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Transcription profiling in hexaploid wheat for identification of candidate genes contributing to resistance to *Fusarium* head blight by cDNA-AFLP method

A diploma thesis

by

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The little prince crossed the desert and met with only one flower.
“Good morning”, said the little prince.
“Good morning”, said the flower.
“Where are the people?” the little prince inquired politely.
The flower had one day seen a caravan passing.
“I caught a climpse of them several years ago.
But you never know where to find them. The wind blows them away.
They have no roots which makes their life rather trying”.

The Little Prince (Antoine de Saint-Exupery).

ABSTRACT

The fungal pathogen *Fusarium graminearum* infects wheat florets at anthesis and causes *Fusarium* head blight (FHB).Several quantitative trait loci (QTL) with major and minor effects on resistance to FHB have been characterised by QTL mapping. In a mapping population of CM82036/Remus two QTL with major effects were identified. While *Qfhs.ifa-5A* on chromosome 5A is primarily associated with resistance to initial infection, *Fhb1* on chromosome 3BS is associated with resistance to spread of disease within the head and detoxification of the virulence factor deoxynivalenol (DON).

By making use of the QTL mapping population, we aimed to characterise the effects of the two QTL at gene expression level. Beside the parents, two closely related sister lines of the mapping population were subjected to point inoculation experiments and cDNA-AFLP fingerprinting. Sister line DH1 shows resistance and carries the resistant alleles on *Fhb1* and *Qfhs.ifa-5A*, whereas line DH2 is susceptible and carries the susceptible alleles on both QTL. In addition, four near isogenic lines of the susceptible parent with resistant alleles on the QTL regions were included in the experiment.

Transcript derived fragments that showed altered expression levels after *Fusarium* inoculation and differential expression between the genotypes were cloned and sequenced. Functional annotation of 70 differentially expressed sequence tags (ESTs) revealed gene candidates such as members of the serine/threonine protein kinase family, PR-1 protein, PR-5 protein, and a member of the terpene cyclase multigene family.

In this comparative transcription profiling experiment, the question of what renders plants resistant or susceptible was addressed within the same genetic background. Furthermore, assignment of putative function to gene candidates may provide the foundation for further functional genomic studies and contribute to the elucidation of key mechanisms in this intriguing plant–pathogen interaction.

Keywords: *Fusarium* head blight, *Triticum aestivum*, plant–pathogen interaction, transcription profiling, cDNA-AFLP method.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
AUDPC	area under disease progress curve
CC-NB-LRR	coiled coil-nucleotide binding-leucine rich repeat
cDNA	complementary deoxyribonucleic acid
CHS	chalcone synthase
DH line	doubled haploid line
DON	deoxynivalenol
dpi	days post inoculation
EST	expressed sequenced tag
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FHB	*Fusarium* head blight
GFP	green fluorescent protein
hai	hours after inoculation
JA	jasmonic acid
LRR-RLK	leucine rich repeat-receptor like kinase
MAS	marker assisted selection
mRNA	messenger ribonucleic acid
NB-LRR	nucleotide binding-leucine rich repeat
NIL	near isogenic line
NIV	nivalenol
PAMP	pathogen-associated molecular pattern
PAL	Phenylalanine ammonia lyase
PR-proteins	pathogenesis-related proteins
PRR	PAMP recognition receptors
PTI	PAMP-triggered immunity
QTL	quantitative trait loci
RLP	receptor like protein
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic aquired resistance
TDF	transcript derived fragment

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1 INTRODUCTION

The fungal pathogen *Fusarium graminearum* infects wheat florets at anthesis and causes *Fusarium* head blight (FHB) in susceptible cultivars. As *Fusarium graminearum* produces mycotoxins such as deoxynivalenol, high infection levels lead to contamination of food and animal feed. Several epidemics in Asia, Europe, and North America have posed a threat to global food supply over the last centuries (Bushnell et al. 2003). In recent years, mycotoxin contamination is of particular concern for people who live in poverty and can not afford to participate in the global food market. People who depend on their local harvest every season have no free choice in food quality and cannot count on food safety policy.

Resistant cultivars provide the foundation of crop protection, in particular, in low input subsistence farming systems. Wheat cultivars with good to moderate field resistance have been reported from all wheat growing areas. In particular, the Chinese wheat cultivar Sumai3 and its derivatives are considered to be the most effective and durable resistance source available to date (Bai et al. 2004, McKendry, 2008).

Resistance to *Fusarium graminearum* was first phenotypically described by Schröder and Christensen in 1963. Based on differences in visible symptoms, resistance was dissected into resistance to initial infection (type I) and resistance to spread of disease within the spike (type II) (Schröder and Christensen 1963). Furthermore, resistance to kernel infection and resistance to DON accumulation in kernels have been established as resistance components more recently (Mesterhazy 2003).

In addition, advanced techniques in microscopy have offered insight into the early events of wheat–*Fusarium graminarum* interaction at ultra-structural level (Kang and Buchenauer, 2000b, 2002, 2008). It was shown that physical barriers, in first place, may restrict fungal colonisation in resistant cultivars. Apart from these observations, further resistance mechanisms still remain to be elucidated.

Resistance to *Fusarium graminearum* was genetically characterised to be of quantitative and polygenic nature. Therefore, mapping of quantitative trait loci (QTL) has been the method of choice in molecular plant breeding for the last ten years (Waldron et al. 1999, Anderson et al. 2001, Bürstmayr et al. 2002, 2003, Lemmens et al. 2005). In addition, several expression profiling experiments have been performed over the last ten years, in order to determine which genes are expressed when and where over time course of infection (Boddu et al. 2006, 2007; Golkari et al. 2007, 2009). It was aimed to identify candidate genes that contribute to resistance and to elucidate the mechanisms underlying this intriguing plant–pathogen interaction.

1.1 AIMS AND OBJECTIVES

Extensive QTL mapping in the CM82036/Remus population provided the foundation for this comparative transcription profiling experiment:

In the QTL mapping population that derived from a cross between resistant line CM82036, a Sumai3 derivative, and susceptible wheat cultivar Remus, two QTL with major effects on resistance to *Fusarium* head blight were identified. While, *Qfhs.ifa-5A* on chromosome 5A was primarily associated with resistance to initial infection (Bürstmayr et al. 2001, 2002), *Fhb1* on chromosome 3BS was associated with resistance to spread of disease within the spike. *Fhb1* was furthermore associated with resistance to DON and the ability to detoxify DON (Lemmens et al. 2005).

Based on information obtained from extensive QTL mapping the following objectives were aimed to be addressed:

1) up- and downregulation of gene transcripts over time course of infection
2) differences in expression between resistant and susceptible phenotypes
3) differences in expression between genotypes in relation to the two QTL with major effects — *Fhb1* and *Qfhs.ifa-5A*
4) prediction of putative function for gene candidates
5) identification of gene candidates associated with the conversion of DON into DON-3-O-glucoside (as described in Lemmens et al. 2005 and Berthiller et al. 2005)

These objectives were addressed by cDNA-AFLP fingerprinting that allows for detection of allelic variation at transcription level. CM82036, Remus, and two closely related sister lines of the mapping population were subjected to point inoculation experiments. Sister line DH1 showed resistance and carried the resistant alleles at *Fhb1* and *Qfhs.ifa-5A*, whereas sister line DH2 was susceptible and carried the susceptible alleles at both QTL. In addition, four near isogenic lines sharing the susceptible genetic background of Remus, but with allelic variation at *Fhb1* and *Qfhs.ifa-5A* were included in the experiment. Transcription profiling was performed following inoculation with *Fusarium graminearum* or water at three time-points at early stage of infection. Expression patterns were screened for differences between treatments, genotypes, and timepoints. Sequence information of differentially expressed transcripts was used to predict putative gene function by homology search in the NCBI database.

2 STATE OF RESEARCH

2.1 WHEAT–*FUSARIUM GRAMINEARUM* INTERACTION

2.1.1 *Fusarium graminearum* causing *Fusarium* headblight

Fusarium head blight (FHB) is caused by various species of the genus *Fusarium* that are capable of infecting florets of small grain cereals such as wheat, barely, oats, and rice (Goswami et al. 2004). FHB is characterised by brown, purple, water soaked lesions shortly after infection followed by bleaching of the infected spikelet. As time course of infection progresses, symptoms of wilting and blight spread through the entire head indicating premature senescence of infected heads (Goswami et al. 2004). Under humid conditions pinkish to orange mycelium harbouring sporodochia can be identified by the naked eye on outer surfaces of spikelets. As a result of infection, the number of kernels that develop to normal size is reduced. Instead, shrivelled tombstone-like kernels are produced (Bushnell et al. 2003; Ruckenbauer et al. 2001; Goswami et al. 2004). In addition, aspects of grain quality important for the milling, baking, and brewing industry are reported to be negatively influenced. Apart from a loss in processing quality, *Fusarium* species produce a wide range of toxins. Nivalenol (NIV) and deoxynivalenol (DON) belonging to the group of trichothecenes are considered as the main toxins in regard to FHB.

Several *Fusarium* species have been described to cause FHB. In the wheat growing areas of Southern and Middle Europe, *Fusarium graminearum* Schwabe referred to as *Gibberella zeae* Schwein (Petch) in its sexual (teleomorph) stage is the predominating causal agent of FHB (Ruckenbauer et al. 2001).

FHB infection is considered of being monocyclic under natural conditions with infections only taking place during anthesis and early stage of kernel development (Bushnell et al. 2003). *Fusarium graminearum* mycelia overwinter on plant debris in the soil, where they exhibit a saprophytical lifestyle (Goswami et al. 2004). In most plant production systems, crop residues of maize, small grain cereals, and rice are the main source of primary inoculum accommodating the fungus (Bai and Shaner 2004; Mc Mullen et al. 2008). In order to establish itself for saprophytical overwintering and propagation, *Fusarium graminearum* spreads from infected heads downwards and colonizes the culm pith cavity and vascular tissue of living stem tissue during later stages of infection. By spreading radially hyphae reach the parenchyma tissue and outer cell layers and substomatal cavities (Guenther and Trail 2005). Pereyra and Dill-Macky (2004) suggested that *Fusarium graminearum* as a virulent pathogen may take a major advantage in competition with other soil living saprophytes, by colonizing the living host from inside and thus being already well established in the tissue by the time of tissue senescence. Located in the culm substomatal cavities, dicaryotic hyphae are formed by the homothallic fungus without the need of crosses (Guenther and Trail 2005). From dicaryotic hyphae perithecia initials derive that remain dormant until environmental conditions induce the maturation of perithecia in spring (Guenther and Trail 2005). Mature perithecia consist of asci that release ascospores with enormous force into air (Figure 1a).

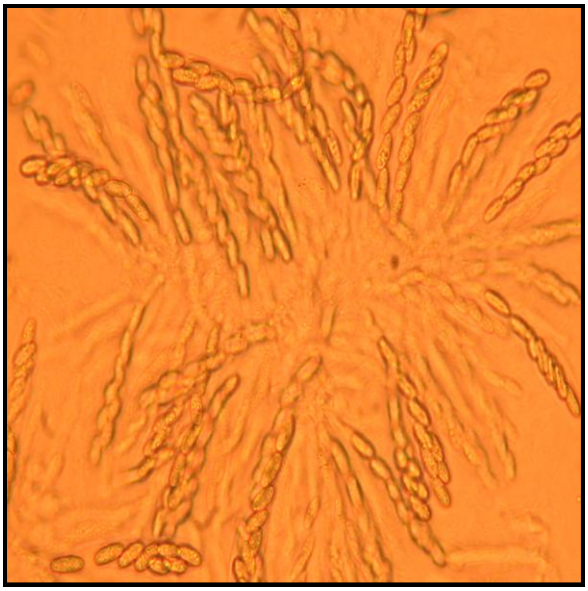



Figure 1a Asci containing ascospores released from perithecia; Figure 1b Macroconidia.

Released ascospores are dispersed upwards to the heads mainly by air current and partly by means of water splash, where they land on aerial parts of the heads and adhere owing to sticky components in the fungal walls (Bushnell et al. 2003). Under humid conditions, ascospores that land on florets open for flowering can germinate and develop hyphae. Subsequently, hyphae grow into the florets consuming tissue of floral parts and of developing kernels (Miller et al. 2004).

Fusarium graminearum exhibits a shift from biotrophic to necrotrophic lifestyle over time-course of infection. At early stages of infection the fungus grows in the intercellular space with plant cells remaining alive. As soon as the fungus has established itself and has built up a dense hyphal network, it invades plant cells directly by intracellular growth (Jansen et al. 2005). Depending on the chemo-type, *Fusarium graminearum* strains produce the mycotoxins DON or NIV that are phytotoxic. There is good evidence that NIV and DON support the shift from biotrophic to necrotrophic lifestyle and are, thus, considered as the main virulence factors (Lemmens et al. 2005, 2008; Kang and Buchenauer 2000). In susceptible plants, the fungus manages to spread internally through the entire spike via the vascular bundles and causes blight of florets up- and downwards from the site of initial infection. By contrast, in resistant plants, the fungus is restricted to the initial site of infection and the majority of spikelets remain healthy (Bai and Shaner 2004; Steiner et al. 2009).

FHB outbreaks that are considered as epidemics, are reported in years with warm and humid conditions during spring time and at flowering time and occur in all wheat growing areas worldwide. In addition to favourable weather conditions, high amount of primary inoculum and high abundance of susceptible hosts give rise to epidemic outbreaks. Applying the concept of the disease triangle “host – pathogen – environment”, environment has to be considered as the most unpredictable component. As a consequence, only a better understanding of the host–pathogen interaction may prevent epidemic outbreaks even under weather conditions favouring the pathogen (Bai and Shaner 2004; Bushnell et al. 2003).

2.1.2 Hexaploid wheat (*Triticum aestivum)* — the host

Hexaploid wheat is the crop with the largest growing area (217, 432 668 ha in 2007) worldwide according to FAO statistics. Taking into consideration that the growing areas of wheat, maize, barely, and oats all being potential hosts of *Fusarium graminearum* (Goswami et al. 2004) represent about one third of the global arable land, it is very likely for this pathogen to encounter a host plant.

In addition, current agronomic practice provides favourable conditions for the pathogen. Crop rotations with wheat following maize and wheat promote development of vast amount of inoculum that is harboured by crop debris in the soil year by year. In addition, survival of the overwintering saprophytical stage on crop debris is facilitated by the cultural practice of minimum soil tillage, where crop debris are not destroyed or buried as in the process of ploughing or chisel ploughing (Mc Mullen et al. 2008; Dill-Macky 2008; Pereyra and Dill-Macky 2004). As a consequence, levels of inoculum build up even in years with no epidemic outbreaks. More recently, the large scale introduction of transgenic maize expressing the *Bacillus thuringiensis* toxin that is effective against European corn borer (*Ostrinia nubialis*) larvae appears to contribute to increased *Fusarium graminearum* infections. It has been observed that plant residues of "Bt-maize" decompose more slowly resulting in increased amount of debris for fungal colonisation (Dill-Macky 2008).

To date, chemical control by fungicides or natural plant protection products is limited as application of these products is confined to a narrow time window and spraying on vertical heads at anthesis is still a challenging application technique (personal communication, M. Lemmens 2009).

In this context, it is most crucial that plants are able to defend themselves. Incompatible interactions with *Fusarium graminearum* restrict the fungus in its development and lifecycle and help to keep inoculum at low level. Low levels of inoculum in the soil is the most efficient way to prevent epidemics even in years with humid and warm weather conditions during flowering time. Therefore, resistant cultivars can be considered as the foundation of sustainable integrated crop management approaches (Mc Mullen et al. 2008).

2.1.2.1 Anthesis of wheat

Wheat heads consist of about 20 to 25 spikelets per spike that are distributed along the rachis alternating from side to side. The rachis as the spindle-like central part contains the xylem and phloem vessels that extend from the culm. Each spikelet has a short stalk termed rachilla that provides the connection between the vascular tissue of the rachis and the spikelets. A spikelet consists of three fertile florets. Each floret is composed of the inner palea and the outer lemma overlapping the palea (**Figure 2**). Prior to flowering, lemma and palea tightly enclose the reproductive organs. At flowering the lodicules located at the base of the florets swell and push palea and lemma apart and open the floret for pollination. The period of open flowering lasts for about 35 minutes per floret. Flowering of the wheat head starts two third up from the base of the head, from where it proceeds in basi- and acro-petal direction. Flowering of an entire wheat head takes about three to five days. In this period airborne spores, that land on open florets, can get access to the host easily (Bushnell et al. 2003; Miller et al. 2004).

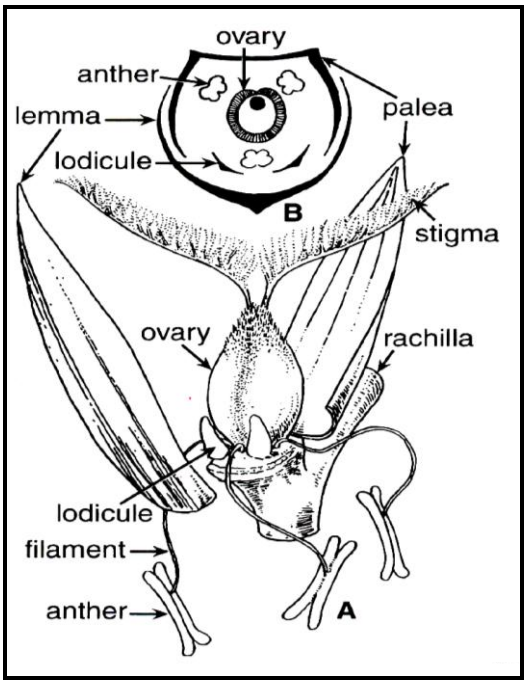


Figure 2 Tissues and organs of a floret at anthesis (Bushnell et al. 2003).

2.1.3 Infection and colonisation

2.1.3.1 Floret organs under attack

Under natural conditions, ascospores land on extruded anthers, stigma, or the tips of floral bracts when florets open up at anthesis. Subsequently, stomata in the chlorenchyma tissue, extruded and partially trapped anthers, and the base of the glume consisting of thin-walled epidermal cells are potential entry sites of the fungus (Bushnell et al. 2003). At anthesis and shortly after pollination, the ovary and respectively the developing caryopsis with endosperm and embryo are not very well protected as protective layers such as testa and pericarp differentiate only ten to twenty days after pollination in the milk ripe to soft dough stage (Bushnell et al. 2003). For this reason, pollen, anthers and ovaries are the major targets in the initial stage of infection. By contrast, outer surfaces of lemma and palea consist of sclerenchyma-like cell layers with lignified cell walls and silicon deposits. According to Miller et al. (2004) these tissues appeared to be comparably harder to penetrate and to consume. They observed that vegetative tissue maintained its basic structure, whereas ovary and anthers were completely consumed by the fungus 21 days post inoculation (dpi).

2.1.3.2 Time course of infection and colonisation

Irrespective of the method of inoculation and the organ they land on, macroconidia germinate within six to twelve hours after infection (hai) under favourable environmental conditions. An unbranched germination hypha develops and extends in order to contact tissue surface and stomata within the first 24 hai (**Figure 3**).

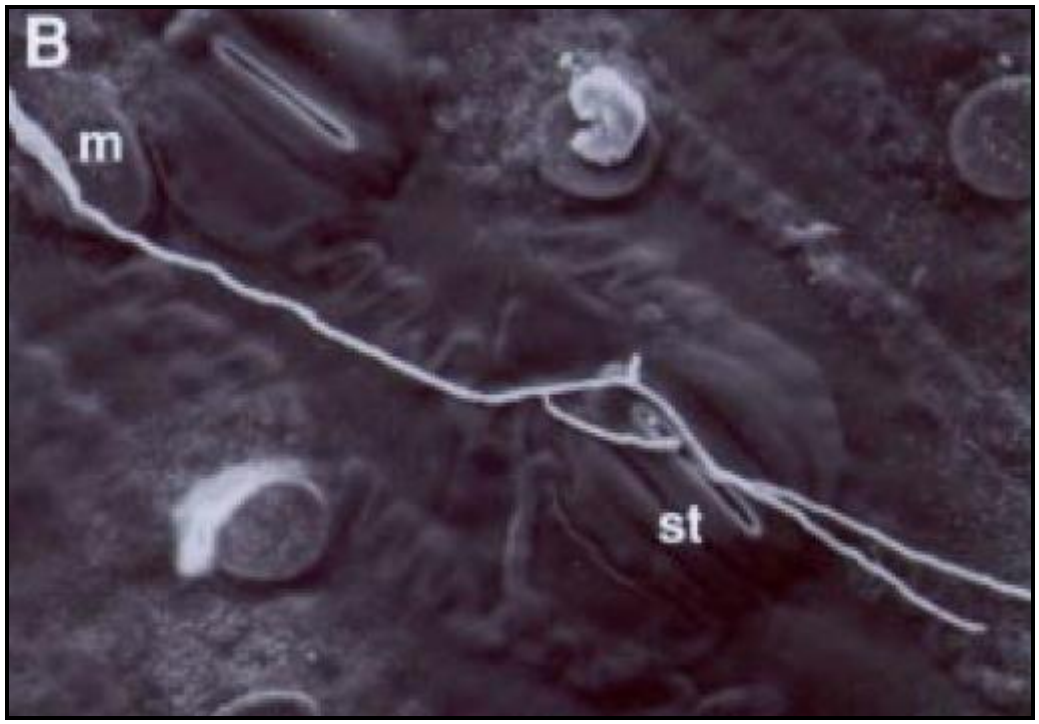


Figure 3 Macroconidia with germination hypha contacting stomata 12 hai (Pritsch et al. 2000).

Pritsch et al. (2000) observed hyphal penetration through stomatal openings into the substomatal chamber and suggested that this site may be the entry point for intercellular growth in parenchyma as well as for subcuticular growth.

Entering the bracts via stomata, the fungus gained access to parenchyma cells circumventing epidermal cells that are thick-walled and consist of highly silicified cell lumina. Subsequently,

the fungus invaded parenchyma cells directly by penetration pegs and spread into the protoplast shifting from a biotrophic lifestyle to a necrotrophic (Figure 4b).

Within 48 hai the fungus had developed a dense hyphal network on the inner surfaces of lemma and palea, consisting of thickened, branched hyphae. At this time-point, increased fungal growth, was particularly observed in the chlorenchyma tissue. In some cells, chloroplasts appeared swollen, stroma was loosely arranged and chlorophyll started to degrade. This is in agreement with Kang and Buchenauer (2000) who reported changes in infected tissue within 48 to 72 hai. Plasmalemma was distorted and the periplasmic space extended between cell wall and plasmalemma. These structural changes were related to the mycotoxin DON that increases rapidly in concentration at this time-point. After four dpi, collapsed host cells with disintegrated cytoplasm and organelles were observed, however, it appeared that only collapsed cells were invaded by the fungus (Kang and Buchenauer 2000).

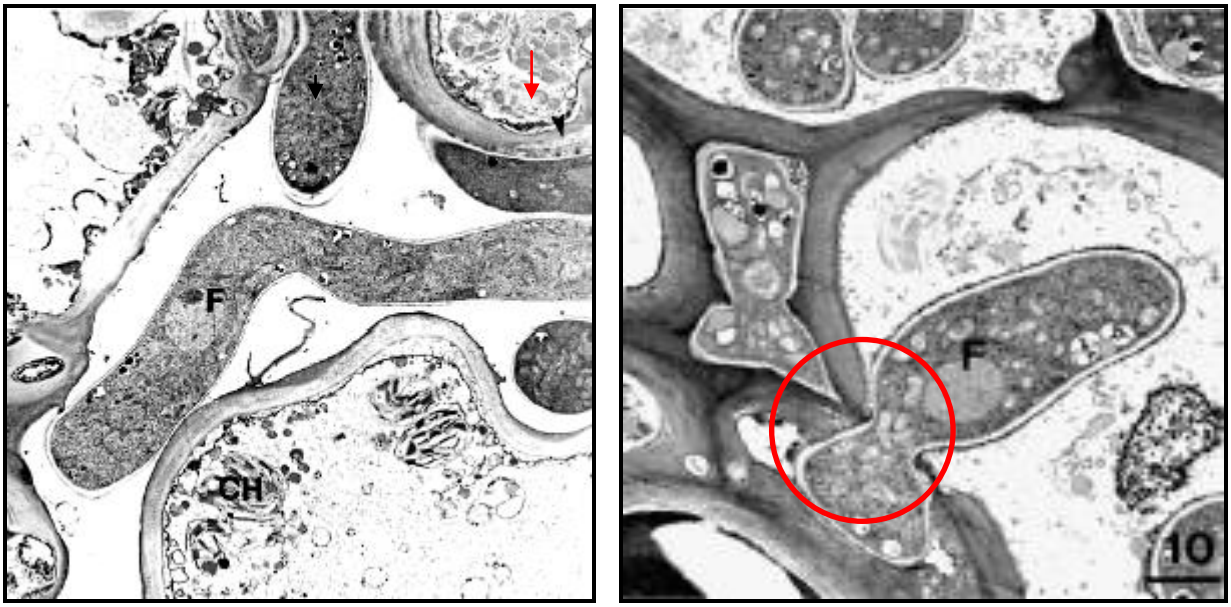
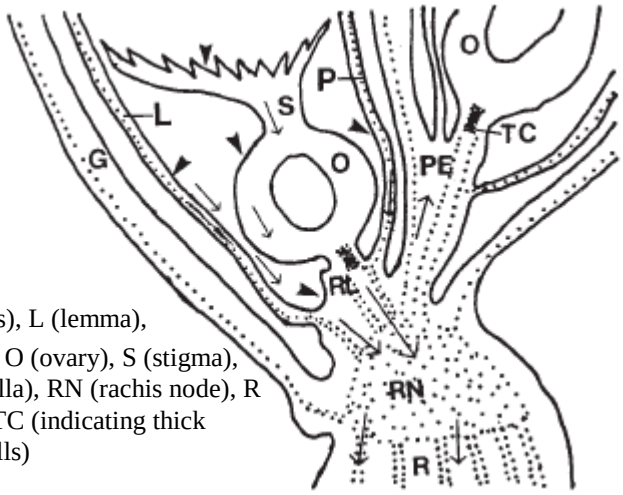


Figure 4a Intercellular growth of hyphae (F); periplasmic space between cell walls and plasma lemma with small dark appositions; cytoplasm, chloroplast (CH) degenerated, cell walls degraded in close contact with hypha (indicated by an arrow); Figure 4b Intracellular growth, a hyphae directly entering a cell (Kang and Buchenauer 2002; Kang et al. 2005).

Three to four dpi, hyphae reach the base of the floral bracts. Subsequently, hyphae spread downwards through the rachilla and rachis node into the rachis and thus beyond the initially inoculated spikelet (Figure 5). Intriguingly, this step of colonisation is delayed in Sumai3 compared to susceptible cultivars: While the fungus reached the vascular tissue of the rachis on the fourth to fifth day post inoculation in the susceptible cultivar, it took double in time in resistant cultivars (Buchenauer and Kang 2000). This observation is in agreement with Miller et al. (2004) who reported resistance in Sumai3. Hyphae were observed in the vascular tissue of the node at the base of the floret seven dpi. Miller et al.(2004) noted, that the reason for the delay in Sumai3 was not visible in the microstructure of uninfected tissue, indicating that there are no additional preformed physical barriers in Sumai3.

In respect to differences between resistant and susceptible cultivars, neither Pritsch et al. (2000) who examined spray inoculated spikelets of resistant Sumai3 nor Kang and Buchenauer (2000) who examined point inoculated spikelets of resistant Frontana, Arina, and Sumai3 (Kang and Buchenauer 2008) observed any differences in timing and development of infection and initial spreading between resistant and susceptible cultivars. However, three dpi, significant

differences in colonisation patterns were observed between susceptible and resistant cultivars. Fungal spreading was delayed and restricted in resistant cultivars. This may be owing to the fundamental differences observed between susceptible and resistant cultivars in how cells responded to direct fungal penetration: Penetrated cells of susceptible cultivars disintegrated, cell walls degraded and only thin layers of cell wall reinforcements were detected, whereas resistant host cells responded with extensive formation of thick layers of wall apposition to direct penetration (Kang and Buchenauer 2000; Pritsch et al. 2000).



G (glumes), L (lemma), P (palea), O (ovary), S (stigma), RL (rachilla), RN (rachis node), R (rachis), TC (indicating thick walled cells)

Figure 5a Diagram of two florets in a spikelet, arrows indicate direction of fungal spreading (Kang and Buchenauer 2002; Figure 5b *Fusarium* strain expressing GFP passing through rachis node (Miller et al. 2004).

Apart from moving downwards, the fungus spreads horizontally by disrupting palea and lemma tissue from inside. This step of colonisation causes first symptoms such as brown, water soaked lesions and the bleaching of glumes and lemma. In addition, as soon as the outer surfaces have been reached, conidiophores harbouring macroconidia are formed (Figure 6). Thus, the fungus is able to complete its first asexual cycle within 76 hai (Pritsch et al. 2000). Interestingly, Miller et al. (2004) did not observe the fungus to break through the surface in Sumai3 although the fungus was present inside.

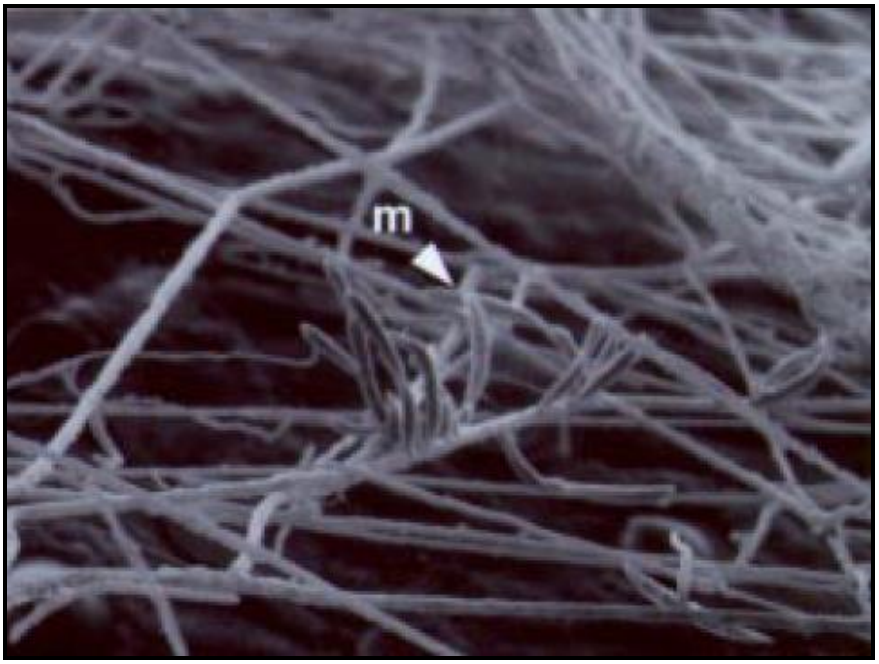


Figure 6 Macroconidia (m) developing on conidiophores (76 hai) (Pritsch et al. 2000).

