3	4	5
	1	2	3	
1	2	3	4	5
6	7	8	9	10
6	7	8	9	10
6	7	8	9	10
11	12	13	14	15
		6	7	
11	12	13	14	15
16	17	18	19	20
16	17	18	19	20
16	17	18	19	20
21	22	23	24	25
	11	12	13	
21	22	23	24	25
26	27	28	29	30
26	27	28	29	30
26	27	28	29	30

Distribution of plants chamber 2

3	6	3	6	33	15	15	15	36	9	12	9	12
2	5	2	5	32	14	44	15	35	8	11	8	11
1	4	1	4	31	14	14	14	34	7	10	7	10
42	3	6	3	6	43	13	45	9	12	9	12	39
41	2	5	2	5	13	13	13	8	11	8	11	38
40	1	4	1	4				7	10	7	10	37
18	21	18	21	48				18	18	21	18	21
17	20	17	20	47				17	17	20	17	20
16	19	16	19	46				16	16	19	16	19
19	24	27	24	27				24	27	24	27	51
20	23	26	23	26				23	26	23	26	50
21	22	25	22	25				22	25	22	25	49
28	22	29	23	30				28	24	29	25	30
28	52	29	53	30				28	54	29	55	30
56	57	58	59	60				26	27	28	29	30

Legend:
- TS1
- TS2
- FB1
- FB2
- WT
- NIL

6.2. Disease scoring excel sheets

The following figures represent the collected phenotypic scoring data, which the diagrams in chapter 4.5 are based on. They were entered in excel sheet and transformed into heat maps for visualizing purposes. Initial spike infection was counted as 1 (= one infected spikelet) and initial rachis infection was scored as 0.5 (= intersection between rachis and spikelet is infested; every additional symptom was counted as 1). Individuals with physiological damages (broken spikes, kinked stem) were excluded from scoring. As plants were split into two groups, their disposal is accordingly.

6.2.1. Climate chamber 1

CS1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	1	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	1	1
4	0	0	0	0	0	0	0	1	1
5	0	1	1	1	1	1	1	1	1
6	0	0	0	0	0	0	0	0	0
7	1	1	1	1	1	1	2	2	2
8	0	0	0	0	0	0	0	0	0
9	0	1	3	4	8	8	8	10	10
10	0	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1
12	0	1	1	1	1	1	1	1	1
13	0	0	0	0	0	1	1	1	1
14	0	0	1	1	1	1	1	1	1
15	0	1	1	1	1	1	1	1	1
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	1	1	1	1	1	1	1	1	1
20	0	0	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1
22	0	0	0	0	0	0	0	0	0
23	1	1	1	1	1	1	1	1	1
24	0	0	0	0	0	1	1	1	1
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	1
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0

CS1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0.5	0.5
5	0	0.5	1	1	2	2	3	3	3
6	0	0	0	0	0	0.5	0.5	0.5	1
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	2	6	6	8	9	11	11	11
10	0	0	0	0	0.5	0.5	0.5	1	1
11	0	2	2	2	3	3	3	3	3
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0.5	0.5
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0.5	0.5	0.5	0.5
29	0	0	0	0	0	0	0	0	0

CS2 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	1	1	1	1	1	1	1
2	0	1	1	1	1	1	1	1	1
3	0	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	2	2
5	0	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	1
9	0	1	1	1	1	1	1	1	1
10	0	0	0	0	1	1	1	1	1
11	0	0	0	0	0	0	0	1	1
12	0	0	0	0	0	0	1	1	1
13	0	1	1	1	1	1	1	1	1
14	0	1	3	4	6	7	7	9	12
15	1	1	1	1	1	1	1	1	1
16	0	0	0	0	0	0	0	0	0
17	0	0	1	1	1	1	1	1	1
18	0	0	0	0	0	0	0	1	1
19	0	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	0
21	1	1	1	1	1	1	1	1	1
22	0	0	0	0	0	0	0	0	0
23	1	1	1	1	1	1	1	1	1
24	0	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1
26	1	1	1	1	1	1	1	1	1
27	0	1	1	1	1	1	1	1	1
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	1	1	1	1
30	0	1	1	1	1	1	1	1	1

CS2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0.5
4	0	1	1	1	2	2	2	2	2
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0.5	0.5
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0.5	0.5	0.5
13	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
14	0	1	3	4	13	13	14	14	14
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0.5	0.5	0.5	0.5	0.5
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0.5	0.5	0.5
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0.5	0.5	0.5	0.5
24	0	0	0	0	0.5	0.5	0.5	0.5	0.5
25	0	0	0	0	0	1	1	1	1
26	0	0.5	1	1	1	1	1	1	1
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0.5	1	1	1
30	0	0	0	0	0	0	0	0	0