Once in the rachis, hyphae colonize phloem sieves, xylem vessels and paratracheal parenchyma, thereby extending preferably downwards to the next spikelets.Vascular elements were packed with hyphae in the susceptible cultivar eleven dpi, whereas in Sumai3, hyphae were present but did not occlude the vascular bundles. This timing is in agreement with Bai and Shaner (2004), who reported that susceptible cultivars developed an entirely blighted head within ten days. Clogging of vascular tissue in the rachis deprives non-infected parts of the spike from water and nutrients and leads to premature ripening of the head and poorly developed grain (Bai and Shaner 2004). In addition, the fungus sends hyphae downwards colonizing the vascular tissue and the pith cavity of the culm ten to twelve dpi (Guenther and Trail 2005). While susceptible cultivars show entirely blight heads at this time-point, in Sumai3 brown, electron dense deposits forming pronounced spots were observed in the vascular tissue at microstructural scale (Miller et al. 2004). Guenther and Trail (2005) identified the deposits as pectin containing compounds by a pectin staining method. It was suggested that *de novo* synthesised papillae may occlude and clog the vascular bundles very early. As a result, movement of DON and hyphal growth through the vascular tissue is prevented. Miller et al. (2004) concluded that the brown deposits act as a physical barriers in the vascular tissue of Sumai3 that restrict the spread of the fungus within the spike. Therefore, the ability to actively clog the vascular tissue by deposits is suggested to be one component of resistance to spread of disease. Guenther and Trail (2005) followed stem colonisation of a strain constitutively expressing GFP by fluorescent microscopy. They found that sixteen to eighteen dpi hyphae spread by inter- and intracellular growth through xylem vessels and parenchyma cells reaching the epidermal layers and the substomatal chambers of stem and sheath tissue. There, perithecia initials were formed leading to the next generation of ascospores. Evidence was provided that the fungus established itself fairly soon after initial infection of living plants for its saprophytic lifestyle on dead matter.

However, in Sumai3, florets above and below the initially infected floret were still intact after 21 dpi (Miller et al. 2004). This is in agreement with visual symptoms observed in the resistent line CM 82036 that derived from Sumai3 compared with the susceptible cultivar Remus (Figure 7) (Steiner et al. 2009).

Figure 7 Left head: resistant CM82036, a Sumai3 derivative, right head: susceptible Remus both evaluated 21 dpi by Steiner et al. (2009).

2.1.4 The plant immune system

Unlike mammals that have specialised cells, such as macrophages, circulating in the blood for recognition of infectious non-self (pathogens) or manipulated self, plants do neither have specialised cells for defence nor cells capable of circulating around. As a consequence, every single plant cell needs to be capable of recognising pathogen attack and of self defence. Therefore, every single cell has to be equipped with a system of recognition, signal transduction, and rapid defence. In addition, plants use signalling compounds to systemically alert cells distant of the infection site (Zipfel 2008).

In the first place, plants have constitutive preformed physical and chemical barriers such as waxy cuticles and rigid cell walls to keep pathogens at bay. Virulent pathogens such as *Fusarium graminearum* in wheat are able to penetrate and overcome these preformed barriers. In this case, the ability to recognize the pathogen as early as possible is pivotal to the outcome of plant pathogen interaction. In incompatible interactions the plant recognizes the invader and responds with induction of the *de novo* synthesis of physical and chemical barriers. Induced defence response relies on recognition of infectious non-self, signal transduction, and alteration of gene expression (Zipfel 2008; Dowell and Simons 2007). In compatible interactions either recognition, signal transduction or *de novo* synthesis of barriers fail to keep the intruder at bay.

2.1.4.1 A model for plant–pathogen interactions

Summarising research results obtained in the field of plant–pathogen interactions over the last decades, Jones and Dangl (2006) deduced a four phased “zigzag model” to describe general mechanisms of plant–pathogen interaction. The zigzag model integrates the gene for gene model postulated by Harold H. Flor more than 50 years ago. It takes the co-evolutionary arms race into account explaining virulence and avirulence on the pathogen side and resistance and susceptibility on the plant side.

In phase 1, the plant recognises the approaching, penetrating pathogen by common pathogen associated molecular patterns (PAMPs), such as fungal cell wall components. Therefore, plants apply PAMP recognition receptors (PRRs) that are located in the plasmamembrane. Early and successful recognition of PAMPs induces plant defence mechanisms such as reinforcement of cell walls and synthesis of antimicrobial compounds that can stop colonisation. This defence response is referred to PAMP triggered immunity (PTI). In a next step, pathogens defeated by PTI try to interfere with components of PTI to regain access to the host plant, in order to compensate for being recognised by PAMPs. For this reason, pathogens develop specific virulence factors also referred to as effectors. Effectors are proteins or secondary metabolites that interfere with PTI. For example, effectors are reported to mimic signals that suppress defence or impair gene expression and metabolism. A successful effector renders a plant susceptible despite the recognition of pathogens via PAMPs, also referred to as effector triggered susceptibility — ETS. In response to ETS, plants deploy specific receptors to either directly interact with the effector compound or to indirectly sense the changes that are caused by an effector on the host target. Recognition by specific immune receptors triggers a signal cascade that activates an array of defence mechanisms resulting in effector triggered immunity — ETI. Response in ETI is reported to be stronger than in PTI, but deploys overlapping mechanisms.

Pathogens defeated by ETI can evolve by developing new effectors. For example, in bacteria, several effectors were reported to be used by one single strain. Pathogens can either abolish the recognised effector, diversify the effector, develop other effectors that suppress and interfere with ETI triggered by recognition of the original effector. While ETI drives natural selection for new effectors in the pathogens, ETS drives natural selection for more sophisticated corresponding recognition genes in the plant host (Figure 8) (Jones and Dangl 2006).

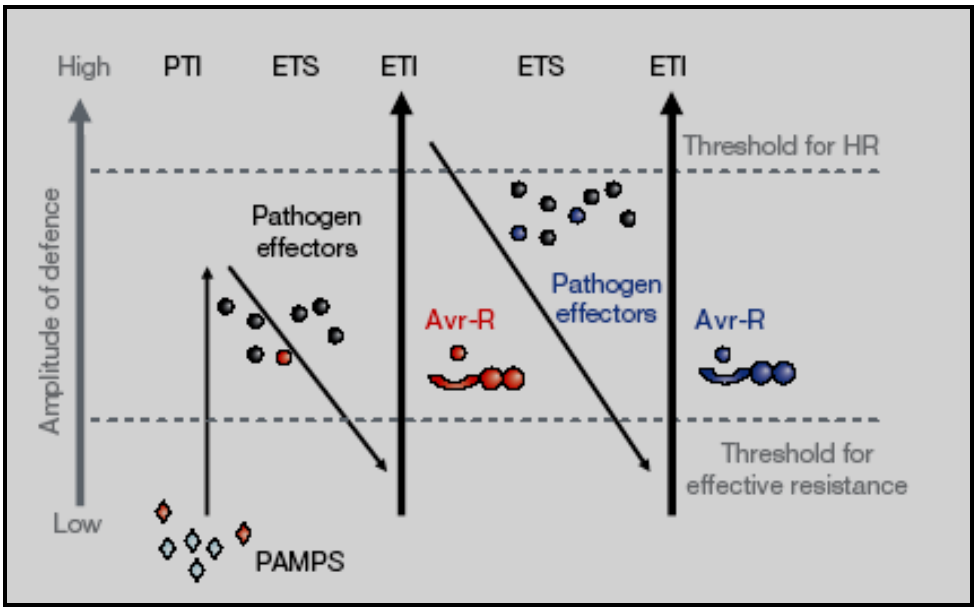


Figure 8 The zigzag model illustrating the quantitative output of immune response by Jones and Dangl (2006).

2.1.4.2 Recognition — the first step of defence

Extracellular recognition of pathogen associated molecular patterns

Pathogens that are able to overcome preformed host defence barriers are recognised by receptors located in the plasmamembrane due to PAMPs. According to Schwessinger and Zipfel (2008), PAMPs represent molecules that confer fundamental functions for survival and fitness to pathogens. Therefore, they are considered as "invariant epitopes" and cannot easily be abolished by pathogens. Mutation or deletion of the patterns would very likely cause a loss in fitness. Fungi-derived PAMPs include cell wall components, such as chitin and β-glucan or ergosterol in the plasmamembrane. Furthermore, cellulose-binding lectins, that are thought to be important for adhering to the cell surface of the host during infection, are reported to function as PAMPs. As they are vital for survival and fitness of pathogens, they are highly conserved across species and as a matter of co-evolution, they are, on the other hand, recognised by a wide range of hosts. In addition, plants are able to recognize "modified self": These are host molecules that are released as a consequence of wounding or pathogen attack, for example, break down products of host cell walls (Zipfel 2008).

PAMPs are detected by leucine rich repeat-receptor like kinases (LRR-RLKs) and receptor like proteins (RLPs) that are associated with the plasmamembrane. LRR-RLK are single pass transmembrane proteins composed of a LRR ectodomain, a transmembrane domain, and a cytoplasmic serine/threonine protein kinase domain. It has been shown that the leucine rich repeat ectodomain directly binds to the PAMPs causing a conformational change that activates the kinase domain leading to activation of downstream signalling pathways (Figure 9). Experiments with different PAMPs have indicated that response to PAMPs leads to a limited number of downstream signalling pathways and a common set of defence responses, which are conserved across plant families. In the downstream signalling pathway, the MAP kinases are reported to play a key role as negative and positive regulators of signalling (McDowell and Simon 2007).

In addition, receptor-like proteins (RLPs), transmembrane proteins with leucine-rich repeat domains and a short cytoplasmic tail are involved in PAMP perception. In rice, a receptor-like

protein with high affinity to chitin has been identified. Furthermore, there is evidence that receptors and receptor like proteins cooperate and that there is crosstalk between them (Schwessinger and Zipfel 2008). In regard to this, it is still an unsolved question whether PRR signals can activate different signal cascades in response to different classes of pathogens leading to specific defence reactions. It would be of great interest whether PRRs can differentiate between biotrophic and necrotrophic fungal pathogens, and consequently trigger the most efficient signalling pathway (Jones and Dangl 2006; Zipfel 2008).

It is thought that plants regulate their defence response tightly in the absence of pathogens by constitutive negative regulators. Following PAMP perception negative regulators are sequestered or degraded and lead to downstream signalling cascades and induction of defence response. It has been observed that seconds to minutes after PAMP treatment physiological changes are induced such as ion flux across the plasmamembrane, increased intracellular Ca^{2+} concentration, stomatal closure, oxidative burst, MAP kinase activation, protein phosphorylation, and protein–protein interactions. Members of the large family of MAP kinases and WRKY transcription factors are downstream regulators leading to changes in gene expression within 30 minutes (Figure 9). PTI defence response comprises cell wall fortification, callose deposition and production of antimicrobial compounds such as pathogenesis related proteins or secondary metabolites (Schwessinger and Zipfel 2008).

As mentioned above, Kang and Buchenauer (2000) did not observe any differences between resistant and susceptible cultivars in their preformed constitutive physical barriers. But, they observed induced reinforcement of cell walls, cell wall appositions and formation of papillae within 48 hai in resistant but not in susceptible cultivars. From this observation it can be concluded that in resistant cultivars, PAMP triggered immunity is efficient, whereas in susceptible cultivars PTI seems to be suppressed. This is further supported by Jansen et al. (2005) who observed that susceptible cultivars are able to build up cell wall appositions and physical barriers against *Fusarium graminearum* strains that are deficient in the production of phytotoxic DON, but not against the virulent wild-type.

From an evolutionary point of view, it is suggested that PTI is the first line of active plant defence that determines the host range of pathogens. According to Zipfel (2008), it is therefore the “primary driving force” of plant–pathogen co-evolution.

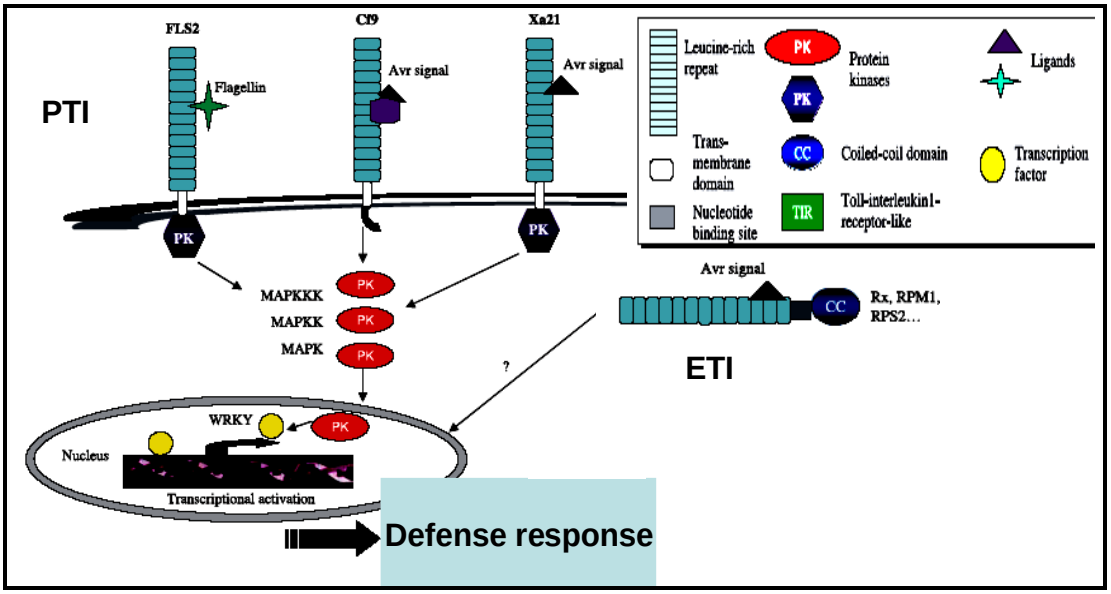


Figure 9 Overview of PAMP and effector triggered immunity (modified after Sanabria et al. 2008).

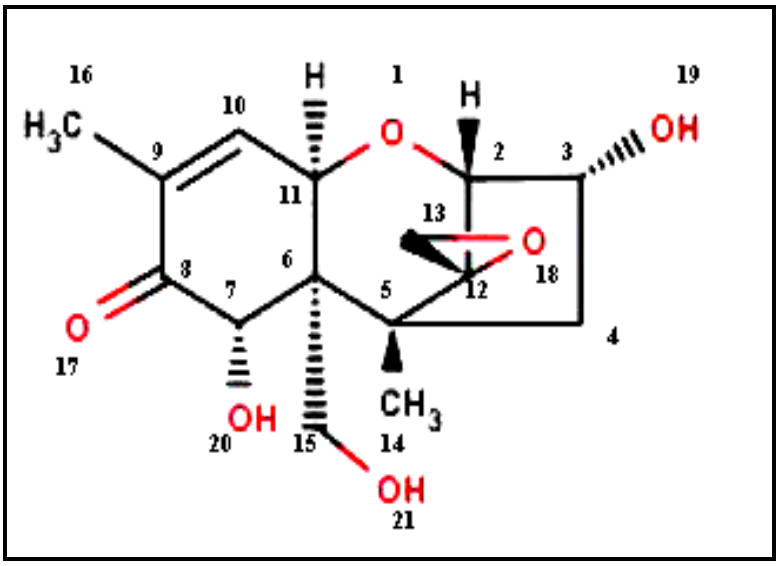
2.1.4.3 Virulence factors in the wheat–*Fusarium* interaction

As a consequence, pathogens are under high selection pressure to circumvent or to suppress PTI. As PAMPs are too essential to be subjected to frequent changes, pathogens follow the strategy to shift the evolutionary battle to specific effector molecules (Zipfel 2008). Effectors are proteins and secondary metabolites secreted by pathogens suppressing PTI. Effector molecules can interfere with recognition, defence signalling pathways, and metabolism. Furthermore, effectors degrade physical barriers and detoxify antimicrobial compounds.

In the wheat–*Fusarium* interaction, the phytotoxins DON and NIV are reported to be virulence factors supported by a secreted lipase (FGL1) (Lemmens et al. 2005; Voigt et el. 2007), and can thus be considered as effectors. For both groups of virulence factors evidence is provided that they are induced by components of the host and are not constitutively expressed in the fungus (Ilgen et al. 2008a; Voigt et al. 2007).

Deoxynivalenol as the major virulence factor

DON belongs to the group of trichothecene mycotoxins and is produced as a secondary metabolite by the fungal species *Fusarium graminearum* and *Fusarium culmorum.* DON is a non-volatile sesquiterpene of low molecular weight that has amphiphilic character. According to Rocha et al. (2005), its toxicity is mainly based on the epoxide at C12 or C13 and a double bond between C 9 and C10 (Figure 10). DON structure and function is preserved under acidic and neutral pH value and it is heat stable. (Rocha et al. 2005; Berthiller et al. 2005).



Deoxynivalenol

12,13-Epoxy-3,4,15-trihydroxy-trichothec-9-en-8-on.

Molecular formula:

$C_{15}H_{20}O_6$

Molar mass:

296,32 g/mol

Figure 10 Chemical structure of the virulence factor DON (modified after Berthiller et al. 2005).

According to Rocha et al. (2005) who reported on effects of trichothecenes in eucaryotic organisms, DON is phytotoxic and causes wilting, chlorosis, necrosis, and seedling inhibition. Owing to its amphiphilic nature, DON can interact with membranes, alter their structure and permeability. Furthermore, it is capable of crossing the plasma membranes of host cells, where all organelles are exposed to its detrimental effects. This is also confirmed by the observations of Kang and Buchenauer (2000) who reported degeneration of cytoplasm and disorganisation of organelles in relation to DON. On molecular level, synthesis of proteins, RNA and DNA is inhibited by DON. It is reported to interfere with the active site of peptidyl transferases on the small ribosome subunit inhibiting elongation and termination of amino acid chains. This is also

supported by the findings of the immunogold-labelling assays by Kang and Buchenauer (2002), where DON showed high affinity to ribosomes. Consequently, it can be concluded that all enzyme activity, the foundation of all pathways, is severely affected. For example, severe effects on mitochondria function and the process of cell division have been reported as well as degeneration of chloroplasts. Thus, it can be expected that the presence of DON may also impair induced defence response of host cells. According to Desmond et al. (2008a), strong evidence is provided that DON induces production of reactive oxygen species (ROS). Reactive oxygen species are supposed to trigger programmed cell death in plant cells facilitating the necrotrophic lifestyle of *Fusarium graminearum* on the one hand and inducing cell signalling cascades resulting in host defence, on the other hand.

DON as a virulence factor

Apart from cutinases, cell wall degrading enzymes such as cellulases, pectinases and xylanases, DON is reported to be extracellularily excreted in the process of host cell penetration. In particular for DON, strong evidence has already been provided to act as a virulence factor in the wheat–*Fusarium graminearum* interaction: Lemmens et al. (2005) found that application of purified DON causes similar visual symptoms as observed in fungal infection, e.g. bleaching of the heads and failure to develop kernels. Mutants of *Fusarium graminearum* with disrupted biosynthesis of DON caused less severe disease symptoms and were, in particular, restricted to the floret of initial infection (Jansen et al. 2005). This is in agreement with Ilgen et al. (2008b) who observed that DON is primarily induced in the rachis node. Taken together these findings resulted in the hypothesis, that DON may have less impact on initial infection, but may strongly facilitate spreading of disease from floret to floret within the spike.

In order to monitor where and when DON production is induced during colonisation, Ilgen et al. (2008b) used a transformed *Fusarium graminearum* strain harbouring a *GFP* reporter gene under the control of the *TRI5* promoter. The *TRI5* promoter naturally acts on the *TRI5* gene encoding a key enzyme of DON biosynthesis. Three to five dpi, DON was predominantly detected in the rachis node, and one day later DON was found in xylem vessels, phloem sieves and parenchyma cells adjacent to the vasculature.

While Ilgen et al. (2008b) followed induction of DON at transcriptional level, Kang and Buchenauer (2002) followed the chemical compound itself by immunogold-labelling. DON was detected in hyphae as soon as they got in close contact with host cells at about 36 hai. Seventy two hours after infection, DON levels increased dramatically indicated by high labelling density. It was shown that DON had already been present in host cells in close vicinity to penetration pegs before they were invaded. At subcellular level, DON was present in cell walls and plasmalemma, in the cytoplasm and the vacuole, in the chloroplasts, the endoplasmatic reticulum, and the ribosomes (Kang and Buchenauer 2002). As DON is water soluble it can be transported in the flow of the vascular bundles and hence was detected ahead of infection in uninfected spikelets (Bushnell et al. 2003).

In order to test the impact of DON as a virulence factor, Jansen et al. (2005) compared the colonisation performance of a DON producing wild-type strain with a transformed *tri5* knockout mutant. For detection of the fungal biomass, both fungal strains harboured the *GFP* reporter gene under control of a constitutive promoter. They could clearly demonstrate that the *tri5* knockout mutant, not being able to produce DON, was restricted by thickened walls of cells in the rachis node, where hyphae incapable of producing DON were trapped in *de novo* synthesised structures acting as physical barriers. By contrast, the wild-type strain managed to pass the rachis node and was able to spread via phloem and xylem vessels of the rachis, as no physical barriers were formed. In regard to these observations, Jansen et al. (2005) suggested, that cell wall thickening and production of physical barriers was based on *de novo* synthesis of pectins, cellulose, xylan

(callose) and phenolic compounds. And, that in the floret infected by the wild-type *de novo* synthesis was inhibited, very likely owing to the presence of DON. They drew the conclusion that particularly in wheat, DON appears to be necessary for fungal passage from the initially infected florets in the rachis and further beyond within the spike, and hence, acts as a virulence factor (Jansen et al. 2005).

Secreted lipase — FGL1

Despite the fact that most research effort has addressed the characterisation of DON and resistance to DON, Voigt et al. (2007) were able to demonstrate that the effect of DON in spreading of disease is supported by a secreted extracellular lipase. Voigt et al. (2005) characterised the *FGL1* gene encoding a secreted extracellular lipase that hydrolytically decomposes triacylglycerols (lipids) into glycerol and free fatty acids as found in membranes. FGL1 was found to be induced at early stage of infection (24 hai) and constantly transcribed over time course of infection. Heterologous expression in *Pichia pastoris* showed that FGL1 was not constitutively expressed and that inducer substrates were needed that are thought to be most likely esterified fatty acids. Furthermore, the degradation products of lipolytic enzymes may act as second messengers during the infection process. It is hypothesized that lipid substrates may be one of the signals that maintain and promote the hyphal growth of the fungus in the host environment.

The role of secreted lipase in the infection process was tested in a gene deletion experiment. Disruption of *FGL1* gene in a transformed *Fusarium graminearum* strain resulted in the lipase deficient mutant *fgl1* that showed reduced extracellular lipolytic activity and reduced virulence in wheat and maize (Voigt et al. 2005). *FGL1* was reported to be already induced at 24 hai, however, it appeared to have an important role at later growth stage for spreading through the rachilla and not for initial infection. Invasion of the *fgl1* mutant was stopped at the rachilla–rachis barrier and no hyphae were observed in the rachis. Moreover, a dark barrier like zone was formed in the transition zone from rachilla to rachis. In respect to these observations, it seemed that the *fgl1* mutant was restricted by the induced physical barriers of plant defence such as cell wall modifications, formation of papillae between plasma lemma and cell wall. It is hypothesised that FGL1 could be involved in host cell wall degrading process and in addition may act as a growth promoter activating other enzymes responsible for fast growth of fungal hyphae in the presence of appropriate host substrate. As a consequence, a loss of lipase activity would result in reduced fungal growth rate, and the plant would have more time to build up physical barriers in the transition zone of rachilla and rachis. As a result, the mycelium growth was about 50 % reduced in the *fgl1* mutant compared to the wild-type after 14 dpi (Voigt et al. 2005).

In order to relate lipase activity to DON, induction of DON was compared between the *fgl1* mutant and the wild-type strain by looking at the transcription level of *TRI5* over time course of infection. In the wild-type, expression of *TRI5* reached its peak 3 dpi when the fungus reached rachilla and rachis. Subsequently, *TRI5* transcription decreased when the fungus successfully passed these structural barriers in order to spread further within the spike. By contrast, in the *fgl1* mutant, *TRI5* expression was strongly increased over all stages of infection resulting in a *TRI5* expression level 5 fold higher than in the wild-type 14 dpi. As a consequence, DON levels found in *fgl1* mutant infected tissue were double as high as in the wild-type infected tissue. Voigt et al. (2007) concluded from these observations that the *fgl1* mutant was trapped at the rachis node, and constantly expressed DON in an attempt to pass through the rachis. They demonstrated that lipase deficient *fgl1* mutants although they produce DON were not able to spread through rachilla and rachis of a susceptible cultivar. And that "even the overproduction of DON could not restore the wild-type virulence in the virulence reduced *flg1* mutant". This is supported by Ilgen et al.

(2008a) who stated that DON and secreted lipase cannot complement each other, and thus mutants deficient in one factor cannot be rescued by the other.

As *fgl1* mutants show restricted growth and do only cause disease symptoms limited to the initially infected spikelet similar to *tri5* mutants, FGL1 can be considered as a virulence factor required for spread of the fungus, beside DON. Voigt et al. (2007) did not only demonstrate the role of secreted lipase as virulence factor, but they were also able to provide further evidence that components of rachilla and rachis may function as strong inducers of DON synthesis.

By testing various fungal disruption mutants on several host species, Ilgen et al. (2008b) found that DON has no influence in the *Fusarium* interaction with maize or barley whereas there is strong evidence for DON to be the major virulence factor in the *Fusarium* – wheat interaction. Ilgen et al. (2008b) stated that trichothecenes represent host-specific virulence factors. Furthermore, they found that secreted lipase (FGL1) appears not to be host specific being important for virulence in all interactions tested (Ilgen et al. 2008a).

2.1.4.4 Recognition of virulence factors

Effector molecules such as DON and FGL1 increase virulence and aggressiveness. Thus, they counteract PTI leading to ETS. However, they also signal the presence of the pathogen. Thus, plants can detect effectors as signals of invasion (Desmond et al. 2008a). Based on effector signals inside the host cells, plants evolved a second line of defence based on resistance genes (*R* genes). Resistance genes directly or indirectly recognize effectors and are responsible for ETI (Jones and Dangl 2006).

R genes were first described by Harold H. Flor in the 1950s who studied the flax–flax rust interaction, which led to the "gene for gene model". The "gene for gene model" predicts that defence is induced when a plant R gene recognizes the product of a corresponding virulence gene from the pathogen. Virulence factors that are recognized by *R* genes can be considered as defeated. Consequently, they are termed avirulence factors resulting in incompatible host–pathogen interaction. The "gene for gene model" can be integrated into the "zigzag model" as avirulent factors are effectors that are perceived by R genes resulting in effector triggered immunity. Most *R* genes encode nucleotide binding-leucine rich repeat (NB-LRR) proteins (Jones and Dangl 2006).

NB-LRR proteins consist of a C terminal leucine rich repeat domain, a central nucleotide binding domain and a N terminal effector domain. Leucine rich repeat domains provide recognition specificity while the nucleotide binding domain is the site of activation that triggers downstream signalling cascades. Within NB-LRR proteins, leucine rich repeat domain is predicted to act as negative regulator of the nucleotide binding domain by suppressing activation of it. As soon as the leucine rich repeat domain interacts with an effector, a conformational change allows for activation of the nucleotide binding domain that binds and hydrolyses ATP. It is thought that NB-LRR proteins allow for permanent signal perception because their conformation is stabilised by heat shock 90 proteins and other receptor co-chaperones in the cytosol (Caplan et al. 2008). The N-terminal domains are responsible for protein–protein interactions (Tameling and Joosten 2007; Caplan et al. 2008). There is good evidence that there is crosstalk between NB-LRR receptors and that they work in a concerted way in signal perception and in downstream signal transduction. It was further found out that for some *R* genes alternative transcripts are produced resulting in a predicted protein with N-terminal domain only, missing the LRR domain. This has also been reported for flax rust receptor M that produces several splice isoforms with the N-terminal domains functioning only. Tameling and Joosten (2007) suggested that these isoforms may function as adaptors in the signalling cascade.

Many of the annotated immune receptors harbour nuclear localisation signals and were found to be associated with the WRKY transcription factor domain in the nucleus. WKRY was identified to play a major role in transcriptional reprogramming during plant immune response. WRKY transcription factors recognize W-boxes of promoters of a large number of defence-related genes. They are involved in signal transduction pathways and are early induced in host–pathogen interactions (Golkari et al. 2007).

The guard hypothesis — direct and indirect recognition

Nucleotide binding leucine rich receptors can interact directly with the effector by physically binding to the effector molecule. For example in the flax–flax rust interaction, the *L* locus of flax rust encodes an effector that directly interacts with specifically corresponding LRR domain in the plant. This locus was used to study *R* genes and to establish the gene for gene model by Harold H. Flor. For the *L* locus many alleles are reported, even so for the corresponding NB-LRR locus, indicating the evolutionary drive to battle against recognition on one side and against effector triggered susceptibility on the other side.

Following the concept of Flor´s gene for gene model, plants would need to deploy thousands of *R* genes to counteract the diversity of effectors and pathogens. According to annotation projects, 150 *NB-LRR* genes and 500 *NB-LRR* genes have been estimated in *Arabidopsis* and rice respectively (McDowell and Simon 2007). This may not be enough to meet the high diversity of effectors. Although, for some *NB-LRR* genes high allelic diversity has been reported, as for the *L* locus in the flax–flax rust interaction. In addition to the very specific direct gene for gene interactions, *R* genes recognize changes of host targets in the cell that are caused by pathogen effectors. This indirect recognition of pathogen induced modified self allows for recognition of a wide range of effector proteins from different pathogens. In indirect recognition, NB-LRR proteins do not bind directly to the corresponding effectors, instead, they guard potential targets of effectors in the host cell. For example, perturbation by pathogens is monitored via cell wall integrity (Ferreira et al. 2007).The strategy to rather watch out for patterns of modified self than to specifically interact with each effector is referred to as “Guard Hypothesis”. The Guard Hypothesis derived from the idea that it is more efficient to look for the limited amount of different target sites in the plant body than to keep up with constantly evolving pathogen effectors that represent an overwhelming diversity. From a co-evolutionary point of view, recognition of pathogen-induced modified self, is considered to confer relatively stable, durable protection for a set of cellular targets (Jones and Dangl 2006).

2.1.4.5 Resistance — effector triggered immunity

In order to trigger the most efficient defence response, it is considered most important that signal perception and transduction allow for differentiation between biotrophic and necrotrophic pathogens. Responding to biotrophic, hemi-biotrophic and necrotrophic pathogens, LRR activation generally leads to salicylic acid and jasmonic acid mediated signalling pathways, respectively. In addition, several plant hormones and secondary messengers alter and fine tune the salicylic acid–jasmonic acid balance (Jones and Dangl 2006).

Response that confers effector triggered immunity (ETI) is reported to be faster and stronger than PAMP triggered response. Though, it was shown by transcription profiling that PTI and ETI triggered gene expression overlap to a high degree. ETI often but not always results in hypersensitive response and programmed cell death, particularly, effective against biotrophic pathogens. In many cases of ETI, it is still unknown what exactly restricts pathogen colonisation (Tameling and Joosten 2007; Jones and Dangl 2006). No specific *R* genes according to the “gene for gene model” have been established for the wheat–*Fusarium* interaction yet, however it may

be the case that the ribosomal protein 3, target of the effector DON, is under guard of immune receptors. There is evidence that DON triggers an immune response that may contribute to ETI (Desmond et al. 2008a; Golkari et al. 2007). Moreover, Lemmens et al. (2005) provided evidence that DON is metabolised into less toxic DON-3-O-glucoside. This would suggest that DON as an effector has already been at least partly defeated in resistant cultivars.

2.1.5 Major components of defence response

Recognition triggers signal transduction and local responses at the site of recognition such as changes in ion fluxes, oxidative burst, mitogen activated protein kinase activity, induction of secondary messengers, and expression of a large number of defence related genes. In addition, systemic response alerts tissue distant from the site of recognition. Systemic acquired resistance (SAR) is considered to be a long lasting, broad spectrum defence response (Buchanan et al. 2000).

2.1.5.1 Oxidative burst

As soon as extracellular receptors recognize PAMPs of penetrating fungal pathogens, Ca^{2+} channels are activated and the Ca^{2+} concentration in the cytoplasm increases. Ca^{2+} activates plasmamembrane-associated NADPH oxidases, pH-dependent peroxidases, and oxalate oxidases that catalyse oxidative reactions and generation of reactive oxygen species (ROS) in the apoplast (Berrocal-Lobo and Molina 2007). Owing to the fact that NADPH oxidases contain Ca^{2+} binding EF hand domains that are located in the cytoplasm, it is thought that oxidase activity is directly regulated through changes in calcium concentration (Buchanan et al. 2000). According to Buchanan et al. (2000), ROS occur within 5 min after challenge, and this rapid generation of massive amounts of ROS is also referred to as oxidative burst.

In the oxidative burst, free radical molecules with an unpaired electron such as the superoxide anion $O_2^{-\cdot}$ or hydroxyl radical $OH^{\cdot}$, and less reactive ROS such as hydrogen peroxide are produced. In general, ROS have enormous reactive but also destructive power in the plant organism (Foyer and Noctor 2009). As oxidative bursts take place in the apoplast, Bollwell et al. (2001) considered them as exocellular events that keep invading pathogens at bay and facilitate cell survival. By contrast, in the hypersensitive response, ROS are generated in the cytoplasm and lead to programmed cell death (Bollwell et al. 2001). In the apoplast, ROS have direct toxic effects on the invading pathogen. Furthermore, they catalyse rapid oxidative cross linking of preformed cell wall hydroxyproline rich glycoproteins to the polysaccharide matrix in order to strengthen the cell wall at the site of penetration (Buchanan et al. 2000). In addition, hydrogen peroxide induces the formation of papillae. Owing to its ability to build up gradients, directed signalling allows for localised formation of papillae exactly at the site of penetration (Bollwell et al. 2001). Furthermore, ROS trigger signalling events that lead to induction of a broad range of defence responses in order to restrict the pathogen, and thus, "the apoplast is the source and the route for most signals" and components in the early plant–pathogen interaction (Bollwell et al. 2001).

Owing to their high reactivity, ROS are not mobile and usually produced at the site where they are needed. As a result, additional secondary messengers are required for signal amplification and transmission in order to induce transcription and translation of defence related genes (Foyer and Noctor 2009). In particular, the MAP kinase cascade is reported to be activated by oxidative signals. For example, up regulation of MAP kinases was observed by Li and Yen (2008) in *Fusarium* challenged Sumai3 24 hai. Furthermore, ROS interact with signal transduction pathways mediated by salicylic acid, jasmonic acid, and ethylene (Foyer and Noctor, 2009).

2.1.5.2 Hypersensitive response

As soon as fungal hyphae can enter the protoplast and are recognised by receptors, generation of ROS is triggered in the cytoplasm. There, ROS have direct effects on hyphae growing in the intracellular space, but also lead to programmed cell death. These and related events are summarized as hypersensitive response (HR). HR triggers signalling and reprogramming in surviving neighbouring cells (Bollwell et al. 2001). Feirrera et al. (2007) reported that within few hours after programmed cell death, a set of defence genes are induced locally and signals are transmitted systemically through the plant. Thus, hypersensitive response triggers systemic signals leading to systemic acquired resistance (SAR). Cells that undergo apoptosis accumulate high levels of antimicrobial phenolic compounds, separate from the living neighbouring cells, and hence, trap and restrict the fungus.

According to Desmond et al. (2008a), DON elicits hydrogen peroxide production, programmed cell death, and defence response in wheat. Desmond et al. (2008a) who performed an infiltration assay with DON in wheat leaves, observed that within 6 hours after infiltration, hydrogen peroxide production was induced, followed by programmed cell death within 24 hours. In order to confirm that DON caused the active process of programmed cell death and not simple necrosis, Desmond et al. (2008a) tested for DNA laddering, an active process that requires *de novo* synthesis of proteins, a hallmark of programmed cell death. Performing a transcription profiling experiment, Desmond et al. (2008a) found that the defence related genes were induced by purified DON in a concentration dependent manner, and that a DON producing strain induced higher levels of defence related transcripts than a DON non-producing strain. Beside the general pathogenesis related proteins, interestingly, peroxidase and germin-like oxalate oxidases, both being involved in oxygen metabolism, were strongly upregulated. By adding ascorbate as an antioxidant, Desmond et al. (2008a) were able to proof that defence related genes are, in fact, induced by ROS, and that ROS act as signalling molecules in the wheat–*Fusarium* interaction.

Hypersensitive response and programmed cell death are frequently reported in incompatible interactions with biotrophic pathogens. As they rely on living tissue for survival, they are deprived of nutrients and trapped in dead matter. Whereas, necrotrophic fungi often produce toxins to induce programmed cell death during infection in order to make nutrients better accessible. Desmond et al. (2008a) hypothesised that DON assisted the necrotrophic phase of *Fusarium graminearum*, but simultaneously elicited and stimulated host defence.

2.1.5.3 The antioxidative system

Plants are equipped with a very efficient antioxidative system in order to maintain their redox balance. It is suggested that the antioxidative system plays a crucial role in plant defence. As it can scavenge ROS, it may counteract induced hypersensitive response and thus protect cells from cell death. It is further suggested that this may be of particular importance in the restriction of necrotrophic fungal pathogens (Foyer and Noctor 2009; Desmond et al. 2008a).

ROS are scavenged by antioxidative compounds and by enzymatic activity. The main antioxidants are ascorbate and reduced glutathione, but also secondary metabolites such as phenolic compounds, flavonoids, and carotinoids are able to protect structures from damage caused by ROS (Foyer and Noctor 2009). Enzymes involved are, for example, superoxide dismutase that converts the superoxide anion into hydrogen peroxide, and catalase that catalyses conversion of hydrogen peroxide into water and oxygen. Ascorbate is the most abundant low molecular antioxidant in plants. In particular, in the apoplast ascorbate plays a major role as redox buffer. It is involved in cross linking of cell wall components. Ascorbate oxidases use molecular oxygen to generate dehydroascorbate whereas ascorbate peroxidases oxidise ascorbate to monodehydroascorbate by converting hydrogen peroxide into water (Foyer and Noctor 2009; Buchanan et al. 2000). The resulting oxidised antioxidants are regenerated by reductases that

require NADPH as a cofactor (Buchanan et al. 2000). For the reduction, enzymes such as NADPH dehydrogenases, dehydroascorbate reductase, and glutathione reductase are needed. In this context, Foyer and Noctor (2009) mentioned the possible role of phytochromes and of enzymes, such as aldo/keto reductases, cytochrome P450s, and glycosyltransferases, contributing to control of secondarily released metabolites (Foyer and Noctor 2009).

2.1.5.4 Signalling pathways and secondary messengers

According to Turner et al. (2002) signalling pathways generally include the following steps: Firstly, pathogens are recognised and perceived by corresponding receptors. Secondly, a signal is transduced locally, and frequently also systemically. Thereby, phosphatases, kinases, and altered ion fluxes are involved in the transduction step downstream of perception. Subsequently, the secondary signal needs to be perceived. Perception of a secondary signal triggers biosynthesis of secondary messengers. The secondary messengers again need to be recognised in order to interact with transcription factor cascades. This, ultimately triggers alteration of gene expression for defence response. In addition, the signals of secondary messengers are integrated into other signalling pathways.

The phytohormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), are reported to play pivotal roles in these signalling pathways (Turner et al. 2002; Chen et al. 2009; Buchanan et al. 2000). Furthermore, auxin (indole-3-acetate) is mentioned to play a role in plant–fungi interactions (Doohan et al. 2008; Charvet-Candela et al. 2002). In addition, ROS, Ca^{++} and small peptides, and proteins are thought to act as secondary messengers (Buchanan et al. 2000; Turner et al. 2002; Lascombe et al. 2008; Foyer and Noctor 2009).

Every single step in the complex process of signal transduction offers several potential sites for regulation and crosstalk in the signalling pathway. Basically, signal transduction can be controlled at the level of perception and at the level of biosynthesis of secondary messengers. In this context, several biosynthetic and catabolic pathways as well as activation and deactivation cascades have been elucidated mainly by testing double and triple mutants of *Arabidopsis* (Buchanan et al. 2000; Turner et al. 2002). Crosstalk between different signalling pathways includes synergistic or repressing effects, positive and negative feed back loops and allows for fine tuning within the signalling network of defence response (Turner et al. 2002; Adie et al. 2007). For example, genes involved in the JA signalling pathway are also JA responsive indicating the presence of a feed back loop (Turner et al. 2002). At present, biosynthesis of secondary messengers is considered to be well established, whereas further research effort needs to be undertaken concerning the regulation of biosynthesis, processes in signal perception, and fine tuning mechanisms. Deciphering the crosstalk between the key phytohormones involved in defence response is considered as the major challenge (Adie et al. 2007). Adie et al. (2007) and Turner et al. (2002) concluded that complex multiple control allowed for high level of flexibility, specificity, and precision in defence response.

Salicylic acid

Salicylic acid (Figure 11) has been associated with hypersensitive response and systemic acquired resistance (SAR). It was demonstrated that SA can alert cells systemically in distant tissues, and consequently, prime defence response rendering distant cells resistant to subsequent pathogen invasion. Furthermore, SA was shown to trigger the expression of several *PR* genes, in particular *PR-1*. Thus, Turner et al. (2002) reasoned that SA is necessary for full expression of both local and systemic acquired resistance. Furthermore, SA has been reported to suppress ET production as it blocks the conversion of the precursor ACC to ET (Buchanan et al. 2000; Turner et al. 2002).

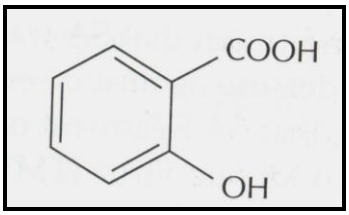


Figure 11 Chemical structure of salicylic acid (Buchanan et al. 2000).

Jasmonic acid

JA has been observed to mediate signals in local and systemic response. Turner et al. (2002) suggested that JA might be synthesised locally and that systemic induction is mediated via the small peptide systemin. JA induces PAL and CHS which are key enzymes of secondary metabolism. Interestingly, apart from signalling in defence response, JA is required for pollen development (Turner et al. 2002).

Jasmonic acid derives from lipids that are released from membranes by phospholipases. These cellular lipids subsequently undergo oxidation steps with ROS. Lipoxygenase (LOX) is classified as PR protein that catalyses the oxygenation of the fatty acids linoleic and linolenic acid in the octadecanoid pathway. Furthermore, allene oxide synthase and allene oxide cyclase are key enzymes in the biosynthesis of JA (

Figure **12**) (Buchanan et al. 2000).

In regard to biosynthesis and signal transduction, COI1, a membrane spanning receptor protein has been identified as a potential key regulator of JA mediated signalling pathway in *Arabidopsis*. The *COI1* gene encodes a protein with an F-box domain and a leucine-rich domain. F-box domains bind to regulatory proteins, for example, E3 ubiquitin ligase that is responsible for ubiquitin mediated protein degradation by proteasomes. Turner et al. (2002) suggested that a key regulator of JA response is recruited by the COI1 protein as substrate for ubiquitination and subsequent degradation. In addition, Turner et al. (2002) considered it very likely that JA response is regulated by ET responsive factors (described below), as microarray data provided evidence that JA responsive genes were associated with ET, SA, and auxine signalling pathways.

In the wheat–*Fusarium graminearum* interaction, results obtained by Li and Yen (2008) suggested that FHB resistance in Sumai3 may be predominantly regulated by JA and ET signalling. Li and Yen (2008) demonstrated that a susceptible Tibetian landrace could be rescued and resistance levels of Sumai3 could be increased by spray application of ET and JA three days before inoculation. By contrast, SA application did not rescue the susceptible plant. These observations are in agreement with several reports that SA-mediated signalling pathway is triggered by biotrophic fungi rather than by necrotrophic ones. Infections by necrotrophic fungi are reported to trigger ET and JA-mediated defence response. However, for *Fusarium graminearum* exhibiting a biotrophic lifestyle at early stage and switching to a necrotrophic lifestyle at later time-points, fine tuning mechanisms might turn out to be more complex.

Furthermore, Li and Yen (2008) performed a transcription profiling experiment of *Fusarium*-challenged wheat lines at the time-point 24 hai. Key enzymes such as allene synthase were found to be upregulated in Sumai3 after 24 hai but not in the susceptible Tibetian landrace. In addition, PR proteins that are indicative for SA-mediated signalling were observed to be upregulated in both lines. Li and Yen (2008) reasoned from their experiment that JA and ET mediated signal transduction pathways may make a significant difference in resistance to FHB.

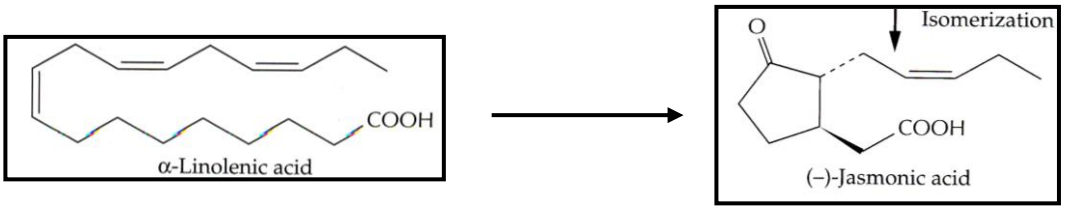


Figure 12 Chemical structure of α–linolenic acid, the precursor of JA (Buchanan et al. 2000).

Ethylene

Several defence responses have been related to the effects of ET: Ethylene is reported to stimulate the vascular gel production for occlusion of xylem vessels, a phenomenon that may be similar to the one observed in incompatible wheat–*Fusarium graminearum* interactions. In addition, it has been reported that ET induces cross-linking of hydroxyproline-rich proteins, and thus induces strengthening of the cell wall. Furthermore, ET is involved in the induction of PR proteins and phytoalexin biosynthesis where it acts as a component and fine modulator in the complex signalling network (Adie et al. 2007). In addition, Ludwig et al. (2005) suggested that ET mediated the cross talk between calcium dependent protein kinases (CDPKs) and mitogen activated protein kinases (MAPKs), both groups being key players in signal transduction pathways.

ET and JA act synergistically in the induction of several PR proteins, for example, PR1-b, PR3, and PR5. It is well established that the JA/ET signalling pathway antagonises SA mediated signalling, however co-operative interactions between ET and SA as well as suppressing effects of ET on SA have been reported. ET is considered as the fine modulator between signalling pathways. ET biosynthesis is tightly controlled by multi-gene families and the strong regulation is considered as an indication that ET acts in a concentration dependent manner rather than in a present/absent dependent manner. Spatial and temporal variations of endogenous ET levels are pivotal in the fine-tuning of the most suitable defence response (Buchanan et al. 2000; Adie et al. 2007).

In respect to the role of ET as a fine modulator, research has focussed on ET responsive transcription factors and their DNA binding sites. ET responsive genes have GCC sequences in their promoter regions, these cis-elements are also referred to as GCC boxes and ET responsive elements. Many PR proteins are reported to have GCC boxes. GCC boxes are important for the recognition by ET responsive factors (ERF). ERF proteins represent transcription factors that specifically bind to GCC boxes present in promoters of pathogenesis related proteins, and thus can activate transcription of *PR* genes. The ERF superfamily is one of the largest gene families of transcription factors in plants implying the importance of regulation of ET mediated response. Furthermore, ERFs have sites for phosphorylation by MAP kinases for activation and deactivation. As an additional regulatory element several ERF proteins have F-box domains that subject them to degradation by the ubiquitin mediated proteasomal pathway.

ERF factors can function as activators or repressors of defence response. Overexpression experiments of certain characterised ERFs resulted in enhanced resistance to specific pathogens while enhancing susceptibility to others, indicating the very precise and fine tuned response to specific stresses. For example, Zhang et al. (2007) obtained a full length cDNA clone of TaERF from Sumai3 challenged with *Fusarium graminearum*. TaERF was characterised to have a nuclear localisation signal and a GCC box binding domain. Transcription profiling by quantitative RT-PCR showed that *TaERF3* was differentially expressed between resistant Sumai3 and a susceptible cultivar at different time-points over time course of infection. *TaERF3*

transcription level was 3 to 6 fold higher in the susceptible cultivar compared with Sumai3 at 12 hai, whereas at later time-points 24 hai and 48 hai *TaERF3* in Sumai3 showed 3 to 9 fold higher transcription levels compared with the susceptible cultivar. When applying exogenous ET and JA to Sumai3, induction of *TaERF3* occurred within 1 to 4 hours respectively and peaked 24 hours after application. From the observation that response to exogenous hormones was significantly faster than to pathogen attack, Zhang et al. (2007) concluded that TaERF3 plays rather a role in defence response by being inducible by the signalling pathways of SA, JA, and ET at different time-points of plant–pathogen interaction.

Based on previous reports that pathogens may exploit the ET signalling pathways of their host plants, and further based on the fact that ET as a phytohormone is associated with senescence, and premature senescence of wheat heads is the outcome of compatible wheat–*Fusarium* interaction, Chen et al. (2009) developed the hypothesis that *Fusarium graminearum* exploits ET signalling for colonisation. *Arabidopsis* mutants deficient in ET biosynthesis, perception, and signal transduction were challenged *with Fusarium graminearum* and all showed reduced disease symptoms and a decrease in production of fungal conidia compared with the wild-type. In addition, mutants were tested that constitutively expressed genes involved in ET signalling pathway. It was found that they showed increased susceptibility — greater lesions and conidial production. These observations in the dicotyledonous model plant *Arabidopsis* laid the foundation for further bioassay experiments with detached wheat leaves and wheat heads. Chen et al. (2009) used the susceptible cultivar Bobwhite and a transgenic derivative. The transgenic line was impaired in expression of the *EIN2* ET signalling gene by means of RNAi-induced gene-silencing technique. In the transgenic line EIN2 expression was reduced by 50 % compared with the parental line Bobwhite. Firstly, exposure of detached wheat leaves to DON showed less severe DON induced cell death in the transgenic line impaired in ET signalling than in Bobwhite. Chen et al. (2009) concluded from this result that ET signalling influenced HR and programmed cell death. Furthermore, Chen et al. (2009) performed inoculation experiments in the wheat lines and found that under conditions of spray- and point inoculation the transgenic line showed enhanced type I and type II resistance. Disease symptoms, conidial production, and most importantly DON content in the harvested grain were significantly reduced in the *EIN2* gene-silenced line compared with the parental line Bobwhite. Unfortunately, these observations were not supported by transcription profiling data in the publication by Chen et al. (2009).

Experiments conducted by Zhang et al. (2007), Li and Yen (2008), and Chen et al. (2009) exemplify the effort undertaken to entangle the complex role of ET as a fine modulator in the signalling network. Different experimental setups and objectives show the diversity of steps and time-points ET might be involved in compatible and incompatible wheat–*Fusarium graminearum* interactions.

2.1.5.5 Pathogenesis related proteins

Proteins that are induced and upregulated in response to pathogen attack or in related situations are determined as pathogenesis related proteins (PR proteins). In general, PR proteins show high diversity in amino-acid sequence, biochemical function, and site of action. As common features they have low molecular weight and exhibit high thermal and pH stability. In addition, they are reported to be resistant to proteolytic degradation. These common features implicate that PR proteins can persist in hostile environments created upon pathogen attack. In respect to this, PR proteins are reported to mainly accumulate and act either in the intercellular space or are released from the vacuole in response to intracellular growth of the fungus (Tuzun and Somanchi 2006; Ferreira et al. 2007). PR proteins are present in many isoforms (isoenzymes) with different expression patterns and biological functions.

It is reported that *in planta* a combination of different members of one class is induced for defence response acting in synergism. Ferreira et al. (2007) stresses that there is a variation in effectiveness of closely related PR proteins and isoforms against different pathogenic fungi.

According to their amino acid sequence, biochemical properties, and biological function (Muthukrishnan et al. 2001) and the site of action (Ferreira et al. 2007), PR proteins are categorised in classes and subclasses. Up to now 17 classes have been established (Ferreira et al. 2007), thereof some classes are frequently upregulated in response to fungal penetration and colonisation: PR-1 proteins have antifungal activity, the group of PR-2 comprises β-1,3-glucanases, PR-3 and PR-4 proteins are different classes of chitinases and chitin binding proteins, and PR-5 are thaumatin-like proteins with antifungal properties. The class of PR-9 consists of peroxidases that induce lignin formation, first described in tobacco. Furthermore, PR-14 class proteins belong to the group of lipid transfer proteins, first described in barley (Barley LPT4). PR-15 and PR 16 are germin-like oxalate oxidases and oxalate oxidase-like proteins, also first described in barely. Furthermore, the classes of PR proteins were originally divided into two subclasses, an acidic subclass that is found in the intercellular space and reported to be induced by SA and a basic subclass found in plant cell vacuoles and is induced by ET and JA (Ferreira et al. 2007).

According to Tuzun and Somanchi (2006), PR proteins accumulate in compatible and incompatible interactions the same way, and thus do not determine the resistance response. This is in agreement with Christensen et al. (2004) who reported that the majority of *PR* genes were induced in compatible as well as in incompatible wheat–pathogen interactions.

The signalling events that lead to expression of PR proteins are diverse, complex, and only partly elucidated. Second messengers such as ROS, JA, ET, and SA have been shown to induce and regulate expression of PR proteins. For example, osmotin and PR-1 in tobacco were reported to be upregulated by JA and ET (Ferreira et al. 2007). Downstream of the second messengers, transcription factors and elements in the promoters of PR proteins are required for transcription and translation of PR proteins. For example, the GCC box is an ET responsive element that has been identified in many PR protein promoters indicating that these genes may be involved in ET mediated defence response. The GCC box was found in PR-5 protein gene promoters, and in the *Arabidopsis* promoter of PR-1 where it exhibited jasmonate responsiveness (Ferreira et al. 2007).

PR proteins are reported to contribute to defence response in two fundamentally different ways: Firstly, they have direct lytic or toxic effects on pathogens. And secondly, they are involved in the signalling cascade by creating elicitors, acting as secondary messengers or signal molecules by themselves (Muthukrishnan et al. 2001). Acting in a concerted way, PR proteins are capable of targeting different structures of the fungal pathogen that are exposed to the plant–fungus interface. First, the cell wall is targeted that consists of the polymers chitin, β-1,3-glucan, and glycoproteins. Cell wall synthesis can be inhibited and polymers can be degraded into oligomers. In a second step, fungal membranes can be disrupted by pore and channel formation leading to efflux of cellular components and changes in potential (Ferreira et al. 2007).

Hydrolytic enzymes such as chitinases and β-1,3-glucanases are mainly categorized in the classes PR-2, PR-3, and PR-4. They have dual function in defence response: Firstly, they have antimicrobial effects against invading pathogens. Secondly, they act as enhancers of defence response as they increase the concentration of PAMPs by releasing oligosaccharides from fungal cell walls. This is supported by the observation that hydrolytic enzymes are reported to be early induced after pathogen attack. According to Muthukrishnan et al. (2001), this results in enhanced oxidative burst or HR (Tuzun and Somanchi 2006), and induces expression of PR proteins and enzymes involved in the phenylpropanoid pathway. The acidic subclasses of these enzymes are mostly found in intercellular spaces and interact with fungal hyphae. Whereas, the basic

subclasses that are present in the vacuoles may act as soon as hyphae grow in the intracellular space, destroy cell integrity, and cause the protoplast to burst. Chitinases and glucanes act together to digest chitin-glucan fibre mixtures of fungal cell walls. Chitinases and β-1,3-glucanases are reported to have synergistic effects. Anand et al. (2003) showed enhanced resistance to *Fusarium graminearum* in transgenic wheat by overexpression of chitinase and β-1,3-glucanases in the greenhouse but these transgenic wheat lines failed to maintain resistance in the field. For wheat in particular, Caruso et al. (2001) identified wheat win3 and wheat win4 belonging to the PR-4 chitinase family to have very distinct antifungal activity. The class of PR-4 wheat win proteins was also found to be upregulated 24 hai in Sumai3 and near isogenic derivatives being susceptible to *Fusarium graminearum* in the microarray experiment performed by Golkari et al. (2009).

The classes PR-1 and PR-5 are reported to interact with fungal plasmamembranes and to inhibit fungal growth. According to Ferreira et al. (2007), PR-1 and PR-5 are thought to create transmembrane pores and thus the two classes are also termed permatins. The family of PR-1 is a highly conserved protein family found in all plant species examined to date. In the rice genome, 39 different genes with homology to *PR-1* have been annotated (Ferreira et al. 2007). Members of the PR-1 protein family have been frequently observed in defence reactions and they tend to be upregulated to high levels, reaching 1–2% of total leaf protein. The mechanisms are not elucidated yet. However, PR-1 proteins have been demonstrated to have antifungal activity *in vivo and in vitro.* Thaumatins and thaumatin like proteins (PR-5 proteins, osmotins) have antifungal activity and are thought to permeabilise fungal cell membranes and cause leakage of cytoplasmic material and rupture of hyphae. Some fungi have glycoproteins in their cell walls that can immobilize osmotins. Overexpression of these glycoproteins in *Fusarium oxysporum forma specialis nicotianae* being susceptible to osmotin rendered the fungus resistant to it (Tuzun and Somanchi 2006). Chen et al. (1999) introduced thaumatin like proteins of rice under the control of a constitutive maize ubiquitin promotor and a chitinase under control of the CaMV 35S promoter to the susceptible wheat line Bobwhite. In transformed wheat plants outbreak of FHB was delayed, but TLP did not restrict the fungus in later stages of infection indicating that it does not play a decisive role in resistance to *Fusarium graminearum* (Muthukrishnan et al. 2001). Moreover, Ferreira et al. (2007) reported that osmotin in tobacco has been reported to stimulate a MAP kinase acting in signal transduction. Pritsch et al. (2001) who performed a Western Blot of *Fusarium* infected tissue observed that transcript accumulation of PR-4 and PR-5 was earlier (6 hai) and stronger in Sumai3 than in the susceptible cultivar. Although, there was no difference in colonisation progress between the resistance and the susceptible cultivar at these time-points. Conducting histological work, Pritsch et al. (2000) were able to demonstrate that induction of *PR* genes coincided with conidia germination and first contact of germination hyphae with the surface and stomata. Highest transcript accumulation was observed between 36 and 48 hai, coinciding intracellular colonisation. This is in agreement with the cDNA microarray data of Golkari et al. (2007): PR protein expressed sequence tags (ESTs) were detected 6 to 12 hai with a peak at 36 to 48 hai. More recently, Golkari et al. (2009) even provided evidence that members of the PR-5 protein family could be associated with the QTL on 3BS as they were exclusively upregulated in Sumai3 but not in its susceptible near isogenic derivatives lacking the resistant Sumai3-alleles in this QTL region.

Specific peroxidases that are upregulated after pathogen attack have been classified as PR-9. Peroxidases are reported to be mainly found extracellular in the apoplast. They catalyse processes in lignin biosynthesis and thus play a major role in induced cell wall reinforcement and formation of physical barriers. During lignin polymerisation, free ROS (oxygen anion O_2^-, hydroxyl anion OH^-) are released that derive from the reduction of molecular oxygen. In the presence of a hydrogen donor, peroxidases can convert ROS into hydrogen peroxide. Thus, PR-9 peroxidases

are involved in protection of cells against ROS. According to Golkari et al. (2007) who performed transcription profiling experiments with DON and *Fusarium graminearum* challenged barley, ascorbate peroxidases belonging to the PR-9 class are reported to be associated with the JA signalling pathway. Pritsch et al. (2000) observed that peroxidase levels remained high in early time course of *Fusarium graminearum* infection in Sumai3, whereas they significantly earlier declined in the susceptible cultivar Wheaton.

In addition, PR-15 and PR-16 families are involved in the generation of hydrogen peroxide. For example, germin-like oxalate oxidases are stable glycoproteins that were initially discovered in cereals where they were induced in response to fungal infection (Ferreira et al. 2007). Germin-like oxalate oxidases catalyse degradation of oxalic acid into hydrogen peroxide and carbon dioxide (Foyer and Noctor 2009). Race non-specific resistance to cell wall penetration by fungi was found to be accompanied by the accumulation of H_2O_2 in several plant species (Christensen et al. 2004). Hydrogen peroxide induces plant defence response and enhances cell wall strengthening (Ferreira et al. 2007). In addition, the generated hydrogen peroxide may have direct toxic effects on penetrating fungi (Tuzun and Somanchi 2006; Muthukrishnan et al. 2001).

TaGLP4 in wheat represents a subfamily of germin-like protein (*GLP)* genes and encodes a protein with superoxide dismutase activity. *TaGLP4* in wheat was found to be expressed in epidermal cells under attack of a pathogen and the corresponding proteins are supposed to accumulate in the apoplast. Plant superoxide dismutases are reported to scavenge ROS together with catalases, thereby generating hydrogen peroxide. The generated H_2O_2 is thought to be involved in the cross linking of cell wall polymers that are responsible for *de novo* development of local cell wall fortification structures (Christensen et al. 2004). In addition, transient overexpression and gene-silencing experiments functionally characterised HvGLP4 as a functional superoxid dismutase in barley. Furthermore, it was demonstrated that *TaGLP4* contributes to disease resistance in wheat and was found to be exclusively upregulated in incompatible plant–pathogen interactions (Christensen et al. 2004).

Lipid transfer proteins categorized as PR-14 proteins are induced in response to infection and are thought to be involved in SAR. In general, lipid transfer proteins (LTPs) are small proteins which are characterised by the formation of a hydrophobic pocket or a tunnel-like cavity where lipids and their derivatives can be bound to, harboured, and transferred. Lipid transfer proteins exhibit great variety in ligand specificity, and LTPs capable of forming complexes with a broad range of lipids and even organic molecules others than lipids are termed non-specific lipid transfer proteins. PR-14 proteins are reported to have signalling peptides that localise them in close vicinity to the plasmamembrane or to the cell wall. Moreover, they are reported to act in the extracellular space and on exposed surfaces, for example, in the vascular tissue (Kirubakaran et al. 2008).

Lipid transfer proteins are organised in multi-gene families of enormous complexity. For example, Boutrot et al. (2008) identified 156 putative non-specific *Ltp* genes in wheat. Most work was done to characterise the pathogen inducible *DIR1* (deficient induced response) wild-type gene encoding a putative lipid transfer protein in *Arabidopsis thaliana.* Maldonado et al. (2002) identified an *Arabidopsis* mutant (*dir1-1*) that showed good local resistance to pathogen attack but was deficient in systemic required resistance (SAR). Maldonado et al. (2002) reasoned that in *dir1-1* mutants that show same local defence response as wild-type plants systemic acquired resistance should be triggered, but that either the production of the mobile signal, the transmission or the perception in distant leaves must be impaired. In addition to these findings, the gene responsible for SAR deficiency was assigned as a putative non-specific lipid transfer protein by homology search. Later, Lascombe et al. (2008) found out that the wild-type DIR1 protein exhibited higher affinity to lipids that are released under the action of secreted lipases

(mono-acetylated lipids). They reasoned that such lipids released under pathogen attack may play a role in signal transduction.