HL1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	1	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	1	1	1	1	1	1	1	1	1
4	0	2	2	5	6	8	8	8	10
5	1	1	1	1	1	1	1	1	1
6	0	1	1	1	1	1	1	1	1
7	1	1	1	1	1	1	1	1	1
8	0	1	1	1	1	1	1	1	1
9	0	1	1	1	1	1	1	1	1
10	0	0	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1
12	0	1	1	1	1	1	1	1	1
13	1	1	1	1	1	1	1	1	1
14	0	0	0	0	0	0	1	1	1
15	0	0	0	0	0	0	1	1	1
16	0	0	1	1	1	1	1	1	1
17	0	0	0	0	0	0	0	0	0
18	1	1	1	1	1	1	1	1	1
19	0	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1
21	0	0	1	1	1	1	1	1	1
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	1	1	1	1	2	2	2	2	2
25	0	0	0	0	0	0	0	0	0
26	0	1	1	1	1	1	1	1	1
27	0	0	0	0	0	1	1	1	1
28	0	0	0	0	1	1	1	1	1
29	0	0	1	1	1	1	1	1	1

HL1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0.5	0.5	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	0	1	4	5	9	9	9	13	13
4	0	1	3	8	9	14	14	14	14
5	0	0	0	0	0.5	0.5	0.5	0.5	0.5
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0.5	0.5	0.5
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0.5	0.5	0.5	0.5	0.5
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0.5	0.5	1	1
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0.5	0.5	0.5	0.5	0.5
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0.5	0.5	0.5
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0.5	0.5	0.5
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0

HL2 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	1	1	1	1	1	1	1
2	0	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	0	0	0	0	0	0	1	1	1
5	0	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1	1
7	0	0	0	0	1	1	1	1	1
8	0	1	1	1	1	1	1	1	1
9	0	1	1	1	1	1	1	1	1
10	0	1	1	1	1	1	1	1	1
11	0	0	0	0	0	0	0	0	0
12	0	1	1	1	1	1	1	1	1
13	0	1	1	1	1	1	1	1	1
14	1	1	1	1	1	1	1	1	1
15	0	0	0	0	0	0	0	0	0
16	0	0	1	1	1	1	1	1	1
17	0	0	1	1	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1
19	0	0	1	1	1	1	1	1	1
20	0	0	0	1	1	1	1	1	1
21	0	0	0	0	0	0	0	0	1
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	1	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	1	1	1	1	1
28	1	1	1	1	1	1	1	1	1
29	0	0	1	1	1	1	1	1	1
30	0	0	1	1	1	1	1	1	1

HL2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0.5	0.5	0.5	0.5	0.5
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	1	1	1	1	1	1
18	0	0	0	0	0	0	0.5	0.5	0.5
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0.5
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

WT1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	2	2	2	3	9	11	12
2	0	0	1	1	1	1	1	1	1
3	0	0	0	0	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
5	0	1	2	5	6	6	7	8	14
6	0	1	1	1	1	1	1	1	1
7	0	1	1	1	1	1	1	1	1
8	0	0	0	1	1	1	1	1	1
9	0	0	0	0	0	0	1	1	1
10	0	0	0	0	0	0	0	0	0
11	1	1	1	1	1	1	1	1	1
12	0	0	0	0	0	0	1	1	1
13	0	0	1	1	1	1	1	1	1
14	0	0	0	0	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1
16	0	0	0	0	0	0	0	0	0
17	0	1	1	1	1	1	1	1	1
18	0	0	0	0	1	1	1	1	1
19	0	1	1	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	1	1
23	0	0	0	1	1	1	1	1	1
24	0	1	1	1	1	1	1	1	1
25	1	1	1	1	1	1	1	1	1
26	0	0	0	1	1	1	1	1	1
27	0	1	1	1	1	1	1	1	1
28	0	1	1	1	1	1	1	1	1
29	0	0	0	1	1	1	1	1	1
30	0	0	1	1	1	1	1	1	1

WT1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	2	6	8	12	14	14	16	16
2	0	0	0	0	0	0	0	0.5	0.5
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0.5	0.5	0.5	0.5	0.5
5	0	0.5	2	7	9	11	13	13	13
6	0	0	0	0	0.5	0.5	0.5	0.5	0.5
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0.5	0.5	0.5	0.5
19	0	0	0	0	0	0.5	0.5	0.5	0.5
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0.5	0.5	0.5	0.5
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0.5	0.5	0.5
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