In addition, Buhot et al. (2004) detected similarity between lipid transfer proteins involved in pathogenesis and small cysteine-rich molecules that are released by Oomycetes and are termed "elicitins". Elicitins are reported to interact with plant receptors and trigger hypersensitive response and SAR. Buhot et al. (2004) demonstrated that a wheat lipid transfer protein showed high affinity to an elicitin receptor in the plasmalemma and competed with the elicitor for binding sites of receptors located in the plasmalemma. In respect to these findings and, furthermore, in order to test the function of the lipid transfer protein, Buhot et al. (2004) tested which ligands show affinity to the tobacco lipid transfer protein LPT1 and whether the LTP1–ligand complex enhanced the affinity of the lipid transfer protein to the elicitin receptor in tobacco. Furthermore, it was of great interest whether binding of the complex to the receptor may trigger any defence response and long distance signalling for SAR. They regarded JA and its precursor linolenic acid as the most interesting ligands owing to their well confirmed role in plant signalling. Buhot et al. (2004) were able to demonstrate that tobacco LPT1 forms complexes with JA. These complexes compete with elicitin in binding to the receptors and by doing so are capable of inducing long distant signalling and SAR in distant leaves of tobacco challenged with *Phytophtera parasitica*. By contrast, lipid transfer proteins or JA on their own were not able to prime resistance in distant leaves. In respect to fungal pathogens Lascombe et al. (2008) also hypothesised that lipid transfer proteins that build complexes with sterols from the fungal plasmamembrane might interact with receptors and trigger plant defence response. In addition, many of the putative PR-14 lipid transfer proteins are reported to show antimicrobial activity *in vitro*. It is hypothesised that the molecules may insert themselves into fungal membranes and cause pores and efflux of ions (Ferreira et al. 2007). However, the exact biological functions of members of the diverse lipid transfer protein family still remain to be elucidated.

2.1.5.6 Secondary metabolites in plant defence

Defence response involves the redirection of carbon flow from primary to secondary metabolic pathways. This is caused by enhanced expression of enzymes that are involved in these pathways such as peroxidases, lipoxygenase, and phenylalanine ammonia lyase (Ferreira et al. 2007). According to their biosynthetic precursors and pathways, secondary metabolites in plants are grouped in terpenoids, phenylpropanoids, and alkaloids. For monocots, some terpenoids and phenylpropanoids are reported to play pivotal roles in plant–pathogen interaction. For example, it was shown that the triterpenoid "avenacin" determines the resistance of oats to the root infecting fungus *Gaeumannomyces graminis var. tritici* causing take-all disease in wheat (Osbourn 2003).

According to their function in plant defence, secondary metabolites with low molecular mass and antimicrobial and antifungal activity can be categorised into preformed phytoanticipins and induced phytoalexins. They can be considered as the backbone of chemical defence. In addition, structural components for physical barriers in plant defence such as polymeric phenolic compounds derive from secondary metabolism (Buchanan et al. 2000; Richter 1996).

Terpenoids

The precursor of all terpenoids is isopentenyl diphosphate, a 5 carbon unit (isoprene unit) that derives via the mevalonic acid pathway. Joining of isoprene units results in monoterpenes with 10 C, sesquiterpenes with 15 C, diterpenes with 20 C, and triterpenes with 30 C atoms. Terpene cyclases catalyse cyclisation steps from basic multiple 5 C units of isoprene. The basic skeleton produced by terpene cyclases is secondarily modified by oxidation and conjugation steps. To introduce additional oxygen atoms, hydroxylations, and epoxidations are performed. In these

steps various highly stereo-selective and substrate specific cytochrome P450 and $NADP^+$ dehydrogenases that oxidise alcohols to ketones are involved (Buchanan et al. 2000; Richter 1996).

The group of triterpenes (30C) comprises phytoalexins and compounds with antimicrobial properties such as the saponine avenacin. The basic structure is a flexible 30 C chain with double bonds referred to as squalene. Squalene is oxygenised by mono-oxygenases, and several cyclisation steps catalysed by membrane associated cycloartenol synthases lead to cycloartenol that serves as a precursor for phytosteroles such as brassinosteroids (Figure 13) (Richter 1996).

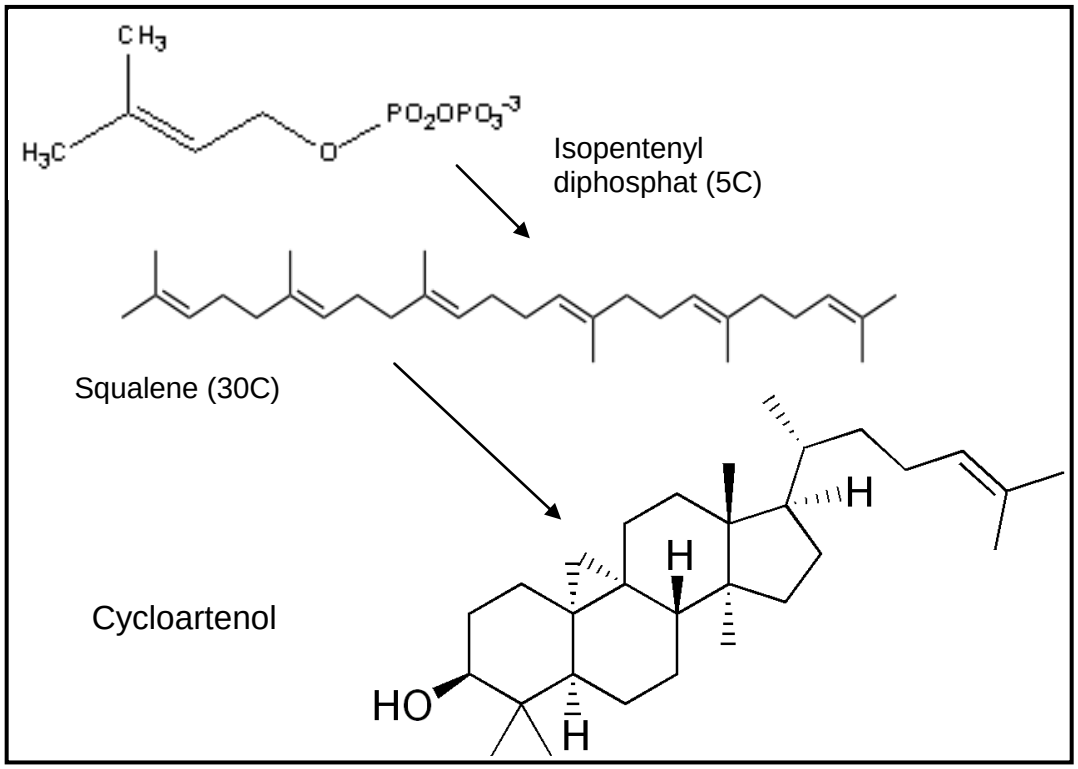


Figure 13 Key intermediate compounds in biosynthesis of triterpenes (Buchanan et al. 2000).

Phenylpropanoids – phenolic compounds

Most phenylpropanoids (6 membered C ring structure with an aliphatic 3 C chain) derive from the aromatic amino acids phenylalanine and tyrosine via the phenyl-propanoid, phenyl-propanoid-acetate pathway. Phenolic compounds are hydrophilic, as acidic hydroxyl groups are attached to the aromatic phenyl-ring of phenylpropanoid.

Phenylalanine ammonia lyase (PAL), as the key enzyme in the phenylpropanoid pathway, directs the carbon skeleton from phenylalanine and tyrosine to the synthesis of phenyl-propanoid. PAL converts the amino acids phenylalanine into cinnamic acid and tyrosine into p-coumaric acid by releasing an ammonium ion (Figure 14).

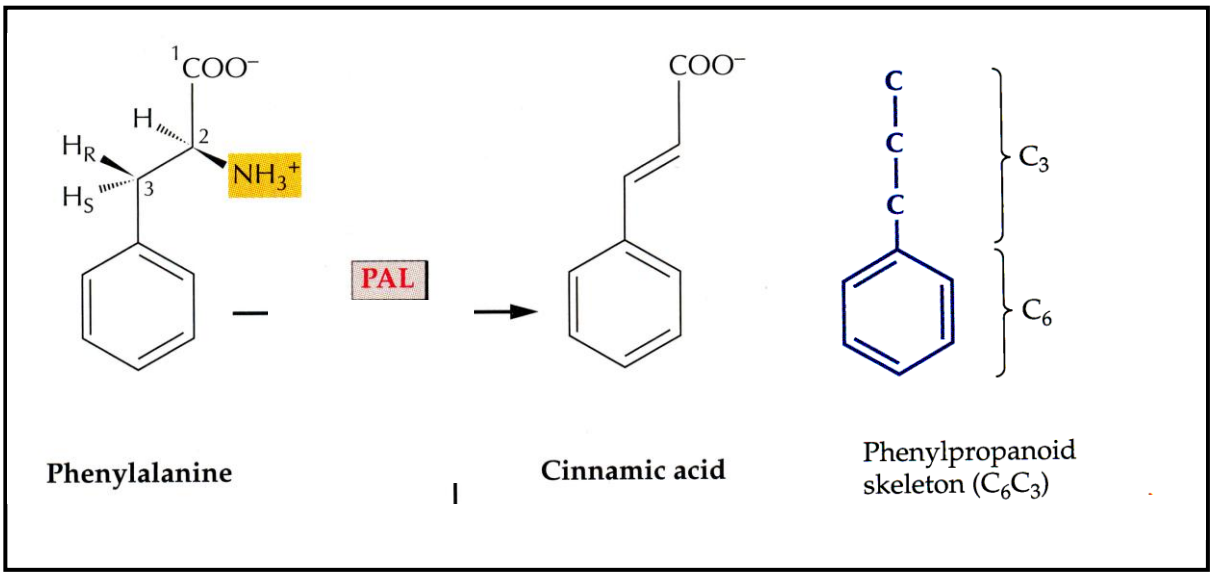


Figure 14 Phenylpropanoid pathway (modified after Buchanan et al. 2000).

In general, it is reported that strong induction of PAL is associated with hypersensitive response. And in particular, there is evidence that enzymes involved in the phenylpropanoid pathway are induced in the wheat–*Fusarium graminearum* interaction: Desmond et al. (2008a) showed that PAL and chalcone synthase (CHS) were upregulated in the resistant wheat cultivar. This is in agreement with Steiner et al. (2009) who found PAL and CHS to be upregulated in resistant CM82036, and with Golkari et al. (2007) who found cinnamate-4-hydroxylase and polyphenol oxidase upregulated in the resistant cultivar after 24 hai. More interestingly, Golkari et al. (2009) found PAL to be strongly up-regulated 24 hai in Sumai3 but not in its susceptible near isogenic derivatives lacking the Sumai3 alleles in QTL regions on chromosome 3BS and 2AL. Based on these observations, Golkari et al. (2009) concluded that compounds of the phenylpropanoid pathway may make a major difference in resistance.

Lignin — a polymeric phenolic compound

Cinnamic acid is converted into monolignols. The basic aromatic skeleton is changed by enzymatic reactions such as aromatic hydroxylation, O-methylation, and NADPH-dependent reductions. Enzymes involved in these conversions are NADPH-dependent, oxygen-requiring cytochrome P450s and hydroxylases that introduce additional hydroxyl groups into the skeleton, and furthermore, reductases and alcohol dehydrogenases. The well concerted activity of an array of enzymes leads to formation of the monolignols p-coumaryl, coniferyl, and sinapyl alcohols. Monolignols are the precursors for lignin and lignans. For oxygenation of monolignols to polymeric lignins by interunit linkage, ROS such as hydrogen peroxide are needed (Figure 15). In the lignification step, hydrogen peroxide is converted into water by peroxidases. Non-woody plants such as wheat contain lignins formed from a mixture of monolignols and hydroxycinnamic acids.

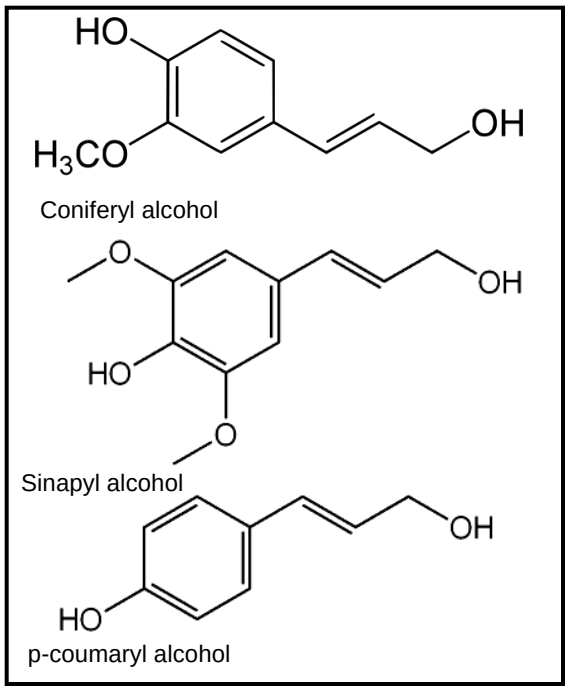


Figure 15 Lignin formation via interunit linkage based on monolignols as building blocks (modified after Buchanan et al. 2000).

In particular in non-woody plants, early and rapid deposition of lignin into plant cell walls provides an effective barrier against pathogens (Buchanan et al. 2000). Camera et al. (2004) hypothesised that plasmodesmata were closed down and clogged by lignin depositions and the cell to cell connection was interrupted for toxins and pathogens.

In addition to lignification, the process of suberization plays a major role as a physical barrier in plant defence. Suberin is characterised by alternating layers of polyaliphatic compounds such as long chain lipids and polyaromatic layers (hydroxycinnamate-derived substances). These layers are formed as wound healing layers and form an impenetrable barrier for pathogens (Buchanan et al. 2000).

Free phenolic compounds

According to Siranidou et al. (2002), free phenolic compounds were found in the floral bracts already prior to *Fusarium* infection in the cultivar Frontana that shows good resistance to initial infection and spread of disease. Analysis of phenolic compounds by high performance liquid chromatography clearly demonstrated that in Frontana constitutive phenolic compound content was more than double of susceptible cultivar Agent. Free phenolic compound levels remained high at all time-points after infection compared with susceptible wheat lines.

The significant increase of p-coumaric phenolic compound 24 hai, indicated that phenolic compounds are not only constitutively present, but also induced in response to fungal attack. Cell wall apposition and lignin content increased in resistant Frontana at regions close to the infection site 48 hai. Siranidou et al. (2002) concluded that phenolic compounds may play a crucial role in the resistance of Frontana to *Fusarium culmorum*. Ferulic and p-coumaric acid are the most abundant phenolic compounds in green tissue of wheat spikes. Free and cell wall bound phenolic compounds showed antifungal activity *in vitro* against *Fusarium graminearum* causing retarded mycelium growth (Siranidou et al. 2002).

In addition, a broad range of secondary metabolites derive from the phenylpropanoid pathway such as phytoalexins with antimicrobial activity such as cumarins and isoflavonoids (Camera et al. 2004). Activation of PAL also leads to biosynthesis of SA (Buchanan et al. 2000).

2.1.5.7 Physical barriers

Waxes, cutin, and the main components of the plant cell wall such as cellulose, β-1,3-glucan, pectins, and cell wall proteins are the constitutive and preformed structures penetrating fungi encounter as first physical barriers.

During fungal penetration additional structural barriers can be induced rapidly. These processes comprise cross-linking of cell wall glycoproteins, lignification, deposition of additional cell wall material, and formation of papilla at the site of penetration. Additional structural barriers are an attempt to confer resistance against physical penetration of hyphae and cell wall degrading enzymes. Furthermore, they help to block diffusion of toxins and other effectors to the host protoplast, and reduce nutrient uptake from the host cell (Kang and Buchenauer 2008).

The first reaction triggered upon recognition of the pathogen is cross-linking of glycoproteins in the cell walls caused by ROS in the event of oxidative burst. Further strengthening of the cell wall is achieved by lignification. Proline-rich proteins are involved in lignification and may direct the deposition in the cell wall spatially and temporally (Buchanan et al. 2000). In addition, lignin precursors, as phenolic compounds, may have antimicrobial activity themselves (Kang and Buchenauer 2002). Additional deposition of wall material provided in secretion vesicles leads to cell wall appositions (Bollwell et al. 2001). Papillae are tuber-like structures built in the periplasmic space. They are highly localised structures opposing the site of hyphal penetration in order to trap the fungal hyphae. Papillae are composed of cross linked proteins, phenolic compounds and β-1,3-glucans (callose). Callose is accompanied by hydroxyproline-rich proteins and phenolics (Kang and Buchenauer 2002). The formation of papillae is a highly localised process guided by the signalling compound hydrogen peroxide. It includes changes in cytoplasmic streaming and de-polymerisation and polymerisation events of the cytoskeleton in order to direct the cytoskeleton towards infection site (Bollwell et al. 2001). In this context, Ferreira et al. (2007) stressed the fact that rapid production and secretion of wall material requires localised exchanges and communication between cell wall and cytoplasm via the plasmamembrane.

In addition, amorphous, electron dense material was incorporated in the cell walls and was also found in the intercellular space and vascular bundles, in particular in resistant cultivars. The electron dense material that has not been further specified by Kang and Buchenauer (2000, 2002), was described as phenolic compounds by Bollwell et al. (2001), whereas Guenther and Trail (2005) identified them as pectins.

According to Jansen et al. (2005), physical barriers may slow down hyphal growth even if they are not completely efficient to stop fungal spread. As a consequence, the plants gain time for initiation of defence response.

No differences in preformed physical barriers have been observed between resistant and susceptible cultivars. Whereas, induction of physical barriers is significantly increased in resistant cultivars. Resistant cultivars develop extensive cell wall appositions, form papillae, and show higher labelling density in immunogold-labelling experiments with antibodies against lignin and callose than susceptible cultivars. Susceptible cultivars only show thin layers of cell wall appositions in the periplasmic space and loose labelling density (Kang and Buchenauer 2000, 2002, 2008; Pritsch et al. 2000; Sirandou et al. 2002). The general observation that only resistant cultivars show efficient induction of physical barriers could address the question what really makes a difference in resistance to *Fusarium graminearum* infection.

Apart from physical barriers induced in living cells, plant cells that undergo programmed cell death create a physical barrier themselves (Bollwell et al. 2001). Cells that undergo apoptosis are reported to physically separate from living neighbouring cells. Plasmodesmata are closed in dead cells, and as a result, the endomembrane system between cells is disconnected. Thus, hyphal growth, transfer of toxins and effector molecules is prevented. In addition, phenolic compounds that are produced during hypersensitive response are excreted into the wall. The phenolic compounds build a toxic barrier to invading necrotrophic pathogens and trap biotrophic ones. Bollwell et al. (2001) suggested that these walls had an electron dense appearance owing to phenolic compounds. By contrast, in the surviving neighbouring cells paramural bodies with material to seal the interphase towards the dead cells were observed.

2.1.5.8 Coping with the phytotoxin DON

Significant differences between DON concentration in resistant cultivars and susceptible cultivars at an early stage of 36 to 48 hours were observed by Kang and Buchenauer (2002, 2008) by immunogold-labelling with antibodies against DON. After 3 dpi labelling density was significantly higher in susceptible cultivars than in resistant cultivar Sumai3 (Kang and Buchenauer 2008). Due to higher DON levels, cells appeared collapsed and compartments disintegrated in susceptible host cells, whereas cells of resistant Arina and Frontana were still alive (Kang and Buchenauer 2002; Pritsch et al. 2001).

In general, there are several strategies to deal with toxins. Plants can prevent influx by physical barriers and keep toxins at bay by clogging of plasmodesmata and vessels. Furthermore, they can enhance efflux or sequestration via transporters such as ABC transporters. Moreover, plants can metabolise toxins and detoxify them by binding or cleaving functional groups. In order to increase tolerance, plants can change or protect targeted sites of effector molecules. In the case of DON, RLP3 domain in the small subunit of ribosomes would be concerned. In addition, truncated and thus non-functional proteins derived from interference of DON with RLP3 need to be degraded. According to Lucyshin et al. (2008) ubiquitination for protein degradation and recycling can be considered as a crucial pathway to counteract the effects of DON and to maintain a pool of functional proteins in cells. In this context, Boddu et al. (2007) found putative proteins containing U-box and F-box domains upregulated upon *Fusarium graminearum* infection that may be involved in ubiquitination process or in the proteasomal complex. Lucyshin et al. (2008) hypothesised that DON may cause a depletion of ubiquitin by overproduction of non-functional truncated proteins, and thus further disturb and weaken metabolism and signal transduction of infected cells. Lucyshin et al. (2008) suggested to follow up this pathway in the search for tolerance to DON.

The fact, that DON concentration was lower in resistant cultivars has led to the presumption that DON influx may be blocked, its efflux or sequestration may be enhanced or it may be degraded and metabolised. Observations by Lemmens et al (2005) suggested that DON must have been rapidly taken up as only a small proportion of applied DON could be later washed off from treated spikelets. Furthermore, DON must have been transported through the apoplast and xylem vessels because symptoms such as bleaching of the heads and failure of kernel development occurred rapidly after inoculation with pure DON. Berthiller et al. (2005) reported that DON and its glycosylated derivative DON-3-glucoside occured naturally on wheat plants infected by *Fusarium graminearum.* DON 3-glucoside was naturally present between 4% to 12 % of total DON concentration. According to Berthiller et al. (2005) DON-glycosylation transforms DON into a stable, non-reactive form with increased water solubility and the reactive site of the toxin, the epoxide, appearing to be blocked. In this respect, Lemmens et al. (2005) suggested that resistance to DON may rather be the result of detoxification into DON-3-glucoside than reduced uptake.

Furthermore, Poppenberger et al. (2003) were able to isolate a UDP-glycosyltransferase with DON glycosylation properties in *Arabidopsis thaliana*. Characterisation of UDP-glycosyltransferases that was isolated in the transcription profiling experiment performed by Steiner et al. (2009) is under way.

In regard to DON detoxification mechanisms, Lemmens et al. (2005) and Berthiller et al. (2005) were able to demonstrate that DH lines carrying the QTL Fhb1 converted higher levels of DON into DON-3-O-glucoside resulting in a higher DON-glucoside/DON ratio than DH lines lacking Fhb1. In addition, DON-glucoside/DON ratio was significantly negatively correlated with values of area under disease progress curve after inoculation with DON (

Figure 16). Owing to the strong correlation of R2 = 0,84, Lemmens et al. (2005) concluded that DON conjugation to glucose was the primary biochemical mechanism for resistance towards DON.

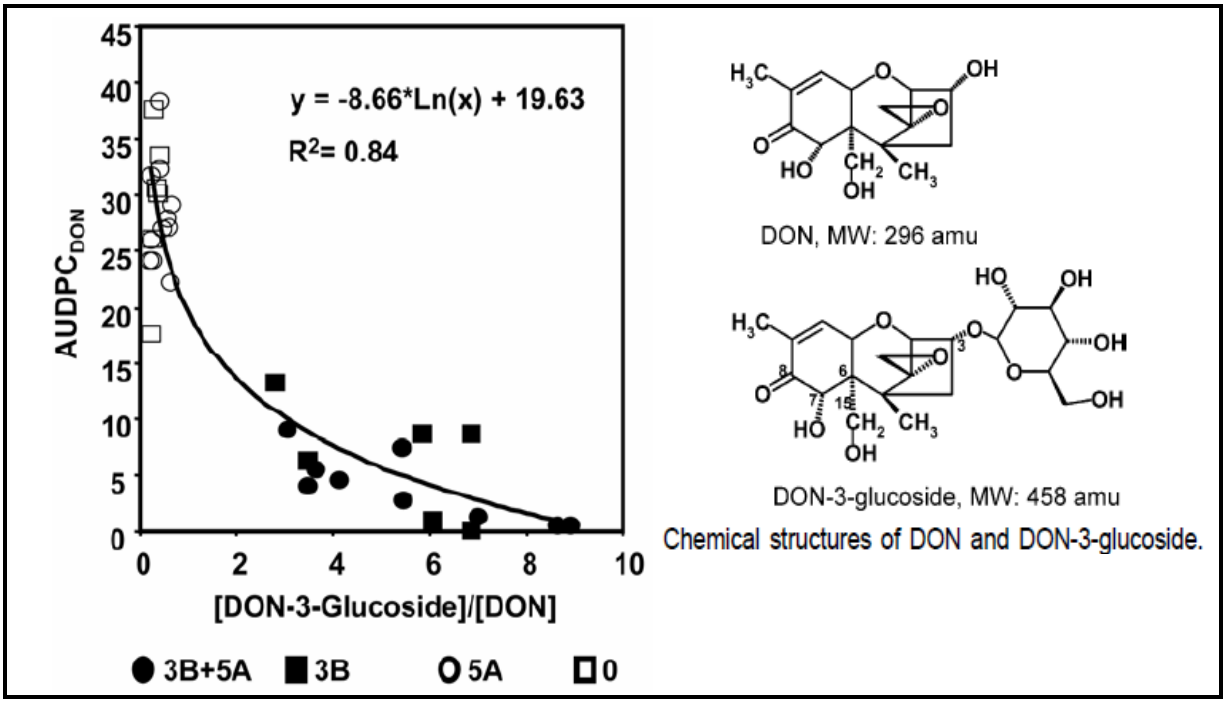


Figure 16 Relation between DON-3-Glucoside/DON ratio and resistance levels (Lemmens et al. 2005).

Furthermore, Lemmens et al. (2005) suggested that a gene or a group of genes located in the region of *Fhb1* either encoded for a UDP-glycosyltransferase or a corresponding regulatory gene. Currently, Lemmens et al. (2008) have been addressing the question whether the characterised putative UDP-glycosyltransferase is substrate specific to DON or can also detoxify related trichothecenes such as NIV.

Kang and Buchenauer (2008) summarized conclusively that protein synthesis in the resistant Sumai3 may not be impaired by DON owing to several related facts: Firstly, DON levels are lower as the fungus is restricted by physical barriers. Secondly, Sumai3 has reported ability to detoxify DON, and thirdly it has been reported, that Sumai3 ribosomes show higher tolerance to DON than that of susceptible cultivars. This allows for fast and strong accumulation of defence related proteins and physical barriers in the time course of infection, rendering Sumai3 less and less susceptible to later accumulated DON. Kang and Buchenauer (2008) concluded that lower DON levels reach the phloem and xylem and thus less DON is dissolved in the water. As a result,

distant tissue may suffer less from physiological effects of DON. They consider this as the foundation of type II resistance.

Implying that detoxification and induced cell wall reinforcements go hand in hand and support each other, Jansen et al. (2005) put forward the question, of whether detoxification of DON via 3-O-acetyltransferase or glycosylation at the C3 position by UDP-glycosyltransferase can prevent inhibition of cell wall reinforcement and confer type II resistance.

Besides detoxification mechanisms of DON, Boutigny et al. (2008) reviewed reports that secondary metabolites of plants might interfere with trichothecene biosynthesis and thus prevent the formation of DON. Boutigny et al. (2008) refers to antioxidants such as phenolic compounds that might reduce the rate of DON biosynthesis by impairing the oxygenation steps and epoxide formation. This idea is based on the hypothesis that trichothecene biosynthesis involves several oxygenation steps that require molecular oxygen. Thereby, pro- and antioxidant compounds in the intercellular environment of the host plant are thought to modulate fungal trichothecene biosynthesis. Boutigny (2008) reviewed evidence and hypothesised that presence of increased hydrogen peroxide may enhance biosynthesis of DON. As a consequence oxidative burst and the generation of ROS might even have enhancing effects on DON accumulation. This would support the observations by Mesterhazy (2003) who reported that infection levels of *Fusarium graminearum* do not always correlate with DON levels. In addition, Voigt et al. (2007) reported that strains restricted and trapped by physical barriers show earlier and stronger induction of genes involved in DON biosynthesis. This would suggest that the stronger the resistance response, the more ROS are available for trichothecene biosynthesis in the invaded tissue.

According to Boutnigy et al. (2008), phenolic compounds may interfere with the oxygenation steps and, thus, impair conversion of the intermediate trichodiene to trichothecenes containing an epoxide group. These reports give rise to the question of whether oxidative burst and the release of reactive oxygen induce DON production and whether DON biosynthesis is impaired in resistant cultivars owing to the presence of phenolic compounds. In particular, kernels contain high levels of phenolic compounds and their antioxidant activity might lower DON levels (Boutnigy et al. 2008).

2.2 RESISTANCE TO *FUSARIUM* HEAD BLIGHT

2.2.1 Phenotypic characterisation of resistance

The most efficient way to resist a pathogen is avoidance — by not encountering the pathogen, particularly, in periods of increased susceptibility such as open flowering and early stages of kernel development. For example, Bushnell et al. (2003) reported disease escape of wheat cultivars that open for flowering in the middle of the day avoiding water splash of dew droplets. Ears of tall phenotypes are less accessible to ascospores harboured in soil debris. In this context, it has been suggested that the length of upper internodes had an influence on incidence of water splash from leaves to the head. In addition, loose heads dry faster than heads with high spikelet density providing less humidity for spore germination. Furthermore, ears without awns are less prone to infection as spores cannot be captured by these structures and make their way down to the floral bracts (Mesterhazy 2003). However, if the plant is under attack it needs to be capable of defending itself actively by the use of an efficient immune system.

Resistance to FHB is an interplay between resistance to initial infection of one spikelet and resistance to spread of disease from the initially infected spikelet through the spike. These two components of resistance were first described by Schröder and Christensen in 1963 as type I resistance to initial infection and type II resistance to spread of disease (Schröder and Christensen 1963). In recent years, resistance to DON accumulation in infected kernels was termed type III resistance (Mesterhazy 2003). Resistance to kernel infection is considered as an additional type of resistance. In addition, tolerance to FHB and DON was also reported as a component in this plant–pathogen interaction. However, from a multitrophic point of view, tolerance to DON may not contribute to food safety.

Type I resistance is estimated by spray inoculation of flowering spikes during anthesis. By spray inoculation all florets are exposed to inoculum and can be equally initially infected. For evaluation, number of diseased spikelets or blighted spikelets are counted, or percentage of blighted spikelets is visually estimated and summarized and depicted in the “area under disease progress curve (AUDPC). By contrast, type II resistance is investigated by point inoculation of a single floret of a spike in order to exclude the effects of resistance to initial infection. For type II resistance, spread of disease from the initially infected spikelet to uninfected, neighbouring spikelets and further through the entire spike is evaluated by counting number of diseased spikelets or by estimating the percentage of wilted heads (Bürstmayr et al. 2002).

In the field, type I and type II resistance are both needed to perform good resistance under high inoculum pressure. On the one hand, cultivars with only type I resistance will occur susceptible when inoculum is highly abundant under favourable weather conditions, with increasing probability that at least one spikelet will get infected. Lacking type II resistance, already one infected spikelet will be sufficient for further spread of infection, leading to an entire blighted ear. On the other hand, cultivars with only type II resistance will restrict infection to the initially infected spikelet, but under high inoculum abundance too many spikelets will become initially infected per spike. As a consequence, the ear will be blighted, although there is no spreading of disease. Type II resistance is reported to be less influenced by infection dose and environmental conditions than type I resistance. Thus, type II resistance is considered to confer resistance in the field even under conditions favouring epidemic outbreaks. Type II is the major resistance type found in resistant land-races and cultivars and it is the predominantly targeted type of resistance in breeding programmes. However, the ultimate objective will be to combine type I and type II resistance (Bürstmayr et al. 2003; Bai and Shaner 2004).

In addition to type I and type II resistance, type III resistance is of major interest as it confers low DON levels in harvested grain and thus contributes to food safety. Lower DON content in harvested grain may result from fewer infected kernels, and thus, resistance to DON accumulation in tissue and kernels is considered to be at least partly the result of type I and II resistance. It is assumed that a fungus restricted to the site of its initial infection may produce less DON in absolute numbers compared with a fungus spreading through the whole spike. Results obtained by Mesterhazy et al. (2003) indicated that fungal biomass is strongly correlated with DON levels in kernels. Furthermore, lower DON levels may be the result of detoxification mechanisms and the fact that DON may remain in the tissue and may not be transported into the developing kernel. However, there are contradicting results about the correlation between disease severity and DON levels in the harvested grain. Firstly, time-point of infection is considered to influence DON concentration. In case of early and severe infection during flowering, the infected ovary may either never develop into a mature kernel or it becomes a severely diseased, small shrivelled kernel that is excluded during threshing and milling. Thus, severe and early infection, eliminates kernels with high DON concentration, whereas late infection may result in kernels of normal size and weight with increased DON levels. As a result, severe symptoms may be visible on the heads, but will be correlated to lower DON levels in the harvested grain, while moderately diseased and late infected cultivars may contribute to higher DON levels in the harvest (Mesterhazy 2003). This may be owing to the fact, that in moderately resistant cultivars, infected kernels will develop and grow to normal size, for example, owing to faster corn filling rate. Kernels normal in size and weight are not separated by threshing and thus increase DON levels in harvested grain (Bai and Shaner 2004). Apart from that, it is reported that DON production is induced in *Fusarium graminearum* under stress conditions. As a result, *Fusarium graminearum* will accumulate higher DON levels in partially resistant cultivars, which can build up physical barriers, than in completely susceptible cultivars with no barriers (Voigt et al. 2007).

2.2.2 Sources of resistance for breeding programmes

Sources of moderate and good native field resistance to FHB have been found in locally adapted germplasm of all wheat growing regions on earth. Ernie, Freedom and Truman in North America, Arina, Renan and Fundulea in Europe, and Frontana in South America represent local cultivars with acceptable field resistance. However, the Asian germplasm consists of landraces with extraordinarily high resistance to FHB such as Wangshubai. Unfortunately, these land-races are reported to have undesirable agronomic traits such as low baking quality, small heads, late maturity and they are tall in size. As a result, introgression of these resistance genes into high yielding cultivars by back-cross breeding appeared to be difficult (Bai and Shaner 2004).

Beside highly resistant Asian land-races, the wheat cultivar Sumai3 was released in the seventies of the last century. It arose from a cross of moderately susceptible parents Funo and Taiwanxiaomai by transgressive segregation (Liu and Wang 1990). Mesterhazy (2003) suggested that Sumai3 may comprise of different forms and sources present in the world collections used for breeding programmes and mapping experiments. Sumai3 shows the highest level of type II resistance available to date combined with acceptable agronomic traits. Sumai3 shows high resistance to almost all isolates of *Fusarium graminearum* worldwide and resistance is reported to have been proven persistent in a wide range of environments and in various genetic backgrounds. Therefore, Sumai3 serves as a resistant donor parent for implementation of FHB resistance to high yielding cultivars on all continents. Sumai3 and its derivatives are currently considered as the best available source of FHB resistance and serve as crossing partners in many wheat breeding programmes and research projects. As Sumai3 shows multiple infection sites under high disease pressure, and thus, some weakness in type I resistance, breeding efforts focus on combining type I resistance with Sumai3 in order to breed for derivatives with overall superior resistance performance. For example, Mc Kendry (2008) introduced resistance of

Sumai3 into the American soft red winter wheat cultivars Ernie and Truman, and their offspring and siblings in order to pyramide Sumai3 resistance upon the "solid foundation of native FHB resistance", as according to Mc Kendry (2008) "both, type I and type II resistance are required for a functional level of resistance under heavy disease pressure".

2.2.3 Genetic characterisation of resistance to FHB

Resistance to FHB is reported to be multigenic also termed horizontal, quantitative and polygenic. FHB resistance in wheat is reported to be not species- or strain-specific. It is assumed that a host unspecific pathogenicity of *Fusarium graminearum* meets a non-isolate specific resistance mechanism in wheat (Bai and Shaner 2004). In general, quantitative resistance is based on multiple plant genes and does not require matching *R* and *avr* genes for a timely resistance response (Tuzun and Somachi 2006). Phenotypes within populations exhibit gradual disease severity indicating the quantitative and polygenic nature of resistance compared to monogenic resistance characterised by phenotypes that follow the pattern of the yes or no classification — healthy or severely diseased as observed in wheat–leaf rust interaction (Bürstmayr et al. 2002, 2003). However, genes with major effects on resistance may not only play a role in innate basal host resistance, but also in PAMP triggered and effector triggered resistance which requires a certain level of specificity in the host–pathogen interaction, as it might be the case for DON detoxification according to Lemmens et al. (2005).

Type I, II and several other reported types of resistance describe components of resistance on a phenotypic level and have been used in classical breeding to select for resistant cultivars. However, resistance of wheat to FHB in the field is a quantitative trait under strong influence of environmental factors. As a result, selection for resistance may be environmentally biased, as disease pressure is only severe in years when wet and warm weather meets with flowering time. As a result, many cultivars varying in flowering time may escape disease pressure in uncontrolled field conditions (Bürstmayr et al. 2002). As a consequence, selection tools that are rather based on the genotype than on the phenotype and thus exclude the factor environment have been demanded. As a result, it is aimed to implement marker assisted selection (MAS) to classical selection.

2.2.3.1 QTL mapping — relating phenotypical traits to genomic regions

Most information on genetic characteristics of FHB resistance in wheat has been obtained from QTL mapping so far. In QTL mapping, phenotypic traits such as type I and type II resistance are aimed to be associated with molecular markers that map to a certain genomic region on a chromosome.

In the last decade, QTL mapping for resistance to FHB revealed a major QTL on the short arm of chromosome 3B that is also referred to as *Fhb1* (Anderson et al. 2001; Bürstmayr et al. 2002). *Fhb1* is associated with the phenotypic trait of type II resistance and represented by Sumai3. Apart from *Fhb1*, many QTL associated with FHB resistance have been described in different mapping populations and environments since. In order to summarize these data, Bürstmayr et al. (2008) reviewed 52 papers published on this issue. 100 genomic regions were reported to be associated to FHB QTL, however only 22 were considered consistent and stable as they were confirmed in different mapping populations. QTL associated with FHB resistance were reported for all wheat chromosomes except for 7D, and many QTL are reported to overlap with QTL associated with plant height. The QTL *Fhb1* was described in 26 papers indicating the overall importance of this major QTL. Beside this, Bürstmayr et al. (2008) considered *Fhb2* on 6B and QTL on 5AS (*Qfhs.ifa-5A*) as the most relevant ones.

For example, the wheat lines used in the transcription profiling experiment by Steiner et al. (2009) derived from a population that was originally developed and extensively tested and used

for QTL mapping. The population derived from a cross of resistant CM82036 (Sumai3/Thornbird) and susceptible Remus, a Bavarian spring wheat cultivar. CM82036 showed exceptionally good type I and type II resistance in the field. By applying the wheat by maize pollination technique, 364 doubled haploid (DH) lines were derived from the heterozygous F1 generation. Subsequently, several QTL mapping experiments have been conducted by Bürstmayr et al. (2002, 2003) and Lemmens et al. (2005, 2008):

In order to test for combined type I and type II resistance, a spray inoculation experiment was performed in the field under mist irrigation promoting an epidemic situation. The phenotypic evaluation showed a continuous, close to normal distribution indicating the quantitative nature of FHB resistance in the mapping population. The resistant parent CM82036 showed an average of 9 % of disease severity on 26 dpi, whereas Remus reached 77%. The DH lines carrying both alleles from CM82036 scored on average 22% disease severity whereas lines carrying the alleles from Remus reached on average 58 % bleached heads at the same time-point.

Screening with AFLP and microsatellite markers and subsequent QTL analysis identified two major QTL regions associated with FHB resistance in this population. These are located on chromosome 3B (*Qfhs.ndsu-3BS*) and on 5A (*Qfhs.ifa-5A*). *Qfhs.nsdu-3BS* (*Fhb1*) explained 29 percent of phenotypic variance for visually scored FHB resistance. This agrees with data obtained from a previous point inoculation experiment, in which the QTL on 3BS explained 60 percent of phenotypic variance. Taken together, the results obtained in the mapping population CM 82036/Remus validated the major effect of *Fhb1* on type II resistance (Bürstmayr et al. 2002, 2003). These findings lend further support to the hypothesis stated by Waldron et al. (1999), who predicted that type II resistance would be under control of only few QTL with major additive effects together with an unknown number of minor gene effects. These minor QTL may be able to enhance or suppress the effects of major QTL.

By contrast, QTL on 5A (*Qfhs.ifa-5A*) was shown to be primarily associated with type I resistance as it showed about similar contribution to resistance as *Fhb1* in the spray inoculation experiment, whereas in the point inoculation experiment it showed only limited effects. However, genetic mapping of *Qfhs.ifa-5A* appears to be limited by low recombination rate at this genomic region (Bürstmayr et al. 2002). For both QTL the alleles conferring resistance originated from the resistant parent CM82036. Furthermore, QTL analysis revealed significant additive effects of the two QTL.

In addition, resistance to DON was tested in this mapping population by Lemmens et al. (2005). Effects of DON were evaluated by applying DON drops into the ears of 96 DH lines. DON caused the same visual symptoms as its producer *Fusarium graminearum*. QTL mapping identified a QTL associated with resistance to DON that co-localises with the previously reported resistance QTL on the short arm of chromosome 3BS. This finding is further supported on phenotypic level by the fact that resistance to DON is highly correlated with resistance to spread of disease in *Fusarium* infection experiments. CM82036 showed a low AUDPC of 0,7 units compared with Remus that showed 21 units. Interestingly, DH lines did not show a distribution close to normal for DON resistance compared with FHB resistance in the field, indicating that resistance to DON may be of monogenic qualitative nature, encoded by one gene with a major effect on resistance to DON (Figure 17) (Lemmens et al. 2005).

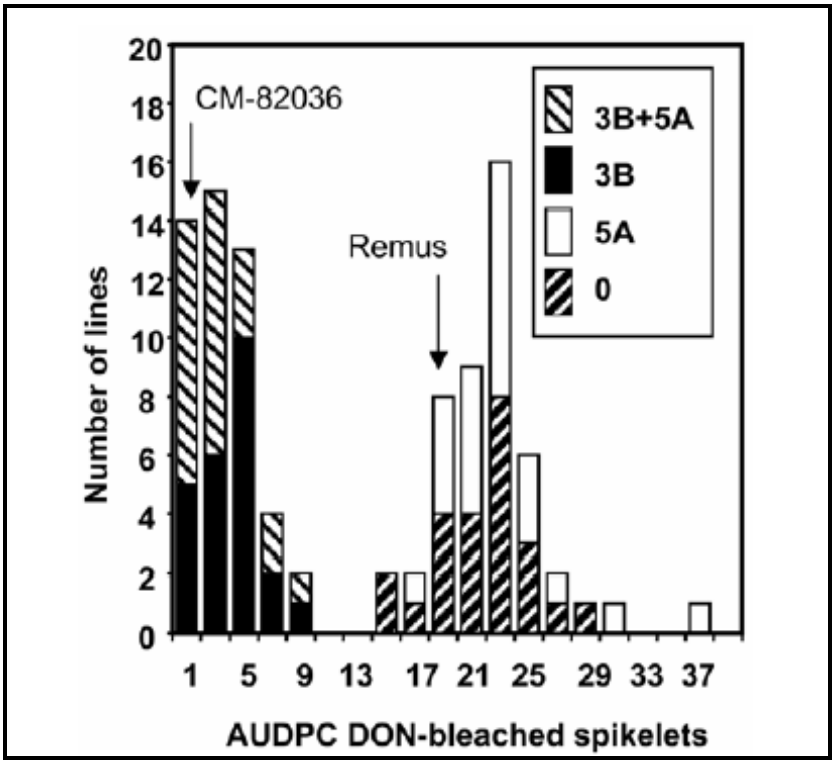


Figure 17 Distribution of effects of DON in the CM82036/Remus mapping population (Lemmens et al. 2005).

Based on the results of Bürstmayr et al. (2002 and 2003) and Lemmens et al. (2005), effects of *Fhb1* and *Qfhs.ifa-5A* were aimed to be tested under susceptible background, therefore near isogenic lines were developed from DH lines by back-crossing with susceptible Remus (Steiner et al. 2009; Lemmens et al. 2008).

In addition to QTL mapping approaches, Liu et al. (2003) evaluated the potential of comparative mapping with rice at the *Fhb1* region based on reported strong synteny between wheat chromosome 3B and rice chromosome S1. Furthermore, in physical mapping, fine mapping and positional cloning approaches, the *Fhb1* region was narrowed down to a 261 kb region with 7 putative genes (Liu et al. 2008). However, none of the 7 genes was significantly induced by *Fusarium* inoculation. In addition, a cosmid library of Sumai3 was explored for clones containing this genomic region. These clones were transformed into a susceptible cultivar but did not rescue the phenotype. Currently, a BAC library of Sumai3 is created for further physical mapping approaches (Liu et al. 2008).

2.3 EXPRESSION PROFILING USING THE cDNA-AFLP METHOD

2.3.1 Preliminary and accompanying research on FHB

As FHB has a major impact on crop production, most research has been conducted in the field of classical plant breeding. Selection for natural variation in disease resistance relies on the presence and absence of symptoms such as blighted spikelets, bleached ears, and shrivelled kernels. Thus, what has been basically examined, is the outcome of compatible and incompatible interactions.

Visual scorring of symptoms provides a resolution of 0.2 mm — the resolution of the human eye. In order to study micro- and ultrastructural changes in the early stages of wheat–*Fusarium graminearum* interaction, fluorescence microscopy, transmission and scanning electron microscopy have been deployed. Ultrathin sections of tissue gave insight into the infection and colonisation process and addressed the question of what happens when and where. In recent years, potential key proteins and metabolites of this interaction were detected at subcellular level by immunocytological methods. Furthermore, biological processes have been studied at subcellular level with different reporter gene contructs that tag inducibility of promoters or expression of proteins such as the green fluorescent protein (GFP). While immunogold-labelling can only be performed on fixed tissue, GFP assays can be performed in living tissue, but require transformation of the organism. For example, Miller et al. (2004) and Jansen et al. (2005) used transformed *Fusarium* strains expressing GFP as a reporter protein to follow infection and colonisation of the fungus through the spikelet and to monitor induction of DON biosynthesis. At the level of single molecules, x-ray christallography elucidates the structure of complex proteins.

At molecular level a wide range of tools in the disciplines of genomics, transcriptomics, proteomics, and metabolomics are applicable to characterise defence response. However, hexaploid wheat poses a major challenge to methods applied in genomics and functional genomics, owing to its large genome (17.500 Mbp) with high content of repetitive elements and the complex structure of the allo-hexaploid genome with a high degree of gene redundancy on homoeologous loci (Lagudah et al. 2001).

Sequencing, fine mapping, transformation, and mutant screening are either considered as not feasible or have only the potential to be performed on small scale compared with high throughput approaches in *Arabidopsis* or rice. Therefore only limited sequence information and annotated genes are available considering the large wheat genome and the enormous importance of this crop species. In this regard, functional genomics in wheat relies on sequence homology to model plants such as *Arabidopsis*, rice, and *Brachypodium* for which full sequence information is available. In particular, the grass model systems rice and *Brachypodium* are exploited in comparative mapping approaches as strong synteny and even colinearity of genes are expected among these grass species (Lagudah et al. 2001).

2.3.2 Transcription profiling

In addition, transcription profiling experiments are considered as valuable tools to obtain sequence information from coding regions of the large and complex wheat genome. Transcript derived fragments (TDFs), also referred to as expressed sequence tags (ESTs) derive from mRNA. In transcription profiling it is assumed that genes are transcribed from the basic genomic template when and where they are needed (Kazan et al. 2004). Therefore, transcription profiling allows for a context dependent analysis of the transcriptome, the complement of mRNA present in a cell at a certain timepoint under defined conditions. According to Mehta et al. (2008), the protein complement defines the actual contribution to cellular function. Several years before, Holtorf et al. (2002) had already reasoned that temporal and spatial expression patterns give an idea on gene function. On the one hand, it can be hypothesised that genes with the same

expression patterns are subjected to co-regulation and are involved in the same biological process. On the other hand, in comparative studies with resistant and susceptible genotypes differences in expression patterns could be related to resistance and susceptibility, for example. According to Mehta et al. (2008), generally good correlation between mRNA and the corresponding proteins have been observed. However, mRNA levels do not necessarily correspond with protein levels owing to post-transcriptional and post-translational regulation, lifetime differences of mRNAs, and differences in translation efficiency. This may be of importance at time-points with high DON accumulation. Kazan et al. (2004) introduced the term "molecular phenotype" that represents the physiological picture at different levels, for example, on transcriptional level. By combining QTL mapping with transcriptional profiling, the "molecular phenotype" could be intergrated into QTL mapping and ESTs can serve as markers.

Owing to the fact that transcription profiling mirrors the state of the cells under certain conditions, the experimental set-up predetermines the outcome of a transcription profiling experiment. Therefore, a tailored experimental set-up allows to address objectives and research questions with high precision. In respect to this, preliminary information about what happens when and where is required and can be considered as a prerequisite. In the wheat–*Fusarium graminearum* interaction, the TEM micrographs of Kang and Buchenauer (2000, 2002, 2008) provided the backbone for transcription profiling experiments. For example, it was clearly demonstrated that tissue sampling before 24 hai and at 48 hai addresses different lifestyles of the fungus. Tissue sampling of point inoculated spikelets addresses the question of local defence response whereas tissue sampling of neighbouring spikelets or the the whole ear includes systemic response. Moreover, different types of resistance can be addressed by the choice of inoculation method.

Furthermore, tissue sampling is considered an important part of the experimental set-up. In inoculation experiments, sampled tissue comprises a mixture of cells with different physiological states. Firstly, tissues consist of different cell types such as epidermal cells and mesophyll cells. Secondly, not all cells are equally affected by colonisation. Apart from uninfected cells, cells that are in close contact with hyphae and influenced by DON, as well as, directly penetrated cells and cells that have undergone hypersensitive response and programmed cell death can be present within the same tissue. As a consequence, low-level transcripts are prone to be obscured, diluted, and masked, although they might play a crucial role in defence response.

Transcriptional studies are often performed as comparative studies. For example, transcriptome data of defence response in resistant and susceptible phenotypes are compared. By integration of data from applied molecular plant breeding, the experimental set-up can be based on well-characterised genotypes from QTL mapping populations. Steiner et al. (2009) compared the parents, closely related sister lines, and near isogenic lines with the genetic background of the susceptible parent harbouring the resistant alleles on the QTL *Fhb1* and *Qfhs-ifa5A.*. By contrast, Golkari et al. (2009) tested near isogenic lines with a resistant genetic background harbouring the susceptible alleles on the major QTL on 3BS and/or 2A. Both transcription profiling experiments aimed to identify candidate genes that may be exclusively associated with the effects of the previously mapped QTL. Furthermore, transcription profiling has been used to monitor chemical assays and bioassays that allow for investigation of major components in defence response. For example, Golkari et al. (2007) compared defence response of a DON deficient *Fusarium* strain with a wild-type strain in order to dissect the basal defence response from the effector triggered response.

Moreover, the experimental set-up needs to be adapted to the method and equipment used for transcription profiling.

2.3.3 The cDNA-AFLP method

The method of cDNA-amplified fragment length polymorphism (cDNA-AFLP) represents a transcription profiling technique that allows for simultaneous detection of thousands of expressed genes. Furthermore, the cDNA-AFLP method is described as an open system that allows for detection of novel not yet described TDFs compared with closed microarray systems that are based on hybridisation events to previously characterised and spotted oligonucleotides (Meyers et al. 2004).

In order to make use of the DNA polymerase chain reaction for amplification, RNA needs to be reverse transcribed into its double stranded complementary DNA (cDNA). As this reverse transcription step is primed with poly-dT oligonucleotides, only mRNA with a poly-A tail should be subjected to reverse transcription. It is expected that reverse transcription results in double stranded cDNA that only contains untranslated regions and coding regions (exons) as messenger RNA has already undergone post-transcriptional processes such as splicing and polyadenylation.

Subsequently, double stranded cDNA strands are digested with two different restriction enzymes that cut cDNA strands within specific restriction sites and thus create fragments of different lengths with the same end sequences corresponding to the restriction sites. Double stranded adapters are ligated to the corresponding restriction overhangs. These constructs serve as primary template for the first amplification step, where the adapters with known sequences serve as primer annealing sites. As the primers are complementary to the adapters, no previous sequence information is necessary to prime the amplification step (Figure 20). Consequently, it can be applied to explore large genomes such as wheat with only little sequence information available.

In a second amplification step selective primers are used that consist of a core sequence corresponding to the adapter, a restriction enzyme specific region, and selective nucleotides extending into the unknown region of the fragment. As a result, only subsets of restricted fragments are amplified with primers extended by one, two or three selective nucleotides at the 3´end (

Table **2**). Amplification of subsets allows for increased resolution in the separation by electrophoresis in a polyacrylamide gel matrix. The PCR amplification step allows for visualisation of originally limited amounts of RNA extracted from dissected spikelets. Therefore, the primer corresponding to the rare cutter restriction enzyme is directly labelled with fluorochromes. Sequence information obtained from cDNA-AFLP method ranges between 50 to 1000 bp (Figure 21) (Vos et al. 1996, Bachem et al.1998).

Differences in gene expression as well as allelic polymorphisms can be detected by the cDNA-AFLP method. Only cDNA of genes that are transcribed can be digested and serve as a template for amplification by polymerase chain reaction. As a result, differences in expression levels can be detected as present/absent differences. According to Bachem et al. (1998), intensity of cDNA-AFLP bands mirrors the abundance of fragments present in the template, and thus allows for some quantitative estimation.

Owing to the sequence-specific digestion step by restriction enzymes, allelic polymorphisms such as single nucleotide polymorphisms and insertion/deletions (INDELs) can be detected. A single nucleotide polymorphism within the recognition site can either create an additional restriction site and an additional fragment that is shorter in size, or can change recognition sites the way that they are no longer recognised and subjected to restriction. As a consequence, a longer fragment will be generated while two shorter ones will be missing.

In addition to polymorphisms that lead to altered restriction sites, polymorphisms in fragment length are generated from INDELs within a fragment. INDELs result in longer or shorter fragments even though the restriction sites are the same (Figure 18).

Differences in expression could derive from allelic variation and differences in the promoter region of a gene or from differences in other regulatory loci (Meyers et al. 2004). Allelic differences can be further investigated at genetic level. In respect to natural selection, Meyers et al. (2004) reported that polymorphisms that alter transcription level of a gene tend to have a higher impact than polymorphisms in the coding region that alter amino acid sequence of the gene product.

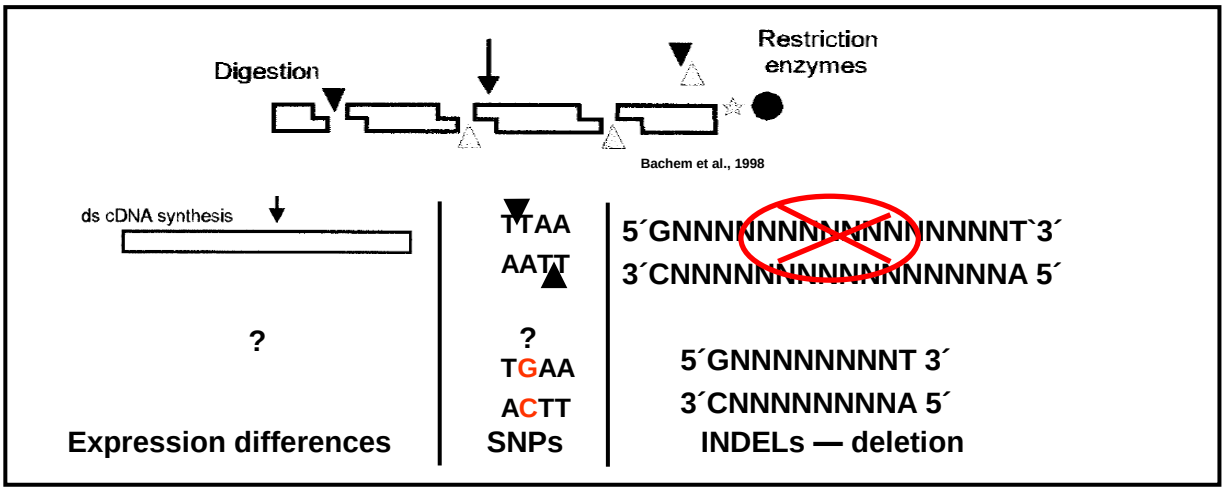


Figure 18 Polymorphisms generated by cDNA-AFLP method (modified after Bachem et al. 1998).

3 MATERIALS AND METHODS

3.1 PLANT MATERIAL

In order to detect differences in gene expression between resistant and susceptible genotypes, four spring wheat lines representing phenotypes with contrasting levels of resistance to FHB were selected. Highly resistant line CM82036 (pedigree: Sumai3/ Thornbird) and the highly susceptible Bavarian spring wheat cultivar Remus (pedigree: Sappo/Mex/Famos) that had previously served as parents in the mapping population, were selected as contrasting phenotypes. In addition, two wheat lines were selected from the mapping population comprising 364 recombinant doubled haploid (DH) lines that had been previously developed to map for QTL with effects on FHB resistance (Bürstmayr et al. 2002, 2003; Steiner et al. 2009).

The two closely related sister lines (DH1 and DH2) of the mapping population were selected as they share 305 of 430 markers indicating high allele similarity. However, DH1 and DH2 were phenotypically evaluated in 5 field experiments with single spikelet inoculation as well as spray inoculation (Bürstmayr et al. 2002, 2003) and showed significantly contrasting phenotypes: Line DH1 exhibits resistance to FHB almost to the level of CM82036 whereas line DH2 is susceptible and develops symptoms similar to the highly susceptible cultivar Remus. At molecular level, CM82036 and DH1 share alleles for resistance on QTL *Fhb1* on chromosome 3BS and on QTL *Qfhs.ifa-5A* on chromosome 5A, whereas DH2 shares the susceptible alleles with Remus at these QTL (Bürstmayr et al. 2002, 2003; Steiner et al. 2009).

In order to test the effects of the two major QTL on chromosome 3BS and 5A separately and in susceptible genetic background, F1 recombinant doubled haploids were advanced to four near isogenic lines by back-crossing with the susceptible parent Remus. These four F1:BC 5 lines share 98.40% of their genotypic constitution with Remus and were selected for the presence and absence of the QTL on chromosomes 3BS and 5A by molecular markers. NIL1 carries the resistant alleles in both QTL — 3BS and 5A, NIL 2 carries the resistant alleles in *Fhb1*on 3BS, NIL 3 carries resistant alleles in *Qfhs.ifa-5A* on 5A, and NIL4 does not carry any of the two QTL and served as a negative control in this experiment (Table 1).

Table 1 Summary of genotypes, QTL, and phenotypes

genotype	QTL *Fhb1* on 3BS	*Qfhs.ifa -5°*	FHB resistance level
CM82036	X	X	highly resistant
DH1	X	X	Resistant
DH2			Susceptible
Remus			Susceptible
NIL1	X	X	moderately resistant
NIL2	X		moderately resistant
NIL3		X	moderately resistant
NIL4			Susceptible

3.2 EXPERIMENTAL DESIGN

In the glasshouse experiment, the four wheat lines (CM82036, DH1, DH2, and Remus) were compared in two treatments — inoculation with *Fusarium graminearum* and water. The experiment was performed in two biological replications. Tissue samples were harvested at three time-points after inoculation resulting in 48 samples. In addition, the four near isogenic lines (NIL1, NIL2, NIL3 and NIL4) were tested in the same experimental set up in one biological replication resulting in 24 samples. As a result, the cDNA-AFLP fingerprinting was performed with 72 cDNA samples in total.

3.3 GREENHOUSE EXPERIMENT

3.3.1 Cultivation and inoculation

Plants were cultivated and point inoculated as previously described by (Steiner et al. 2009). The lines were planted in two biological replications with 30 plants per line and replication. At anthesis, plants were inoculated with either 10 µl of a suspension containing 50 conidia/µl of *Fusarium graminearum* isolate IFA 65 or 10 µl of water serving as a negative control. The 10 µl of inoculum were pipetted between the palea and lemma of the two basal florets of four central spikelets per spike (Figure 19a).

3.3.2 Tissue sampling

For tissue sampling, point inoculated spikelets were harvested at three time points that were previously described as the most crucial time points in the *Fusarium graminearum*–wheat interaction: 6, 24, and 72 hai. In order to evaluate disease development, 125 control heads of the inoculation experiment were scored for symptoms at later time-points.

Lemma, palea, rachilla, rachis node, the subtending section of the rachis of the inoculated spikelets were sampled without the reproductive organs (Figure 19b). According to the evidence that floral bracts and the rachis play a crucial role in type II resistance, these tissues were used for RNA extraction. For each sample, tissue of a minimum of six heads was pooled.

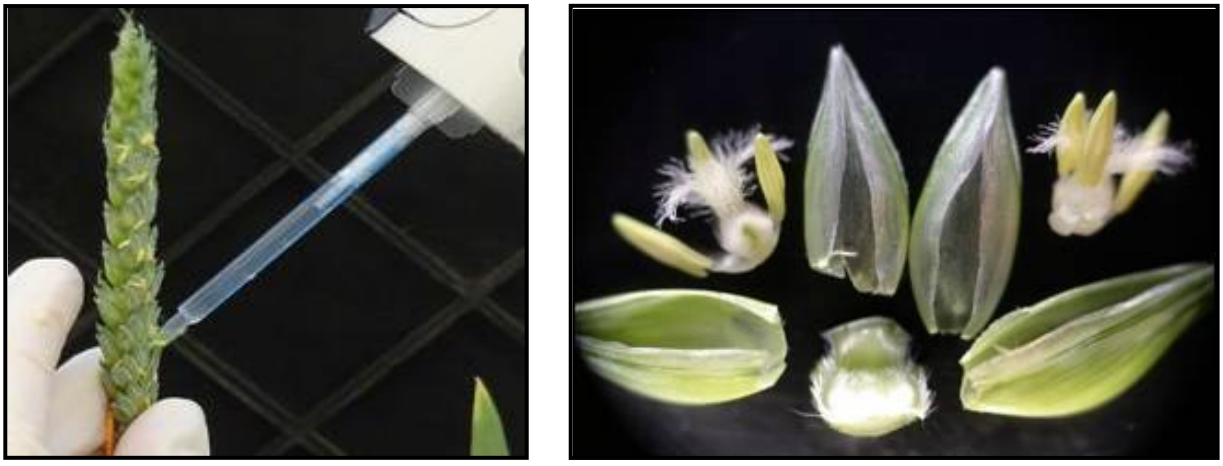


Figure 19a Point inoculation using a pipette; Figure 19b Dissected florets with lemma, palea, rachis node, rachis, and reproductive organs (Steiner et al. 2009).

3.4 cDNA-AFLP FINGERPRINTING

3.4.1 RNA extraction

For extraction of total RNA the Guanidinisothiocyanat/Phenol method (peqGOLD RNAPure, peqLab, Erlangen, Germany) was used, followed by a treatment with DnaseI (Invitrogen, Carlsbad, CA, USA), and a clean up and concentration step with Rneasy MinElute Spin Columns (Qiagen, Hilden, Germany) as previously described by Steiner et al. (2009).

3.4.2 Reverse transcription, digestion, ligation, and preselective amplification

Synthesis of double stranded cDNA from template mRNA was performed as previously described in Steiner et al. (2009). Reactions were performed with First Strand cDNA Synthesis Kit, *E. coli* DNA polymeraseI and ribonuclease H (all Fermentas St. Leon-Rot, Germany) according to the manufacturer's instruction. For reverse transcription, mRNA was fished from 5 µg of total RNA by the use of an oligo dT18 primer — complimentary to the poly A tail of mRNA at the 3´ end. Reverse transcription was carried out using M MuLV Reverse Transcriptase. After reverse transcription of the first strand, the second strand was complemented by *E. coli* DNA polymeraseI. Additionally, the mRNA template strand was removed by *E.coli* ribonuclease H.