NIL1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	1	1	1	1	1	1	1
2	0	1	2	5	6	8	8	9	9
3	1	1	3	4	7	11	13	14	14
4	0	0	0	0	0	0	0	0	0
5	0	1	3	6	6	9	12	14	14
6	0	1	1	4	7	7	8	9	10
7	0	2	2	6	8	10	10	10	10
8	0	1	2	3	5	8	9	10	10
9	0	0	1	1	1	1	1	1	1
10	0	1	1	1	1	1	1	1	1
11	0	2	3	6	8	8	10	10	10
12	0	1	1	2	2	2	4	7	9
13	0	1	3	4	5	8	8	8	10
14	0	2	2	5	7	9	10	11	12
15	0	1	1	4	5	7	9	11	11

NIL 1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0.5	0.5
2	0	1	2	4	6	7	8	8	8
3	0	1	5	8	9	14	17	17	17
4	0	0	0	0	0	0	0	0	0
5	0	1	5	7	9	13	14	14	14
6	0	0.5	2	6	7	11	13	14	14
7	0	1	3	8	10	10	12	12	12
8	0	0	2	4	6	9	16	17	17
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	4	7	12	12	14	14	14
12	0	0	0	0.5	2	5	8	11	15
13	0	2	5	6	11	11	15	15	15
14	0	2	6	6	9	9	11	15	15
15	0	0	2	7	9	13	13	13	13

6.2.2. Climate chamber 2

TS1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	1	1	1	1	1	1	1	1
3	0	1	1	1	1	1	1	1	1
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	1	1	1	1	1	1	1	1	1
7	0	0	1	1	1	1	1	1	1
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	1	1	1
10	0	1	1	1	1	1	1	1	1
11	0	0	1	1	1	1	1	1	1
12	0	0	0	0	0	0	0	0	0
13	0	1	1	1	1	1	1	1	1
14	0	1	1	1	1	1	1	1	1
15	0	1	1	1	1	1	1	1	1
16	0	1	1	1	1	1	1	1	1
17	0	0	0	0	0	0	0	0	0
18	0	1	1	1	1	1	1	1	1
19	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1	1	1
22	0	0	1	1	1	1	1	1	1
23	0	0	0	0	0	0	0	1	1
24	0	0	0	0	0	0	0	1	1
25	0	0	1	1	1	1	1	1	1
26	0	1	1	1	1	1	1	1	1
27	0	0	1	1	1	1	1	1	1
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	1	1	1	1	1	1	1	1

TS1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0.5	0.5	0.5	0.5	0.5	1
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0.5	0.5	0.5	0.5
6	0	1	4	6	6	8	8	9	9
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0.5	2	4	4	4	4	4	5
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0.5	0.5	0.5	0.5
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	2	2	4	5	6	6	6
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0

TS2 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	1	1	1	1	1	1	1
2	0	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	0	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1
6	0	0	0	0	1	1	1	1	1
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	1	1	1	1	1	1	1	1	1
10	0	0	1	1	1	1	1	1	1
11	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	1	1	1
13	0	1	1	1	1	1	1	1	1
14	0	1	1	1	1	1	1	1	1
15	1	1	1	1	1	1	1	1	1
16	0	1	1	1	1	1	1	1	1
17	0	0	1	1	1	1	1	1	1
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	1	1	1	1
20	0	0	0	0	1	1	1	1	1
21	0	0	0	0	0	0	0	0	0
22	0	1	1	1	1	1	1	1	1
23	0	1	1	1	1	1	1	1	1
24	1	1	1	1	1	1	1	1	1
25	0	1	1	1	1	1	1	1	1
26	0	1	1	1	1	1	1	1	1
27	0	0	1	1	1	1	1	1	1
28	0	0	0	0	0	1	1	1	1
29	0	0	0	0	1	1	1	1	1

TS2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0.5	0.5	0.5
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5
4	0	0	0	0	0	0.5	0.5	0.5	0.5
5	0	0	0	0	0	0.5	0.5	0.5	1
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
13	0	0	1	2	2	2	2	2	2
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0.5
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	4	6	6	8	8	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0

FB1 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	0	0	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1
6	0	0	0	0	0	0	0	0	0
7	1	1	1	1	1	1	1	1	1
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	1	1	1	1	1	1	1	1	1
11	1	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	4	9
13	0	0	0	0	0	0	0	0	0
14	1	1	1	1	1	1	1	1	1
15	0	1	1	1	1	1	1	1	1
16	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1
18	0	0	0	0	0	0	0	0	0
19	1	1	1	1	1	1	1	1	1
20	1	1	1	1	1	1	1	1	1
21	0	0	0	0	0	0	0	0	1
22	0	0	0	0	0	0	0	0	0
23	0	1	1	1	1	1	1	1	1
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	1	1	1	1	1
26	0	0	0	0	0	1	1	1	1
27	0	1	1	1	1	1	1	1	1
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0