Two-hundred and fifty ng of the double stranded cDNA were digested with the rare cutter restriction enzyme *Pst*I (Figure 20 — green recognition site, arrows indicating cutting sites) and the frequent cutter *Mse*I simultaneously (Figure 20 — red recognition site, arrows indicating cutting sites). Double stranded adapters (in yellow) corresponding to the restriction sites were ligated to the 5´and 3´ends of the digested fragments (Figure 20 — in blue letters), in order to obtain the primary template for the preselective amplification with the corresponding *Pst*I primer 5´GACTGCGTACATGCAG 3´ and *Mse*I primer 5´GATGAGTCCTGAGTAA 3´ (in orange) that are complementary to the adapters (Figure 20).

Digestion, ligation, and preselective amplification of the primary template were performed according to the protocols and under cycling conditions as described by Steiner et al. (2009).

Figure 20 Restriction sites, adaptors and primers.

3.4.3 Selective amplification

To facilitate the screening of amplified cDNA fragment length polymorphisms, the big pool including all amplified fragments of the preselective amplification was divided into subunits by applying primers with two selective nucleotides. Extension with two selective nucleotides on the

3´ends of the forward and reverse primer resulted in 256 primer combinations to cover all combinations for the four nucleotides.

Experimental work of this diploma thesis project started at this step and covered screening of 160 of the 256 primer combinations (

Table **2**)

Table 2 Screening of 160 primer combinations (indicated with white background)

dyes	Cy3	Cy3	Tam	Tam	Fam	Cy3	Fam	Cy5	Cy3	Cy5	Cy5	Cy5	Cy3	Fam	Cy5	Fam
PstI / MseI	AA	AC	AG	AT	CA	CC	CG	CT	GA	GC	GG	GT	TA	TC	TG	TT
AA	97	113	129	133	137	141	157	161	177	181	185	201	217	221	237	241
AC	98	114	*82*	*81*	*80*	142	*79*	162	*77*	*78*	186	202	*73*	222	*76*	242
AG	99	115	*63*	*62*	*61*	143	*60*	163	*58*	*59*	187	203	*72*	223	*57*	243
AT	100	116	*11*	*33*	*21*	144	*17*	164	*5*	*23*	188	204	*64*	224	*49*	244
CA	101	117	*19*	*8*	*9*	145	*22*	165	*29*	*12*	189	205	*65*	225	*50*	245
CC	102	118	130	134	138	146	158	166	178	182	190	206	218	226	238	246
CG	103	119	*32*	*20*	*6*	147	*30*	167	*40*	*16*	191	207	*66*	227	*51*	247
CT	104	120	*42*	*15*	*25*	148	*13*	168	*41*	*34*	192	208	*67*	228	*52*	248
GA	105	121	*7*	*48*	*31*	149	*1*	169	*45*	*44*	193	209	*68*	229	*53*	249
GC	106	122	*3*	*43*	*18*	150	*36*	170	*10*	*47*	194	210	*69*	230	*54*	250
GG	107	123	131	135	139	151	159	171	179	183	195	211	219	231	239	251
GT	108	124	*96*	*95*	*94*	152	*93*	172	*91*	*92*	196	212	*75*	232	*90*	252
TA	109	125	132	136	140	153	160	173	180	184	197	213	220	233	240	253
TC	110	126	*83*	*84*	*85*	154	*86*	174	*88*	*87*	198	214	*74*	234	*89*	254
TG	111	127	*38*	*37*	*35*	155	*26*	175	*46*	*4*	199	215	*70*	235	*55*	255
TT	112	128	*24*	*28*	*2*	156	*39*	176	*14*	*27*	200	216	*71*	236	*56*	256

For the selective PCR, the secondary template was diluted 1:120, reaction was performed using the following protocol and cycling conditions (

Table **3** and Table 4):

Table 3 Selective cDNA-AFLP PCR protocol (according to Steiner et al. 2009)

	Stock	Final	µl
Biotherm buffer Mg^{2+} 15mM	10X	1X	1
dNTPs	2mM	0,2 mM	1
***Pst*I Primer(NN)**	10 µM	0,3 µM	0,3
***Mse*I Primer (NN)**	10 µM	0,3µM	0,3
Biotherm Taq Polymerase	5 U/µl	0,5 U	0,1
aqua dest. H_2O			4,3
Master-Mix			7
Template – cDNA			3
Total reaction volume			10

Table 4 Selective cDNA-AFLP PCR program (according to Steiner et al. 2009)

selective amplification	**Temp**	**Time**
initial denaturation step	**94°C**	**2 min**
Loop: 10 cycles		
Denaturation	**94°C**	**30 sec**
annealing with gradient -1°C/cycle	**63°C**	**30 secs**
Extension	**72°C**	**2 min**
Loop: 24 cycles		
Denaturation	**94°C**	**30 sec**
Annealing	**54°C**	**30 sec**
Extension	**72°C**	**2 min**

The selective *Pst*I (NN) primers were labelled with one of the fluorochromes Cy5, Cy3, Tam or Fam of different excitation and emission wavelengths to allow for multiplexing in detection (d FAM on one gel simultaneously.

Table 5). As a result, electrophoresis and subsequent detection could be performed for three PCR reactions with differently labelled primers – Cy5, Cy3/Tam, and FAM on one gel simultaneously.

Table 5 Excitation and emission wavelength of fluorochromes for primer labelling

Fluorochrome	**Excitation (nm)**	**Emission (nm)**
Cy5 – red	**649**	**670**
Cy3 – green	**550**	**570**
Tam – green	**555**	**580**
Fam – blue	**435**	**520**

Ten µl of PCR product were diluted with 3X form amide fuchsine loading buffer (Merck, Darmstadt, Germany) and denatured at 96° Celsius for 10 minutes. Electrophoresis was performed in a vertical C.B.S. chamber (C.B.S Scientific Company, Del Mar, US) on a 7% denaturing polyacrylamide gel. 2.2 µl of diluted PCR product were loaded to a 94 shark tooth comb. Gels were run at 55 W and 80 W, for one gel or two gels respectively. In order to detect fragments in the size range between 40 bp and 700 bp, gels were scanned twice after two and a half hours and five hours running time using a Typhoon TRIO imager (GE Healthcare, Freiburg Germany) that allowed for multiplexed detection of the four fluorochromes Cy5, Cy3/Tam and Fam.

Fragment size was estimated with the help of size-markers in the range 70 to 800 bp that were kindly provided by Ms Manuela Diethelm, Bayerische Landesanstalt für Landwirtschaft, Freising. Polymorphisms in banding patterns were visually scored and labelled on the gel images (Figure 21) using standard software.

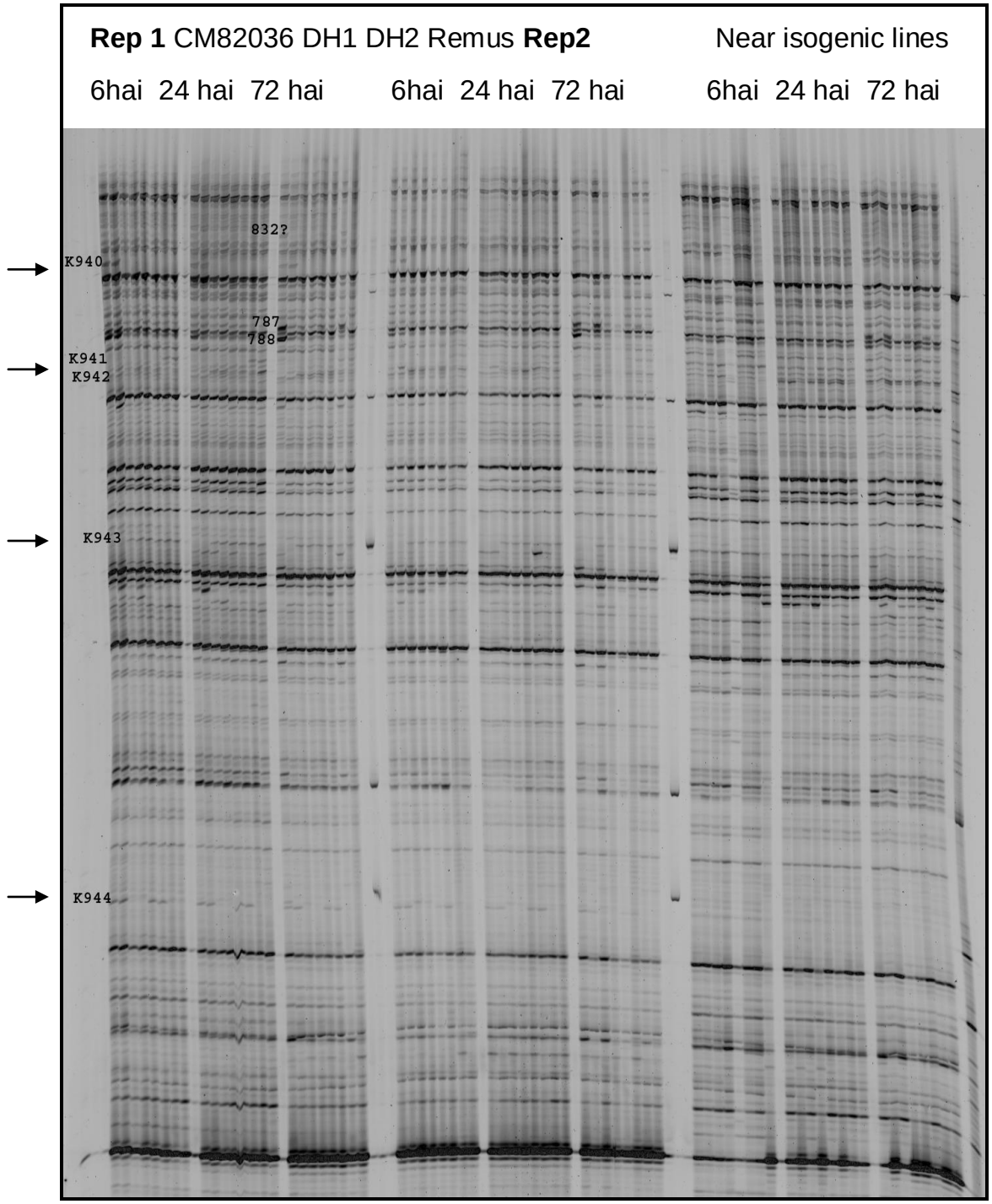


Figure 21 Polyacrylamide gel scanned on Typhoon TRIO imager, 2 replications of the CM86032/Remus experiment and 1 replication of the NIL experiment 3 blocks per replication represent 3 time-points. 8 lanes per block represent 4 genotypes with *Fusarium*(F) and water (W) treatment in the following order: CM-F, CM-W, DH1-F, DH1-W, DH2-F, DH2-W, Remus-F, Remus-W for replication one and two, for the near-isogenic lines: NIL1-F, NIL-1-W, NIL2-F, NIL2-W, NIL3-F, NIL3-W, NIL4-F, NIL4-W; arrows indicating scorred polymorphisms.

3.5 CLONING

3.5.1 Excision of the fragments

PCR products of primer combinations containing targeted polymorphisms were separated a second time for excision of the fragments for cloning. 3 to 4.5 µl of denatured PCR product in loading buffer were loaded on a 7–10% polyacrylamide gel using a 32 well shark tooth comb with 4.5 mm well width. Electrophoresis was performed under the same conditions as described above. Running time was adjusted to the length of targeted fragment and was calculated with 5 to 7 hours. Several control scans were required in order to obtain best separation of the targeted fragment. Before the final scan, the thicker glass plate that had been previously treated with gel repel solution (C.B.S Scientific Company, Del Mar, US) was lifted with the gel adhering to the thinner glass plate. In order to enable alignment of the print out with the glass plate carrying the gel after the scan, positions on the glass plate were labelled with markers of same colour as the detection spectrum of the laser – for Cy5, Cy3/TAM and FAM red, green and black or blue labelling was used, respectively.

The image obtained from the scan was printed in actual size. Then the print out was placed beneath the glass plate and aligned, according to the visible markers. At least two fragments per polymorphism were excised with a scalpel and each separately transferred into 50 µl of aqua dest. in a 1.5 ml Eppendorf tube (Figure 22a and Figure 22b).

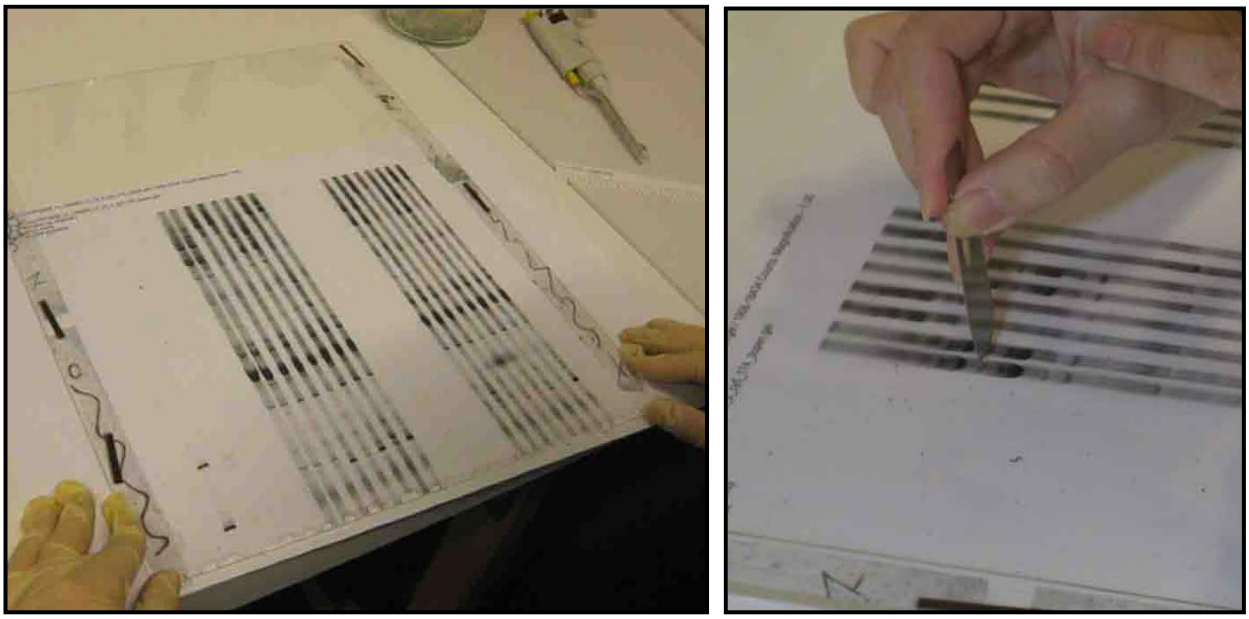


Figure 22a and 22b Excision of fragments from polyacrylamide gel.

Gel pieces the size of about 0.5 mm x 2 mm were incubated overnight to allow diffusion of DNA into the aqua dest. After a centrifugation step at max speed for 3 min, 35 µl of the supernatant were transferred into a new tube. The supernatant was diluted 1:100 for the PCR.

Amplification of the two excised fragments per polymorphism was performed using the cycling conditions of selective PCR as described above and the following PCR protocol (

Table 6) with the corresponding selective *Mse*I primer *Mse*I(NN) and the unselective *Pst*I primer *Pst*I(00) that was not fluorescently tagged.

Table 6 Selective PCR reaction

Selective Amplification	**Stock c**	**Final c**	µl per Reaction
Biotherm buffer			
Mg^{2+} 15 mM	10X	1X	3
dNTPs	2mM	0,2 mM	3
***Pst*I Primer(00)**	10 µM	0,25 µM	0,7
***Mse*I Primer (NN)**	10 µM	0,25 µM	0,7
Biotherm Taq Polymerase	5 U/µl	1 U	0,2
aqua dest H_2O			19,4
Master-Mix			27
template-DNA			3
Total reaction volume			30

To confirm that the amplicon consists of one clear band and to estimate size and quantity, 2.5 µl of PCR product diluted with 1 µl of 6X Massruler Loading Dye Solution (Fermentas, St. Leon-Rot, Germany) and 5 µl of Massruler Express DNA Ladder (Fermentas, St. Leon-Rot, Germany) were loaded on a 2% agarose gel. Electrophoresis was performed at 85 mA for 30 min in 1X tris acetate (TAE) buffer using a horizontal electrophoresis chamber. Fragments stained either ethidium bromide or Sybr green I (Invitrogen, Carlsbad, CA, USA) were detected under UV light using Transiluminator Gel Doc XR 170-8170 (Biorad, Milan, Italy).

In order to ensure that only fragments from targeted bands serve as templates for the cloning, 15 to 20 µl of the PCR product diluted with 6X Massruler Loading Dye Solution (Fermentas, St. Leon-Rot, Germany) were loaded on a 2% agarose gel and separated at 85 mA for 20 to 30 min. For estimation of fragment size and quantity, 10 µl of Massruler Express DNA Ladder (Fermentas,Germany) with a size range of 100 to 1000 bp and quantity range of 10 to 100 ng DNA/10 µl were loaded.

Well-separated single bands were excised from the gel under UV light exposure using Preparation Mode of Transiluminator Gel Doc XR 170-8170 (Biorad, Milan, Italy) (Figure 23a and Figure 23b). DNA was recovered from 1–3 mm x 5–10 mm gel pieces using Wizard SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany) according to the manufacturer's instruction.

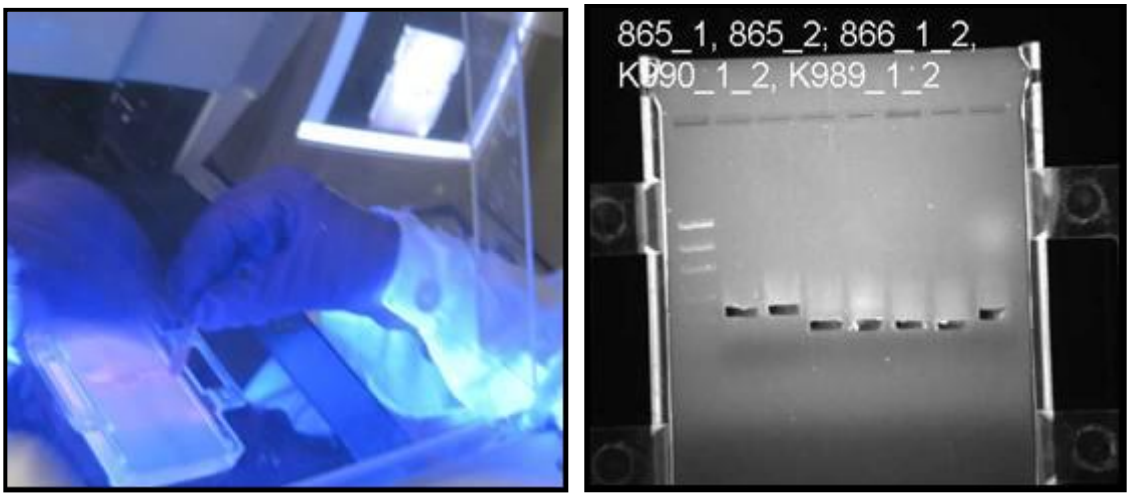


Figure 23a and 23b Excision of fragments from agarose gel under UV light.

3.5.2 Ligation to the Vector

The purified fragments were ligated to the linearised pSTBlue-1 AccepTor Vector (Novagen, VWR International, Vienna, Austria) containing a single 3´dU overhang at restriction site (Figure 24) that allows for ligation of fragments with 3´dA overhangs resulting from unspecific elongation activity of taq DNA polymerase. The cloning site is flanked by T7 promoter and SP6 promoter regions that serve as primer annealing sites for the amplification of the cloned insert in the post colon PCR.

Blue and white screening of the colonies was used as marker for successful ligation of the insert. At the cloning site, the pSTBlue-1 vector harbours an open reading frame for the lacZ alpha peptide that complements the lacZ fragment located on the F´plasmid in the competent cells resulting in active β-galactosidase that enables white colonies to show a blue phenotype in the presence of X-gal substrate and IPGT (both Fermentas, St. Leon-Rot, Germany) for induction. Successful ligation disrupts the open reading frame of lacZ peptide resulting in white colonies on the selective substrate. In addition, pSTBlue-1 AccepTor vector contains an open reading frame for aminoglycosidase 3´phosphotransferase as a selectable marker that confers resistance to kanamycin in transformed cells growing on selective media.

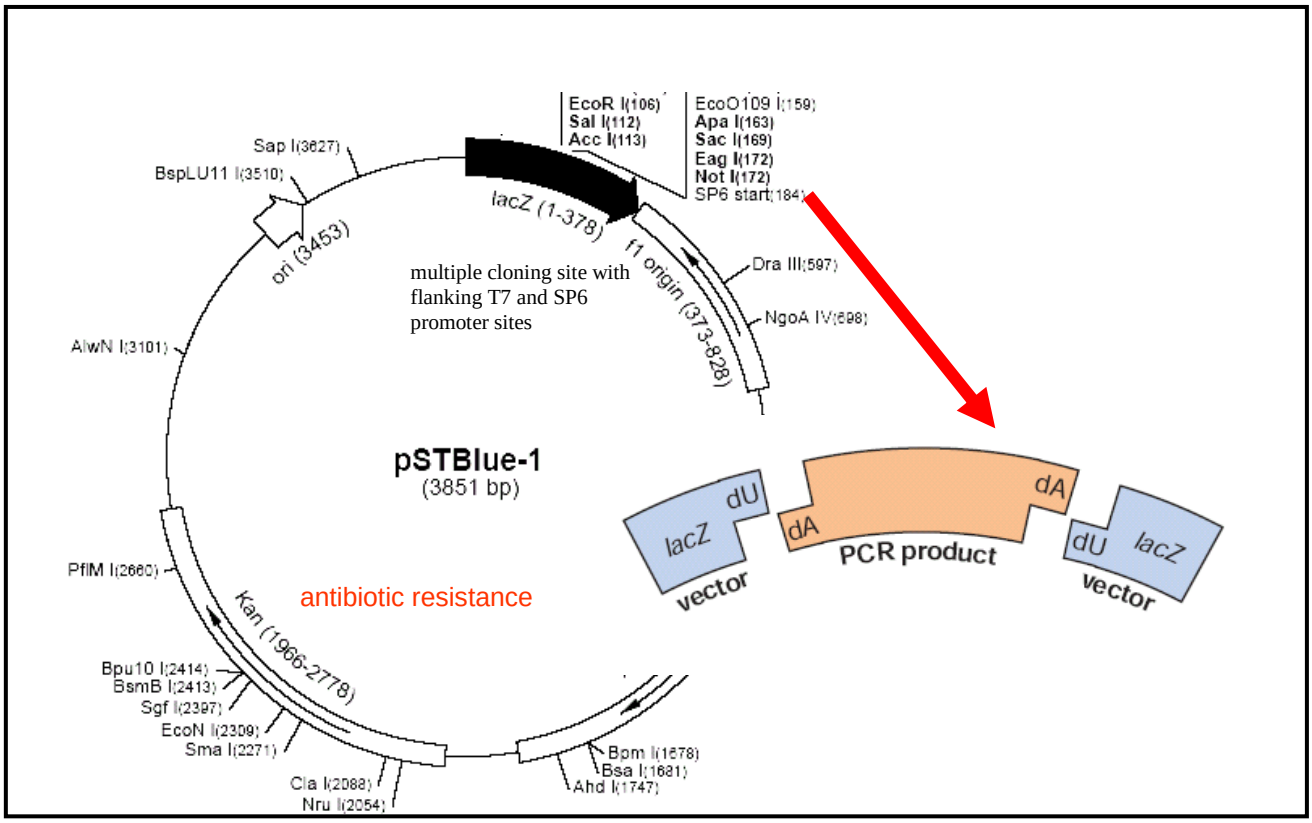


Figure 24 Characterisation of AccepTor Vector pSTBlue-1.

Ligation was performed according to the company ´s protocol using one quarter of the recommended reaction volume: 0.25 µl of AccepTor Vector containing about 12.5 ng or 0.005 pmol of pSTBlue-1 vector and 1.25 µl Clonable 2X ligation mix containing ligase, buffer, and cofactors were assembled to a premix. In order to provide the recommended insert to vector molar ratio of 5:1 for the ligation reaction, amount of template DNA (in ng) was calculated as follows:

$$\text{PCR product of insert (3.5 ng)} = \frac{\text{amount of vector (12.5 ng)} \times \text{size of insert (200 bp)} \times 5 \text{ (ratio)}}{\text{size of vector (3851 bp)}}$$

DNA concentration in the template for ligation ranged between 2.5 ng/µl (weak bands), 5 ng/µl (middle bright bands) and 7 ng/µl (strong bands) according to quantity estimation on agarose gel. Based on this calculation, 0.7 to 1.5 µl of cDNA template were added to 1.5 µl ligation premix in order to obtain the calculated amount of 3.5 ng DNA. Ligation reaction was incubated at 16° C for two hours in the thermo-block.

3.5.3 Transformation

Nova Blue Single Competent cells (Novagen, VWR International, Vienna, Austria) were transferred from –80°C and thawed on ice for 2-5 min. For transformation, 1 to 1.5 µl of ligation product were added to 16 µl of Nova BLUE Single Competent cells (Novagen, VWR International, Vienna, Austria) in 1.5 ml tubes. Reactions were placed on ice for 7 min, incubation on ice was interrupted by heat treatment at 42 °C for 30 second after 5 min. After incubation on ice, 80 µl SOC-Medium (Novagen, VWR International, Vienna, Austria) was added and the suspension was incubated at room temperature for 30 min to allow transformed *Escherichia coli* cells to recover.

Seventy µl of the suspension were plated on one half of a pre-warmed 82 mm LB agar plate (Carl Roth GmbH, Karlsruhe, Germany) containing 50 µg/ml carbeniciline, 15 µg/ml tetracycline (both Carl Roth, Karlsruhe, Germany) 2 µl/ml X-gal and 6.5 µM/ml IPTG (both Fermentas, St. Leon-Rot, Germany) on a sterile work bench. Plates were incubated at 37°C for 24 hours until most colonies were at least between 0.5–1 mm in diameter.

After incubation, white colonies representing transformed colonies with vectors carrying the inserted fragment at their multiple cloning site were selected from the blue colonies on the plates. 8 white colonies per polymorphism — 4 colonies from each cut — were picked and transferred into wells of a 96 deep well plate containing 300 µl LB medium (Carl Roth GmbH, Karlsruhe, Germany) with 50 µg/ml carbeniciline and 15 µg/ ml tetracycline. Adding tetracycline eliminated colonies lacking the F´plasmid and thus appearing white under any condition as they fail to complement the lacZ fragment.

The deep well plates were sealed with breathable film, placed into the thermo-shake incubator at 37°C and shaken at 100 rpm for 24 hours. After incubation, plasmids were isolated from 40 µl of the bacteria suspension by an incubation step at 95°C for 5 min. 1 µl of the suspension served as template for the post-colon PCR performed with primers corresponding to the T7 and SP6 promoter regions flanking the multiple cloning site of the plasmid and thus amplifying the inserted fragment (Table **7**).

Table 7 PCR reaction for post-colon PCR

	Stock	Final	in µl
Biotherm buffer Mg2+ 15mM	10X	1X	2.5
dNTPs (2 mM)			2.5
BSA (250 µg/ml)	2 mM	0,2 mM	2.5
SP6 10 µM	250 µg/ml	25 µg/ml	0.625
T7 10 µM	10 µM	0,25 µM	0.625
Biotherm Taq Polym.	10 µM	0,25 µM	0.1
DNA template	5 U/µl	0,5 U/µl	1
H2O	1 µl		15.15
Total reaction volume	15,15 µl		25

Cycling was performed with a denaturing step at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec, elongation at 72 °C for 60 sec and a final extension step at 72 °C for 5 min. Twenty-five µl of PCR product were supplemented with 10 units of Exonuclease I and 1 unit of Shrimp Alkaline Phosphatase (both Fermentas, St. Leon-Rot, Germany) before sequencing reaction, in order to degrade excess primers and to dephosphorylate remaining dNTPs in the template. ExonucleaseI and Shrimp Alkaline Phosphatase purification was performed at 37°C for 30 min for enzyme activation followed by a temperature step at 80°C for 15 min for enzyme deactivation, according to the manufacturer's recommendation. To confirm DNA quality and similarity of the 8 fragments, 5 µl of purified DNA in 6X loading buffer were loaded on a 100 ml 2 % agarose gel that enables loading of 8 samples in one row for comparison.

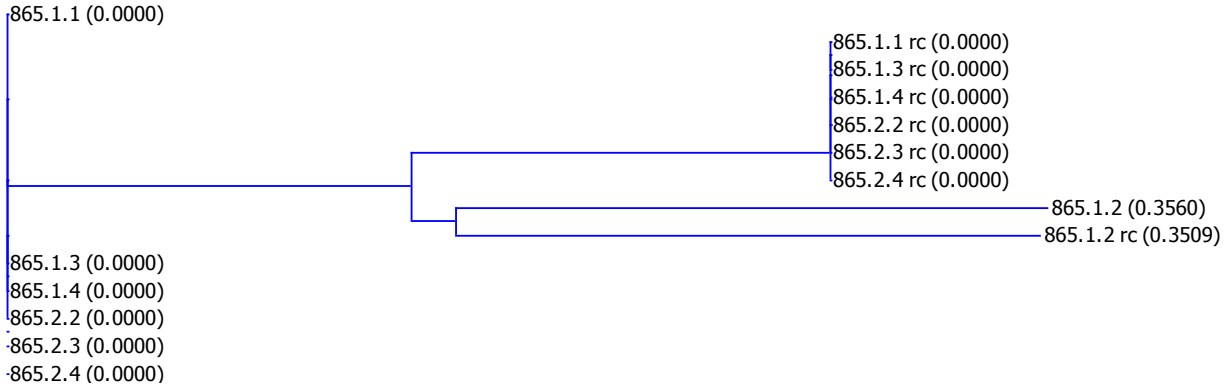
3.5.4 Sequencing — generation of sequence information

Sequencing reaction was performed with the DYEnamic ET DYE Terminator Cycle Sequencing Kit for MegaBace DNA Analysis Systems (GE Healthcare, Freiburg, Germany). Sequencing reaction was assembled according to the manufacturer's protocol. The sequencing reaction was performed in a total volume of 10 µl containing the 4 µl of sequencing reagent premix, 4.5 µl of aqua dest. and 0.5 µl of one of the corresponding primers (T7 or SP6 in 10 µM/µl). The cycling program consisted of 36 cycles of 95°C for 20 sec, 60°C.

After cycling, 5 µl of aqua dest were added to the sequencing reaction resulting in a total reaction volume of 15 µl. For Sephadex column purification, Sephadex G-50 DNA Grade fine powder (GE Healthcare, Freiburg, Germany) was water soaked in Millipore MultiScreen-HV, MSHVN4510, 96 well filter plates with a low protein binding durapore membrane (GE Healthcare, Freiburg, Germany) according to the manufacturer's recommendation. Total amount of sequencing reaction was transferred to the Sephadex columns and centrifuged at 910 g for 5 min into a 96 well sequencing plate compatible to the MegaBace 500 sequencer (GE Healthcare, Freiburg, Germany). Sequencing was performed on MegaBace 500 (GE Healthcare, Freiburg, Germany), electropherograms were quality checked by MegaBace Sequence Analyser software and base calling was performed by Cimarron 4.00 software (both Amersham Bioscience, GE Healthcare, Freiburg, Germany). Subsequently, vector sequences were excised and transformation into FASTA format was performed. Sequences with low quality, in which the vector sequences were not found at 90 % were excluded from further alignments. Alignment and generation of reverse complements were performed in Vector NTI Advance 9 software (Invitrogen, Carlsbad, CA, USA).

3.5.4.1 Sequence alignment and annotation of putative function

Sequence information was accepted and considered as valid, if at least 4 out of 8 sequences were identical or displayed good alignment (Figure 25).




```
                          1                                  40
865.1.1 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
865.1.3 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
865.1.4 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
865.2.2 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
865.2.3 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
865.2.4 rc   (1) ACCATGAGACTGGCGACCAGTAGGACGACAAGGCCAAATC
```

Figure 25 Sequence alignment.

The sequence reads of polymorphisms with good alignment were subsequently aligned with sequences available on the database of NCBI (National Centre for Biotechnology Information, Bethesda, MD, U.S.A) “GeneBank” that comprises an annotated collection of all publicly available nucleotide and protein sequences. Sequence comparison including a calculation of the statistical significance of matches was conducted with the BLASTN 2.2.19 program (Altschul et al. 1997). In order to specify the BLASTN search, divisions of GenBank were used for the queries. For the first query, the non redundant database which represents all GenBank entries except ESTs was selected, whereas in the second query, the est_others database comprising all ESTs of non human and non mouse origin was used. In addition, it was confirmed that sequences were not of fungal origin by performing a BLAST search against the *Fusarium graminearum* Genome DataBase (http://mips.gsf.de/genre/proj/fusarium) using the search parameter “nucleotide, ORF” with an E-value threshold of less than 10 e-10.

BLAST hits resulting from both queries with an E value less than 10 e-5 were accepted as significant hits and subjected to a query against the unigene database. From the unigene database information on gene expression features and co-expressed unigenes were obtained, as this database organises a collection of ESTs and full length mRNA sequences that way that each unigene entry represents a set of transcripts that are supposed to come from the same locus of a unique known or putative gene. In the unigene database a query for protein similarity using the NCBI reference sequence protein database is provided that allows for annotation of gene function. Putative gene functions were summarized in categories using the MIPS FunCat functional annotation scheme (Ruepp et al. 2004) as a guideline. In order to obtain a summary of the gene and its putative function, the full report in the EntrezGene database was used, that gives a gene summary on gene names, genomic regions and gene function. EntrezGene provides links to the Pfam database, a large collection of protein families, and to the literature database PubMed for further reading.

4 RESULTS

4.1 SCREENING FOR POLYMORPHISMS

In the cDNA-AFLP fingerprinting experiment of this diploma thesis, 160 out of 256 primer(NN) combinations were tested (summary of results Table I). Testing of 160 primer combinations with on average 60 fragments per combination in the size range between 40 bp to 700 bp resulted in about estimated 10.000 scored bands in total (Steiner et al. 2009). About 90 % of these TDFs were found to be monomorphic between genotypes, treatments, and time-points. 953 fragments displayed polymorphic banding patterns either between genotypes or and between the two treatments — water and *Fusarium* inoculation. In addition, expression was evaluated over time course of infection at three time-points (6 hai, 24 hai and 72 hai). Only polymorphisms that were confirmed in both replications were considered as reproducible and valid.

Out of 953 polymorphic bands, 687 bands were constitutively expressed after water and *Fusarium* treatment, but displaying polymorphisms between genotypes (

Figure **26**). Constitutive and genotype-specific polymorphisms were classified as group A in Steiner et al. (2009).

In addition, 266 were induced after *Fusarium* treatment, but not after water treatment, and considered as pathogen-responsive fragments. Pathogen-responsive fragments were either upregulated in all 8 genotypes (CM82036, Remus, DH1, DH2, NIL1–4) and termed “pathogen-responsive and genotype-unspecific” (group B according to Steiner et al. 2009) (Figure 27), or they were differentially upregulated between the 8 genotypes and termed “pathogen-responsive and genotype-specific” (group C, according to classification by Steiner et al. 2009) (

Figure 28).

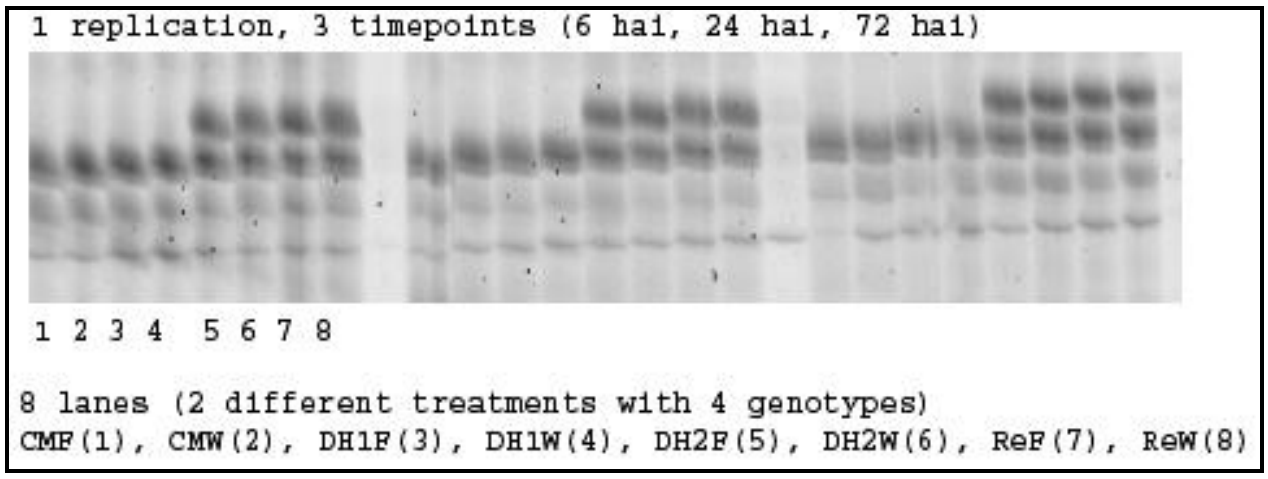


Figure 26 Expression of polymorphism K875, constitutive and genotype-specific expression in DH2 and Remus. F indicating inoculation with Fusarium, W indicating inoculation with water.

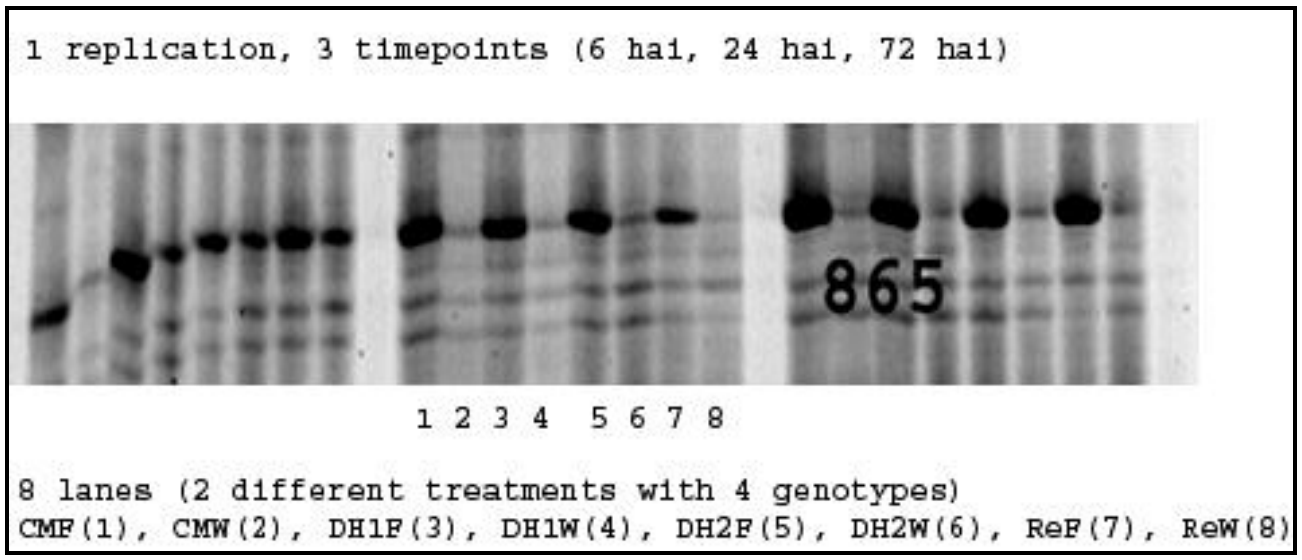


Figure 27 Polymorphism 865, pathogen-responsive, genotype-unspecific and induced 24 hai.
F indicating inoculation with Fusarium, W indicating inoculation with water.

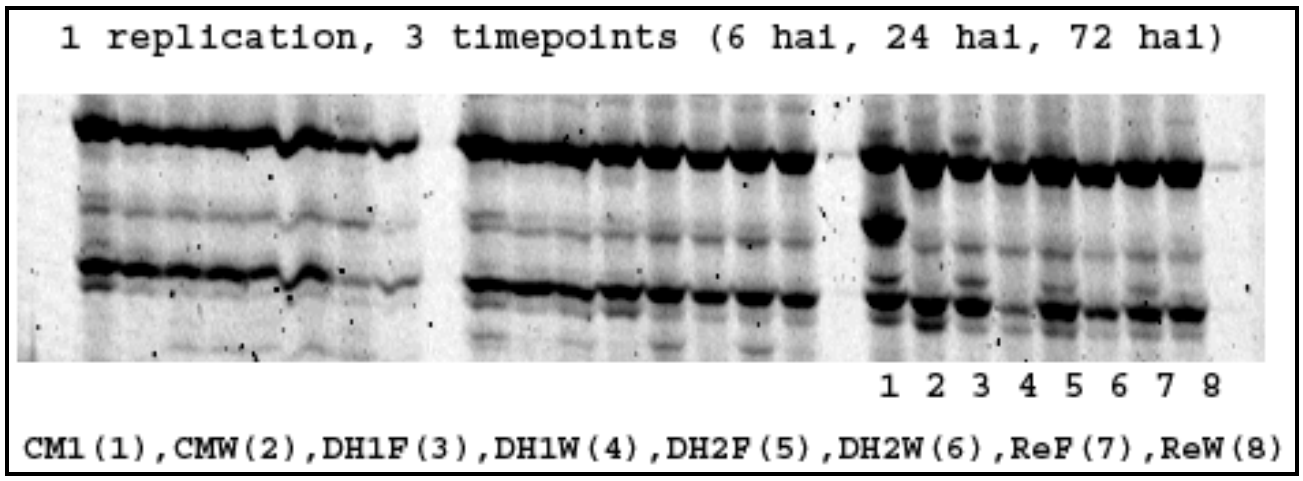


Figure 28 Polymorphism 444, pathogen-responsive, genotype-specific– exclusively expressed in CM82036, F indicating inoculation with Fusarium, W indicating inoculation with water.

4.1.1 Constitutively expressed polymorphisms

687 polymorphisms were genotype-specific and constitutively expressed after water and *Fusarium* inoculation and did not show changes in expression patterns over time course of infection. In resistant CM82036 384 TDFs were constitutively and differentially expressed from Remus. In contrast, 303 TDFs were expressed in Remus but not in CM82036. Out of the 384 TDFs expressed in CM82036, 211 were exclusively present in CM82036. By contrast, out of 303 bands expressed in Remus, 98 bands were exclusively expressed in Remus. Between the F1 recombinant doubled haploid lines DH1 and DH2 200 TDFs were differentially expressed in comparison with 687 TDFs differentially expressed between their parents.

DH1 and CM82036 representing the resistant phenotype, share 86 TDFs. Taken together with the TDFs exclusively expressed in CM82036 (211 TDFs), 297 bands were found in the resistant

phenotypes that were not shared with the susceptible phenotypes. In comparison, Remus and DH2 representing the susceptible phenotype share 60 TDFs and taken together with the TDFs only expressed in Remus, 158 TDFs (23 %) were exclusively expressed in the susceptible phenotypes (Figure 29).

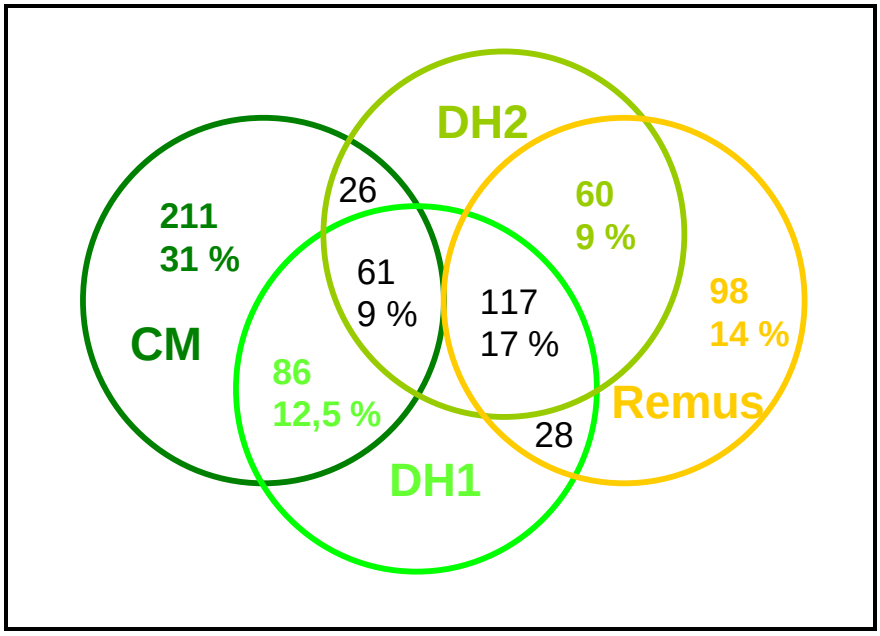


Figure 29 Distribution of 687 constitutive genotype-specific TDFs.

All polymorphic bands expressed in Remus were also present as monomorphic bands in the near isogenic lines carrying 98,4 % of genetic background of Remus. However, 14 constitutive polymorphisms out of 687 were polymorphic in the near isogenic lines. 11 out of these 14 polymorphic bands were not expressed in Remus, but in CM82036 and DH1 indicating that the alleles may have derived from CM82036 and are related to either QTL *Fhb1* on 3BS or *Qfhs.ifa-A5*.

Thereof, 7 polymorphisms (TDFK347, TDFK535, TDFK436, TDFK453, TDFK563, TDFK1031, TDFK945) showed bands in CM82036, DH1 – NIL1, NIL3 indicating association with *Qfhs.ifa*-5A (Figure 30). 4 polymorphisms (TDFK366, TDFK451, TDFK619, TDFK601) were expressed in CM82036, DH1 – NIL1 and NIL2, indicating association with *Fhb1* on chromosome 3 BS (Figure 31). Furthermore, 3 TDFs (TDFK603, TDFK875, TDFK878) were expressed in Remus, DH2 – NIL2, NIL4.

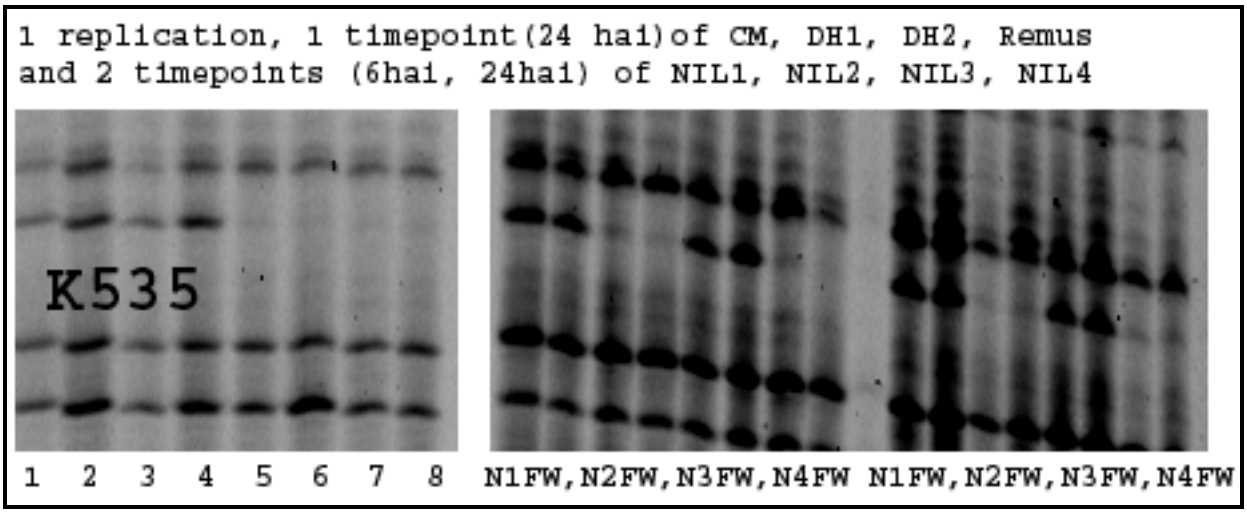


Figure 30 Constitutive polymorphism K535 expressed in the near isogenic lines NIL1and NIL3 carrying the QTL on 5A**;** block 1 showing 1 timepoint: CM (1), CMW(2), DH1F(3), DH1W(4), DH2F(5), DH2W(6), RemusF(7), Remus W(8), block 2 showing 2 timepoints: N1FW, N2FW, N3FW, N4FW, F indicating inoculation with *Fusarium*, W indicating inoculation with water.

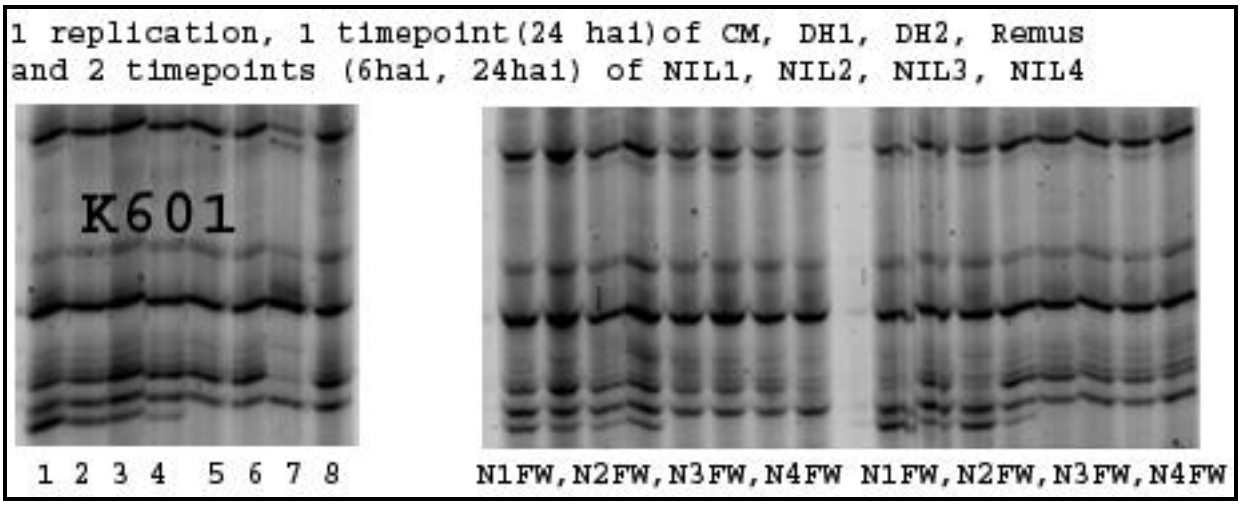


Figure 31 Constitutive polymorphism expressed in the near isogenic lines carrying QTL *Fhb1*on 3BS, block 1 showing 1 timepoint: CM (1), CMW(2), DH1F(3), DH1W(4), DH2F(5), DH2W(6), RemusF(7), Remus W(8), block 2 showing 2 timepoints: N1FW, N2FW, N3FW, N4FW, F indicating inoculation with *Fusarium*, W indicating inoculation with water.

4.1.2 Pathogen-responsive polymorphisms

266 polymorphisms were found to be pathogen-responsive, representing 2,66 % of estimated 10.000 transcript derived bands scored in this experiment. All 266 TDFs were found to be upregulated in response to inoculation with *Fusarium graminearum*, no downregulation was observed as response to the pathogen. Downregulation after water treatment was observed in 56 TDFs (21 % of 266 pathogen responsive) that originally showed constitutive expression patterns at early time-points (Figure 32).

A number of 233 were induced in all genotypes and termed “pathogen-responsive and genotype-unspecific”. Thereof, 213 showed similar quantitative expression levels in all genotypes, whereas 20 showed quantitative stronger expression in CM82036 (Figure 33).

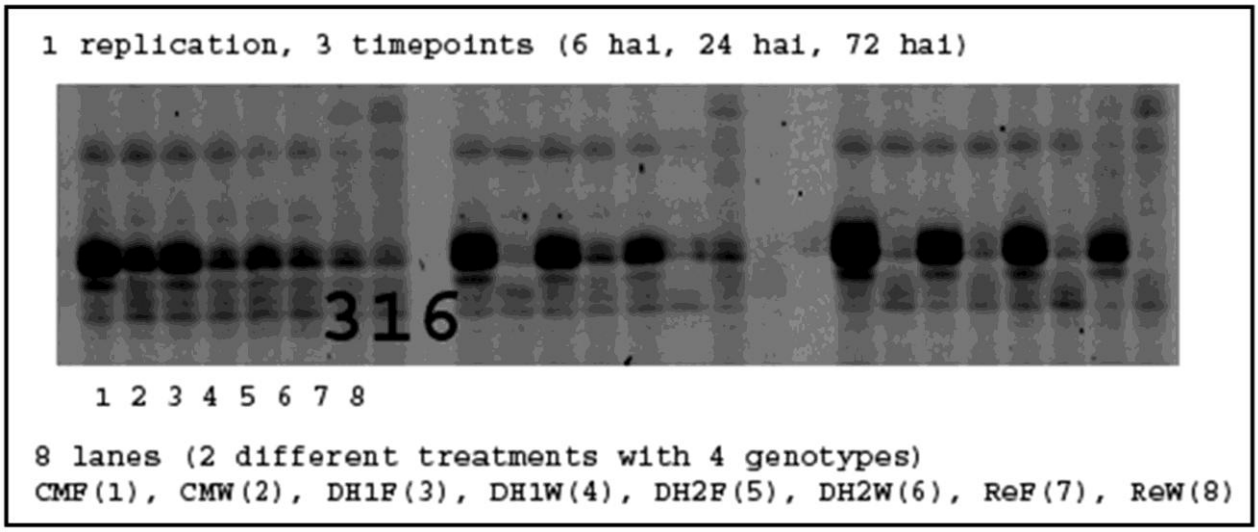


Figure 32 Downregulation after water treatment over time-course of infection.

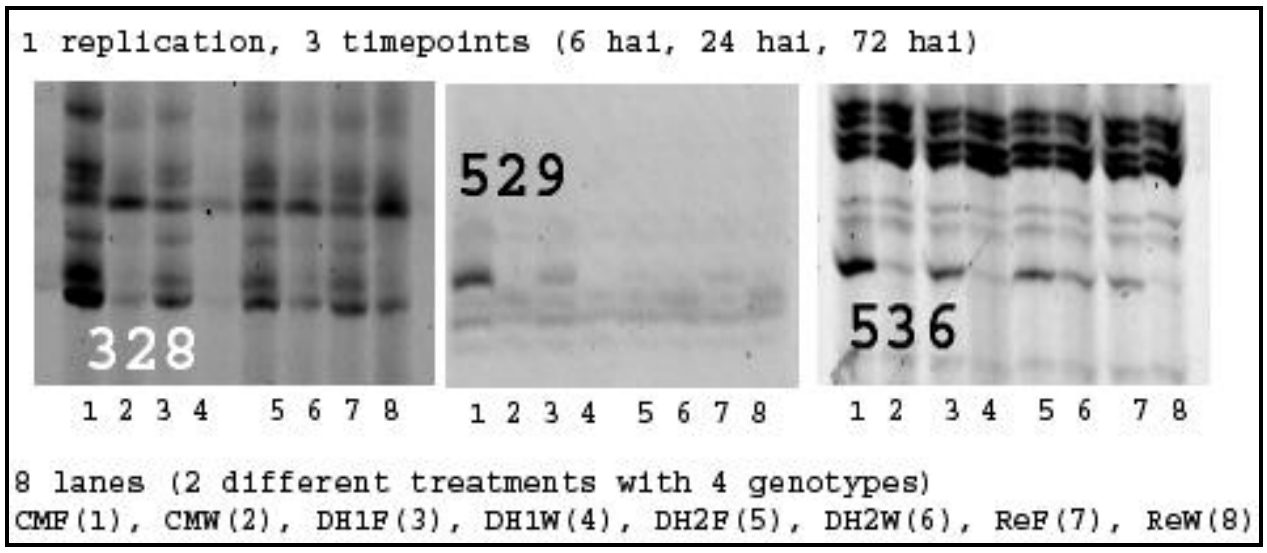


Figure 33 TDF328, TDF 529, and TDF536 showing quantitatively stronger expression in CM82036.

Thirty three TDFs out of total 266 pathogen-responsive TDFs (12 %) showed pathogen-responsive and genotype-specific expression patterns. Thereof, 13 were exclusively upregulated in CM82036 and 5 were upregulated in CM82036 and DH1. Thus, 18 TDFs were exclusively upregulated in the resistant phenotypes that were not expressed in the susceptible phenotypes. In addition, 7 polymorphisms were upregulated in all genotypes except Remus. In total, 25 pathogen-responsive TDFs were found to be expressed in CM82036 that were not found in the susceptible parent Remus.

In the group of susceptible phenotypes, two polymorphisms were upregulated exclusively in Remus, one polymorphism was upregulated in Remus and DH2. Furthermore, 5 polymorphisms were upregulated in all lines except CM82036 (Figure 34).

Out of 233 pathogen-responsive TDFs found, 109 polymorphisms were confirmed in the near-isogenic lines. However, no pathogen-responsive and genotype-specific TDFs were found that showed differential expression patterns between the near isogenic lines.

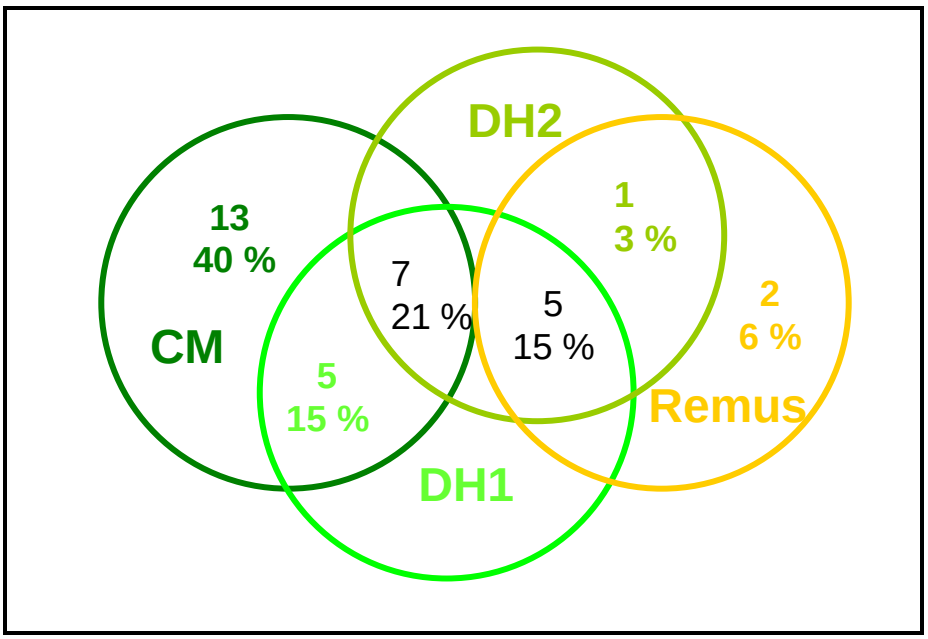


Figure 34 Distribution of pathogen-responsive TDFs.

In the time-course of infection, 5 polymorphisms were found to be early upregulated at 6 hai (TDF865, TDF866, TDF513, TDF487, and TDF321). They are pathogen-responsive and genotype-unspecific, but all 5 TDFs are found to be stronger expressed in resistant CM82036. 50 bands showed induction at an intermediate time-point at 24 hai. Once upregulated, no down regulation was observed at the later time-point at 72 hai. The vast majority of 211 pathogen-responsive TDFs showed late induction at 72 hai.

4.2 GENERATION OF EXPRESSED SEQUENCED TAGS (ESTS)

Out of 953 scored polymorphisms 70 TDFs were excised, cloned and sequenced (summary of results Table II). Twenty-five pathogen-responsive and genotype-specific TDFs and 5 TDFs with quantitative stronger expression in CM82036 were excised, cloned, and sequenced with highest priority. As these TDFs were considered to be most likely involved in resistance to *Fusarium graminearum* infection. In addition, sequence information was obtained for 15 pathogen-responsive and genotype-unspecific TDFs with 4 of them being induced after 6 hai. Selection of pathogen-responsive and genotype-unspecific TDFs was biased and favoured TDFs with clear and strong induction occurring with pathogen-responsive and genotype-specific TDFs in the same primer combination and thus on the same gel.

In the group of constitutively expressed TDFs that were found to be differentially expressed in the near isogenic lines, for 11 out of originally 14 screened polymorphisms sequence information was obtained. In addition, 14 TDFs with constitutive expression pattern being monomorphic in the NILs were excised, cloned and sequenced. In case of the constitutive genotype-specific TDFs, selection was biased favouring TDFs with expression patterns that represented resistant phenotypes (CM82036 or CM82036 and DH1) and that occurred with pathogen-responsive and genotype-specific TDFs in the same primer-combination and thus on the same gel. For the 14 constitutive TDFs, 8 were exclusively expressed in CM82036 and DH1 corresponding to the resistant phenotype and 4 were exclusively found in Remus and DH2 and Remus, corresponding to the susceptible phenotypes.

4.3 FUNCTIONAL ANNOTATION

4.3.1 Pathogen-responsive ESTs

For the 70 ESTs, BLASTN search was performed against the NCBI database divisions “non-redundant” and “est_others, non-human, non mouse” which resulted in further links to unigene entries and reference proteins with putative gene functions. For all 70 ESTs E-values lower than 10 e -5 were obtained in the BLASTN search against the est_others. BLASTN search against non-redundant database division resulted in hits with an E-value lower than 10 e-5. Furthermore, putative gene functions were assigned to 51 ESTs. 18 ESTs did either show no homology to a reference protein or did show homology to a hypothetical protein and were summarized under the category “transcribed locus with unknown function”. For 1 TDF (TDF490) a positive hit in the *Fusarium graminearum* Genome DataBase was obtained and thus was assigned to be of fungal origin. 64 out of 70 ESTs derived from *Triticum aestivum*. Thereof, 20 ESTs showed homology to ESTs reported from *Fusarium* inoculation projects. Apart from that, 4 ESTs showed homology to other grass species such as *Hordeum vulgare, Panicum virgatum, Brachypodium distachyon*. By contrast, information on reference proteins was predominately associated with *Arabidopsis* and rice.

Based on the BLASTN hits, ESTs were summarized in groups according to putative gene function using the “MIPS FunCAT” functional annotation scheme provided under http://www.mips.gsf.de/proj/funcatDB/ (Ruepp et al. 2004) as guideline. Thereby, it was primarily focussed on the pathogen-responsive ESTs.

For 44 pathogen-responsive ESTs BLAST search was performed (Table 8 and Table 9). 19 out of 44 ESTs accounting for more than 40% were found to be related to metabolic processes, such as transferase enzymes and proteins involved in secondary metabolism (members of the cytochrome P450 gene family, cycloartenol synthase and terpene cyclase). Two TDFs were related to transporter and localisation functions.