FB1 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0.5	0.5	0.5	0.5
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0.5	0.5	0.5	0.5	0.5
5	0	0	1	3	3	3	4	6	6
6	0	0	0	0	0	0	0	0	0
7	0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	2	6	8	9	11	12	12	12
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0

FB2 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	1	1	1
4	0	1	1	1	1	1	1	1	1
5	0	0	0	0	0	0	1	1	1
6	1	1	1	1	1	1	1	1	1
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	1	1	1	1	1	1	1	1
13	0	0	0	0	1	1	1	1	2
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	1	1	1	1	1	1	1	1	1
17	1	1	1	1	1	1	1	1	1
18	0	0	0	0	0	0	0	1	1
19	1	1	1	1	1	1	1	1	1
20	0	1	1	1	1	1	1	1	1
21	0	1	1	1	1	1	1	1	1
22	0	0	0	0	0	1	1	1	1
23	1	1	1	1	1	1	1	1	1
24	0	1	1	1	1	1	1	1	1
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	1	1	1	1	1
27	0	0	0	0	0	0	1	1	1
28	0	1	1	1	1	1	1	1	1
29	0	1	1	1	1	1	1	1	1
30	1	1	1	1	1	1	1	1	1

FB2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0.5	0.5	0.5
4	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0.5	1	1	1	2	2
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	3	3	4	0	6
21	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	1	1	1	1	1
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	1	1	1
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0.5	0.5	1	1	1
30	0	0	0	0.5	1	1	1	1	1

WT2 spikelet scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0
3	0	0	1	1	1	1	1	1	1
4	0	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1
6	0	1	1	1	1	1	1	1	1
7	0	0	0	0	0	0	0	0	0
8	1	1	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0	0	0
10	0	1	1	1	1	1	1	1	1
11	0	1	1	1	1	1	1	1	1
12	1	1	1	1	1	1	1	1	1
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	1	1	1	1	1	1	1	1	1
16	0	0	0	0	1	1	1	1	1
17	0	0	0	0	1	1	1	1	1
18	1	1	1	1	1	1	1	1	1
19	0	0	0	1	1	1	1	1	1
20	0	0	0	0	0	0	0	0	0
21	0	0	0	1	1	1	1	1	1
22	0	0	0	0	0	0	0	0	0
23	0	1	1	1	1	1	1	1	1
24	0	0	0	0	0	0	0	0	0
25	0	0	0	1	1	1	1	1	1
26	0	1	1	1	1	1	1	1	1
27	0	0	0	0	1	1	1	1	1
28	0	1	1	1	1	1	1	1	1
29	0	1	1	1	1	1	1	1	1

WT2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0.5
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0
8	0	0	0	0.5	0.5	0.5	0.5	0.5	1
9	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	1
12	0	0	0	0.5	0.5	0.5	0.5	0.5	0.5
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0.5	0.5
22	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0
29	0	0	0	1	1	1	2	2	2

NIL2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	1	1	2	5	6	6	8	9
2	0	0	0	0	1	1	1	1	1
3	1	1	1	1	1	1	1	1	1
4	1	2	3	4	5	8	8	8	8
5	1	1	1	1	1	2	6	8	8
6	0	1	1	1	1	2	2	2	2
7	0	1	3	4	8	8	8	8	9
8	1	1	1	4	5	5	5	5	5
9	0	0	0	1	1	1	1	1	1
10	0	2	2	6	9	9	11	11	11
11	0	1	1	1	1	1	1	1	1
12	0	0	0	0	0	1	1	1	1
13	0	0	0	1	1	1	1	1	1
14	0	1	1	1	1	1	1	1	1

NIL2 rachis scoring

Plant Nr.	2	4	6	8	10	12	14	18	21
1	0	0	0	2	7	8	10	12	12
2	0	0	0	0	0	0	0	2	2
3	0	0	0	0	0	0	0	0	0
4	0	3	3	5	9	9	9	9	9
5	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0
7	0	2	8	9	11	11	11	11	13
8	0	0	2	5	9	9	10	10	10
9	0	0	0	0	0	0	0	0	0
10	0	3	6	8	11	11	11	11	11
11	0	0	0	0	0	0.5	0.5	0.5	0.5
12	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0