Seven ESTs, showed homology to proteins involved in cell communication mainly represented by putative kinases. Interestingly, 4 out of 7 annotated ESTs involved in cell communication belong to the category that shows stronger induction in CM82036. Thereof, TDF321 shows homology to a translation initiation factor SUI1 family protein, TDF529 shows homology to a receptor like kinase1, and TDF536 shows homology to a member of the serine/threonine kinase family.

Eight ESTs could be directly related to defence response such as pathogenesis related proteins and proteins that are reported to be involved in the process of oxidative burst (putative NADP dependent oxidoreductases, blue copper binding proteins). ESTs with a function in defence were exclusively found in the group of pathogen-responsive TDFs. Four of them were genotype-specific and found to be upregulated in all lines except Remus and the near isogenic lines. Four were genotype-unspecific and upregulated in all lines, two of them being early induced.

Ten TDFs accounting for 23% of 44 pathogen-responsive TDFs were summarized in the functional category “unknown function”. These TDFs showed similarity to ESTs in the NCBI database, but either no unigene hit or no homology to a reference protein was found. In addition, TDFs that showed homology to hypothetical proteins were also placed into this category (Figure 35).

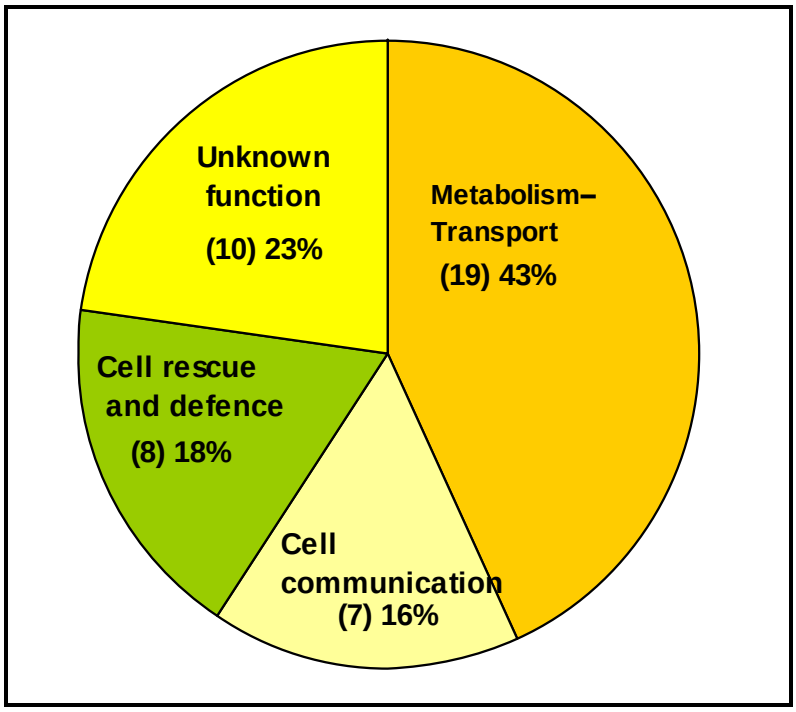


Figure 35 Functional annotation and categorisation of 44 pathogen-responsive wheat ESTs, which sequence information was obtained for.

Table 8 Functional annotation of pathogen-responsive and genotype-specific ESTs

TDF-ID	Size in bp	putative function – gene annotation – EST description	Function Category
		Upregulated in CM82036	
308	135	Non-specific lipid-transfer protein type 1 (LTP 1)	Metabolism
348	146	Ta10c_AAFC_ECORC_*Fusarium_graminearum*_inculated_wheat_head	Transcribed locus
329	280	Cytochrome b5 family protein heme/transition metal ion binding domain.	Metabolism
N301	191	Major Facilitator Super-family, Zinc induced facilitator-like1	Transporter
444	229	Transcribed locus in *Fusarium graminearum* infected spike cDNA library	Transcribed locus
400	250	Transferase family protein	Metabolism
		Upregulated in CM82036 and DH1	
848	83	Terpene cyclase - Ent-kaurene synthase-like protein 1	Secondary metabolism
786	70	Transcribed locus	Transcribed locus
787	370	Cytochrome P450	Metabolism
788	335	Photosystem II protein D1 - photosynthetic reaction centre protein	Metabolism – energy
		Upregulated in CM82036, DH1 and DH2	
757	115	PR-5 Protein, Thaumatin-like protein	Defence
503	107	Putative NADP-dependent oxidoreductase, Allyl alcohol dehydrogenase	Metabolism
432 a	97	PR-1Protein	Defence
432 b	97	Galactosyltransferase	Metabolism
541	169	Zn finger, C3HC4-type RING finger family protein	Cell communication
442	107	PSAL (photosystem I subunit L) Photosystem I reaction centre subunit	Metabolism
372	207	Transcribed locus	Transcribed locus
347	550	Pathogenesis-related protein PRB1-2 precursor	Defence
		Upregulated in Remus and DH2	
409	62	Glycoside hydrolase family 28 protein, pectinase	Metabolism
		Upregulation in DH1, DH2, Remus	
N300	125	Phosphoribosylanthranilate transferase-like protein; Protein kinase C	Cell communication
747	435	Tropinone reductase II	Metabolism
421	123	Transcribed locus	Transcribed locus
407	85	No significant similarity found	Unknown
791	116	EF-hand, calcium binding motif	Cell communication
429	217	MaoC-like dehydratase domain containing protein	Metabolism

Table 9 Functional annotation of pathogen-responsive and genotype-unspecific ESTs

TDF-ID	Size in bp	putative function - gene annotation - EST description	Function Category
		quantitatively stronger in CM82036	
486	186	Transferase family	Metabolism
489	95/85	transcribed locus	transcribed locus
529	94	Protein kinase family protein, Serine/Threonine protein kinases	Cell communication
321	169	Translation initiation factor SUI1 family protein	Cell communication
536	265	Phosphotransferases, Tyrosine kinase, Ser/Tr receptor like kinase1	Cell communication
		early induction	
866	101	Transcribed locus	Transcribed locus
487	260	WW/Rsp5/WWP domain, Wali5 protein	Stress tolerance
865	186	Plastocyanin-like domain, blue copper binding protein	Defence
513	165	Plastocyanin-like domain, blue copper binding protein	Defence
		genotype-unspecific	
316	149	Cycloartenol synthase	Secondary metabolism
437	248	ATUGT85A5, UDP-glycosyl transferase 85A5	Metabolism
401	165	Plastocyanin-like domain, blue copper binding protein	Defence
447	180	ADF5 actin depolymerising factor 5; actin binding	Metabolism
N304	107	Putative NADP-dependent oxidoreductases, Zinc-binding dehydrogenase	Metabolism
420	151	*Fusarium g.* infected spike cDNA library, hypothetical protein	Transcribed locus
490	110	Hypothetical protein, *Gibberella zeae* cDNA clone	*Fusarium graminearum.*
N302	164	Plastocyanin-like domain, blue copper binding protein	Defence
785	54	Transcribed locus	Transcribed locus
408	66	Hydroxyproline rich glycoprotein family protein	Metabolism
528	392	Purple acid phosphatase, serine/threonine phosphatase protein	Cell communication

4.3.2 Polymorphic ESTs in the near isogenic lines

In the near isogenic lines, 7 TDFs out of 11, which sequence information was obtained for, were found to be upregulated in NIL1 and NIL3 corresponding to the QTL on chromosome 5A *Qfhs.ifa-5A*. TDFK347 showed homology to an indoleacetic acid induced protein described to act as a transcription factor. In addition, two TDFs (TDFK436 and TDFK563) with high degree of sequence alignment showed homology to a 2-oxoisovalerate dehydrogenase. TDF_K451 was upregulated in NIL1 and NIL2 corresponding to the QTL *Fhb1* on chromosome 3BS, TDFK451 showed homology to an amino acid transmembrane transporter and TDFK601 showed homology to a transcribed locus that was found in the transcription profiling experiment performed with Sumai3 (Lazo et al. 2004). Furthermore, two TDFs were found in the susceptible lines Remus, DH2, and NIL4 as well as in NIL2. TDFK875 and TDFK878 showed homologies to proteins involved in metabolism (Table 10).

Table 10 Functional annotation of constitutive polymorphisms between the NILs

ID	size in bp	Putative function	FunCAT category
		CM82036, DH1, NIL1 and NIL3 - *Qfhs.ifa-5A*	
K453	142	no significant similarity	no similarity found
K535	188	no significant similarity	no similarity found
K347	345	Indoleacetic acid induced protein 16, transcription factor	Cell communication
K436	77	2-oxoisovalerate dehydrogenase	Metabolism
K563	77	2-oxoisovalerate dehydrogenase	Metabolism
K2412	207	Hypothetical protein	Hypothetical protein
K366	251	Transcribed locus	Transcribed locus
		CM82036, DH1, NIL1 and NIL2 - *Fhb1* on 3BS	
K451	232	Proline transporter	Transporter
K601	204	Transcribed locus, *Fusarium g.* infected spike cDNA library	Transcribed locus
		Remus, DH2, NIL2, NIL4	
K875	315	Cytochrome b5-like Heme/Steroid binding domain	Metabolism
K878	119	Phosphoglycerate dehydrogenase	Metabolism

5 DISCUSSION

5.1 EXPERIMENTAL SET-UP

What renders wheat lines resistant and what renders them susceptible to *Fusarium graminearum*? What candidate genes make a difference in defence response and contribute to or even confer resistance? In order to address these questions, several comparative transcription profiling experiments have been performed over the last ten years (Pritsch et al. 2000, Golkari et al. 2007, 2009; Li and Yen 2008)

Thereby, great diversity in experimental set-up allowed different aspects and components of the interaction to be studied over time course of infection. As a result, vast amounts of ESTs have been generated and made available on public databases. For example, about one third of the ESTs obtained in this transcription profiling experiment showed homology to ESTs on the NCBI database, that derived from previous transcription profiling experiments of *Fusarium graminearum* inoculated wheat heads.

As Sumai3 is considered the most durable resistance source for the time being, most gene expression studies include Sumai3 or one of its derivatives as resistant lines. By contrast, susceptible cultivars in comparative studies tend to have different genetic backgrounds and may represent different, not so well-characterised susceptibility factors.

In the transcription profiling experiment of this project, two closely related sister lines, one being resistant and one being susceptible allowed for comparison of resistance and susceptibility within the same genetic background. Results of extensive QTL mapping studies (Bürstmayr et al, 2002, 2003, and Lemmens et al. 2005) were integrated into the experimental set-up with the aim to identify candidate genes that are associated with the QTL on 3BS and 5A.

The two closely related sister lines DH1 and DH2 were used for a point inoculation experiment. In addition, the near isogenic lines allowed for comparison of the characterised QTL in the genetic background of the susceptible parent Remus and were subjected to the same inoculation experiment.

By dripping inoculum between lemma and palea of single florets without wounding the tissue, it was aimed to assess resistance to initial infection and spread of disease within the spikelets. In the spatial and temporal context, the experiment was narrowed down to the initial site of fungal infection and local defence response at early stage of infection (6 hai, 24 hai, and 72 hai). Therefore, tissue of initially inoculated florets was used for RNA extraction.

For tissue sampling lemma, palea, and rachis were pooled (Steiner et al. 2009), in regard to the results obtained by Golkari et al. (2007) who performed an organ-specific expression profiling experiment, and found that vegetative tissues of the floret show same expression patterns in contrast to anthers and ovary. Thus, it was suggested that averaging and dilution effects in bulked material can be neglected by excluding the generative tissue. Wilson et al. (2004) mentioned that RNA extraction of tissue in earlier developmental stages yielded higher amount of RNA than in later developmental stages. In respect to this, it is also worth separating the embryogenic tissue from the vegetative tissue. As RNA from embryogenic tissue might be over-represented. However, Golkari et al. (2007) sampled at a single timepoint (24 hai). Regarding the fact that most transcripts are induced between 48 hai and 72 hai, more differentially expressed genes can be expected between organs, accordingly. Considering the significant difference in the ability to build up physical barriers between resistant and susceptible cultivars, separate tissue samling of rachis node and rachis may identify gene candidates exclusively associated with resistance to spread of disease.

In respect to dilution effects, Boddu et al. (2007) assumed that repressed genes might be masked by mRNA from healthy tissue, as every tissue contains healthy and infected cells. In

particular, at earlier timepoints such as 6 hai up- and downregulation of genes may be diluted, as only few cells on the surface are challenged by the germinating hyphae. Whereas, at later time points the majority of cells are under attack and in close contact with the spreading and proliferating hyphae.

By using the cDNA-AFLP method for transcription profiling, fragment length polymorphisms are generated based on the sequence specificity of endonucleases. Therefore, not only differences in expression levels can be detected but also polymorphisms such as single nucleotide polymorphisms cSNPs and INDELS. By comparing two closely related sister lines and near isogenic lines with similar genetic backgrounds, detection of allelic variation was considered important as they could contribute to differences in resistance level. Steiner et al. (2009) found one gene upregulated where both corresponding alleles were identified owing to their polymorphisms in fragment lengths TDF59 upregulated in CM82036 and TDF225 upregulated in Remus complemented each other and gave the same BLAST hit. The same phenomenon was observed for TDFK487 expressed in all except Remus and TDFK990 exclusively expressed in Remus.

Generation of ESTs from polyacrylamide gel has its limitations, as excision of bands can be considered as laborious. In particular, strong bands are very likely composed of two or more fragments of the same size. Furthermore, labelling of the primer that corresponds to the restriction site of the rare cutter is advantageous for the readability of gel images, however for excision, it has the major disadvantage that unlabelled fragments may underlie labelled fragments. In both cases, fragments of the same size are obtained that give different results in sequencing.

While microarray profiling allows for global profiling of thousands of genes for which sequence information is available, the cDNA-AFLP method allows for generation of novel ESTs. This is particularly an advantage in the large wheat genome with limited sequence information available.

5.2 RESULTS OF cDNA-AFLP FINGERPRINTING

Interpretation of results was based on the general presumption that genes that play a major role in resistance, may be pathogen-responsive and show stronger and earlier induction in the resistant lines, whereas genes that are significantly upregulated in the susceptible lines may contribute to susceptibility or may reflect the higher level of *Fusarium graminearum* colonisation, in particular at later time-points. In addition, constitutive polymorphisms that showed association with one of the two QTL were regarded as promising candidates.

5.2.1 Constitutively expressed ESTs

Constitutively expressed ESTs that were found to be expressed after water and *Fusarium* treatment could be associated with any process in the floret tissue at anthesis. Apart from general metabolism, however, they could be associated with preformed basal defence, such as cutin, cell wall components, physical barriers, and preformed chemical compounds (phytoanticipins). No preformed differences, for example, in physical barriers were observed between resistant and susceptible cultivars in the TEM micrographs and by immunogold-labelling technique (Kang and Buchenauer 2000, 2002, and 2008). Apart from preformed physical defence, components of recognition and signal transduction such as receptors, receptor like kinases and their regulators may be constitutively expressed. For example, nucleotide binding domains in receptors, kinases or transcription factors may be under strict regulation of constitutively expressed suppressors (Zipfel et al. 2008).

In the resistant phenotypes CM82036 and DH1, 297 constitutive ESTs were found to be exclusively expressed. They can be considered to potentially encode factors that may contribute to resistance. On the other hand, the 158 ESTs were found to be exclusively expressed in Remus and DH2. They may potentially represent susceptibility factors which, for example, repress defense response or render plants more easily accessible to fungal penetration.

From another point of view, 117 bands were expressed in all genotypes except CM82036. These bands may be associated with susceptibility to *Fusarium*, whereas the 61 ESTs that were expressed in all genotypes except Remus may contribute to resistance. As they were expressed in the resistant line DH1 as well as in the susceptible lines DH2, these ESTs can be expected to have only minor supporting or repressing effects on major QTL.

It could be hypothesised that preformed factors and, thus, constitutive ESTs may be associated with resistance or susceptibility to initial infection as they confer a trait that is already present when ascospores land on the floret. Our findings lend support to this hypothesis, as 5 out of the 15 constitutive ESTs sequenced belong to the group of cell communication in our project. Furthermore, a putative trehalase (TDFK1108), galactosyltransferase (TDFK536), acyl-CoA binding protein(TDFK936), and a hypothetical protein predicted to be involved in intracellular trafficking and secretion (TDFK561) were constitutively expressed in the resistant phenotypes. However, as they are not differentially expressed in the near isogenic lines, they may not be associated with the QTL to be characterised.

Regarding the constitutive ESTs that are differentially expressed between the near isogenic lines, it was further confirmed that constitutive ESTs may be involved in resistance to initial infection, because 7 out of 11 constitutive ESTs are related to *Qfhs.ifa-5A*. Among them TDFK347, a putative indoleacetic acid induced protein 16 also assigned as putative transcription factor.

Apart from an oxoisovalerate dehydrogenase, for 5 TDFs associated with *Qfhs.ifa-5A* no putative function could be assigned, inculding TDFK535 for which only very weak similarity to a leucine-rich repeat protein was found. However, data kindly provided by Mrs Apinun Limmongkon confirmed that this fragment maps to the genomic region of *Qfhs.ifa-5A* in the original CM82036/Remus population. Owing to their genetic association with *Qfhs.ifa-5A* and their putative functions TDFK535 and TDFK347 can be considered to be one of the most interesting candidates possibly contributing to resistance to initial infection.

By contrast, for *Fhb1* 4 TDFs were found to be differentially expressed, among them a putative transporter and a transcribed locus previously reported in a *Fusarium graminearum* inoculation experiment with Sumai3 by Lazo et al. (2004).

In regard to effector molecules such as DON, one coping strategy is enhanced and rapid transport either out of the cell or away from the target site into the vacuole, for example. In this context, constitutively expressed transporters such as TDFK451 associated with *Fhb1* and the hyothetical protein involved in intracellular trafficking (TDFK561) expressed in the resistant phenotypes could be considered as interesting candidates.

5.2.2 Pathogen-responsive ESTs within the time course of infection

Comparison of time course of induction and of events gave indication what may act up- and downstream in the signalling cascade and defence response. Because, it can be presumed that earlier expressed genes play a determining role in the question of what genes are expressed at later stages.

No downregulation of transcripts after *Fusarium* inoculation was observed in this experiment. Eckey et al. (2004) reasoned that the cDNA-AFLP method with a two step PCR may mask repressed fragments. However, all in all our findings are confirmed by Boddu et al. (2007) and Golkari et al. (2007, 2009) who performed microarray transcriptome analysis at 48 hai and 24 hai, respectively: Both observed only a minority of genes being downregulated in response to *Fusarium* infection. Golkari et al. (2009) for example, found genes involved in photosynthesis and energy metabolism to be downregulated 24 hai in Sumai3 and susceptible near isogenic lines and reasoned that it might be to do with redox homoestasie and protection against ROS. Steiner et al. (2009) reasoned that downregulation may occur at later time-points. Pritsch et al. (2000), who performed transcription analysis for members of the *PR* genes with Northern Blot observed, downregulation of *PR* genes earlier in the susceptible cultivar than in the resistant cultivar Sumai3. By contrast, Bernardo et al. (2007) observed downregulation of early induced *PR* genes after 48 to 72 hai in the resistant cultivar but not in the susceptible cultivar.

Interestingly, downregulation in the water treatment was observed in 21% of all pathogen-responsive TDFs in this experiment. On the one hand, these TDFs may represent gene products that are not directly associated with pathogen infection and are up- and downregulated over the natural time course, but under pathogen attack they remain upregulated. On the other hand, they may be upregulated in all treatments due to the inoculation experiment, as also dripping water between lemma and palea can be considered as mechanical manipulation. This may cause stress on short notice and trigger an unspecific stress response that is later downregulated after water treatment, but remains upregulated in plants facing the threat of *Fusarium graminearum*. In addition, Pritsch et al. (2000) also observed early accumulation of PR gene transcripts after water inoculation. As this phenomenon was observed in one fifth of all pathogen-responsive TDFs, an additional sampling time-point at 0 hai and an untreated control would have been useful for further interpretation.

5.2.2.1 Early timepoint — 6 hai

Out of 10.000 bands 266 were differentially upregulated between *Fusarium* and water treatment. Thereby, 5 out of 10.000 were differentially upregulated after 6 hai. All 5 were expressed in all genotypes and showed homology to ESTs that had been previously reported in transcription profiling experiments with *Fusarium graminearum* inoculation. Interestingly, two of them (TDF865, TDF513) showed homology to Plastocyanin-like domain containing proteins. In addition, TDF487 showed homology to a wali5 protein associated with stress tolerance and WW and WWP domains that are reported to be involved in mediation of protein–protein interactions in association with the E3 ubiquitin ligase complex. In addition, they mediate binding of proline-rich domains and could be associated with cross-linking in the cell wall. TDF321 showed homology to a translation initiation factor that is associated with protein translation and was quantitatively stronger induced in CM82036. TDF866 showed homology to a transcribed locus. As all 5 TDFs of early response are genotype-unspecific, one could consider them as a genotype-unspecific general PAMP triggered defence response.

Plastocyanin-like domain containing proteins, also referred to as blue copper-binding proteins, are reported in *Fusarium graminearum* transcription profiling experiments, however no clear function has been assigned yet (Desmond et al. 2008b). They are supposed to be involved in binding and transport of electrons. One could deduce from this early induction that they are rather involved in oxidative burst than in hypersensitive response that is reported to be induced by DON about 36 to 48 hai.

5.2.2.2 Intermediate time-point — 24 hai

Following the time course of induction, 50 pathogen-responsive ESTs were upregulated 24 hai. For 5 of them putative gene function was assigned in this project. All 5 ESTs were induced in the resistant phenotypes or genotype-unspecific but quantitatively stronger in CM82036.

Serine/threonine protein kinases (TDF529) and Zn finger ring domains (TDF541) as nucleotide binding domains are involved in signal transduction and may act upstream of later induced ESTs.

Non-specific lipid transfer proteins (TDF308) and pathogenesis related proteins (TDF347) are thought to be involved in signal transduction and are reported to act as signal enhancers in local and systemic response (Lascombe et al. 2008).

Terpene cyclases (TDF848) are involved in secondary metabolism of terpenes, which many of them have antimicrobial effects and belong to the group of phytoalexins. Further evidence is provided for terpene biosynthesis by TDF316, a putative cycloartenol synthase that shows strong induction in all genotypes 72 hai. In this context, early and strong induction of TDF848 in CM82036 and DH1 could indicate the presence of an alternative pathway that may result in a specific compound efficient against *Fusarium graminearum*. Antimicrobial compounds may play a pivotal role at early stages, because slowing down growth and colonisation rate may help to gain time to build up efficient physical barriers in the rachis.

5.2.2.3 Late time-point — 72 hai

In this experiment most transcripts (80%) were induced 72 hai. This is in agreement with Boddu et al. (2007) and many other authors. It has been further visualised by micrographs of Kang and Buchenauer (2000, 2002 and 2008), Jansen et al. (2005), and Pritsch et al.(2000) that the timespan between 48 and 72 hai is the most crucial period in the wheat–*Fusarium graminearum* interaction. At this timepoint, the fungus has already accumulated DON and switches to a necrotrophic lifestyle, invading plant cells by intracellular growth (**Figure 3**).

Out of 266 pathogen-responsive ESTs in total over time course of infection 233 ESTs (88%) were upregulated in all genotypes. Among them, 20 ESTs (8,5%) showed significantly stronger induction in CM82036. 33 (12%) showed pathogen-responsive and genotype-specific expression patterns. This distribution is in agreement with many transcription profiling experiments demonstrating that the majority of genes are upregulated in both cultivars irrespective from their resistance level (Li and Yen 2008; Steiner et al. 2009, Golkari et al. 2009).

For 10 out of 233 pathogen-responsive and genotype-unspecific ESTs sequence information was obtained in this project. Putative functions suggested involvement in general defence response as the candidates are, for example, involved in signal transduction (serine/threonine phosphatise — TDF528) and activation or deactivation of compounds or metabolic processes (UDP-glycosyltransferase — TDF437). Furthermore, NADP-dependent oxidoreductase (TDFN304) and plastocyanin-like domain containing proteins (TDF N302, TDF 401) are possibly involved in generation and transformation of ROS (Noctor and Foyer 2009). In additon, hydroxyproline-rich glycoproteins (TDF408) and actin depolymerising factor5 (TDF 447) are reported to be associated with reinforcement of cell wall structures (Bollwell et al. 2001; Ferreira et al. 2007). Furthermore, cycloartenol synthase (TDF316) provides precursors for sterols and antimicrobial compounds (Desmond et al. 2008a).

Out of 33 pathogen-responsive and genotype-specific ESTs, 13 are exclusively expressed in CM82036 implying that defence response in CM82036 is very distinct even from DH1 and DH2 that carry 50% of alleles from CM82036 according to the Mendelian Rules. This, furthermore, indicates that in CM82036 several additional genes are differentially expressed in response to *Fusarium graminearum* that are not related to *Fhb1* and *Qfhs.ifa-5A*. This may provide further evidence for the fact that QTL with major effects are under the influcene of genes with minor effects. Among, the ESTs exclusively upregulated in CM82036 a lipid transfer protein (TDF308), a member of major facilitor superfamily (TDFN301), a member of a transferase family (TDF400), and a putative cytochrome b5/transition metal ion binding protein (TDF329) were identified.

In addition to the 13 ESTs exclusively expressed in CM82036, 5 ESTs were found to be expressed in CM82036 and DH1, thereof two cytochrome P450 proteins and TDF848, the terpene cyclase expressed after 24 hai. In addition, Steiner et al. (2009) found a phenylalanine ammonia lyase (PAL), a UDP-glucosyltransferase, and a DnaJ-like protein to be upregulated in CM82036 and DH1.

However in this experiment, 18 ESTs are exclusively expressed in the resistant phenotypes, compared with 3 ESTs exclusively upregulated in the susceptible phenotypes. As the 3 ESTs are induced at later time-points, it can be hypothesised that they are induced owing to extensive fungal colonisation leading to cell disintegration and disturbance of physiological processes compared with the resistant phenotypes. Keeping in the concept of Chen et al. (2009) and Desmond et al.(2008a), these TDFs could be involved in the active process of programmed cell death. For example, TDF409 showed homology to a putative glycoside hydrolase/pectinase that could be involved in degradation of cell wall components.

The acummulative effects of the complex network of minor genes addititionally contributing to resistance, apart from *Fhb1* and *Qfhs.ifa-5A* in CM82036 was further demonstrated in the NILs with the susceptible background of Remus. Effects of the two major QTL have been shown to be compromised in susceptible background, as control heads of NILs were moderately to severely diseased, and no pathogen-responsive and genotype specific TDFs could be confirmed between the NILs. Furthermore, only 109 out of 233 pathogen-responsive and genotype-unspecific polymorphisms could be confirmed in the near isogenic lines. A resembling effect was observed by Christensen et al. (2004), who tested the effects of a germin-like protein with reported dismutase activity in wheat. Performing overexpression and gene-silencing studies with this protein in different genetic backgrounds, they found that effects on *Fusarium* resistance were heavily dependent on the genotypic background. For example, in some tested cultivars neither overexpression nor gene silencing had a great effect on resistance, whereas in others both had significant influence.

Direct comparison of pathogen-responsive expression patterns in the parents showed that 25 ESTs derived from the resistant parent CM82036 and 8 ESTs from susceptible Remus. By taking into account the 20 ESTs that were additionally stronger expressed in CM82036, 45 ESTs were differentially upregulated compared with 8 ESTs in Remus. It is in agreement with all comparative transcription studies cited in this contribution, that in resistant lines significantly more ESTs are upregulated in response to *Fusarium graminearum* than in the susceptible lines.

5.3 FUNCTIONAL ANNOTATION OF CANDIDATE GENES

Each of the 45 ESTs can be regarded as a fragment of potential candidate genes that may contribute to resistance.

Boddu et al. (2007) considered the results of the transcription profiling as a hypothesis generating data set, and Leader et al. (2005) considered the list of candidate genes with putative gene functions as the first step towards functional genomics. In order to put the assigned putative gene functions into the context of resistance strategies in wheat, questions need to be asked, ideas need to be developed, and hypotheses need to be discussed.

First, slowing down germination and hyphal growth can be considered as a successful strategy in order to gain time for induction of defence response, in particular, for formation of physical barriers that can trap the fungus. Oxidative burst, free phenolic compounds, and PR proteins with antimicrobial effects may be the hallmarks of early response. As soon as the fungus accumulates DON, the plant has to protect the targeted site at the small ribosomal subunits. Therefore, suppressing DON production, as suggested by Boutigny et al. (2008) would be a good avoidance strategy. However, DON that accumulated in the cytoplasm needs to be eliminated by transporters or detoxified by, for example, transferases.

According to Desmond et al. (2008a), Walters et al. (2008), and Chen et al. (2009) DON causes oxidative stress that leads to hypersensitive response and programmed cell death. Therefore, one of the main strategies might be to avoid ROS to be generated in the cytoplasm. As DON is reported to disturb translation, it might be pivotal for cell survival to maintain protein biosynthesis and to avoid accumulation of disfunctional, truncated proteins.

On the other hand, each of the 8 ESTs that can be related to the susceptible parent Remus may be involved in processes that are exploited by the fungus. According to Desmond et al. (2008a) and Chen et al. (2009) hormone imbalances and disturbance of redox-homoestasie are provoked by the fungus leading to hypersensitive response and programmed cell death. Furthermore, they could be expressed as a result of degradation processes, as the fast spreading fungus extensively consumes the tissue.

Most of the TDFs belong to major multi-gene families and their members are reported to be involved in an array of processes and pathways, such as the protein kinases, the cytochrome P450s, and the UDP-glycosyltransferases.

5.3.1 Cell communication

Incompatible interactions, are frequently characterised by a rapid and strong defence response based on recognition and signal transduction. In addtion, crosstalk between components of different signalling cascades are suggested to allow for a fine-tuned defence response that counteracts the invading pathogen with great specificity and efficiency (Pritsch et al. 2000; Turner et al. 2002; Jones and Dangl 2006)

Protein kinases represent a multigene family, which members are involved in various steps of signal transduction. In this experiment, several putative protein kinases were found to be differentially expressed between treatments and genotypes. They mainly belonged to the group of serine/threonine protein kinases and showed very diverse expression patterns (Figure 36). For example, TDF541 showed homology to the coding sequence of a NBS-LRR-serine/threonine protein kinase protein, which was identifed as a stem rust resistance protein in *Hordeum vulgare*, and to a Zn-finger ring domain containing protein in *Arabidopsis* and rice. By contrast, TDFK617, a putative transmembrane kinase, shows homology to a leucine-rich repeat protein kinase in rice and is constitutively expressed in CM82036 and DH1. In additon, TDFK561/K486 also constitutively expressed in CM82036 and DH1 show homology to a cyclin-dependent

protein kinase regulator. Besides, kinases and phospatases, glycosyltransferases are reported to be involved in activation and deactivation of regulatory components.

Putative kinases	CM_F	CM_W	D1_F	D1_W	D2_F	D2_W	RE_F	RE_W
541								
529								
536								
TDF28								
TDF35								
528								
N300								
K617								
K486								
K561								
K487								
K990								

Figure 36 Expression patterns of putative protein kinases.

5.3.2 Cell rescue and defence

The majority of *PR* genes are reported to be induced in compatible as well as in incompatible wheat–pathogen interactions and are thought to be part of a general defence response (Christensen et al. 2004). Li and Yen (2008) found that *PR* genes are expressed in response to pathogen infection at the same level in resistant and susceptible cultivars. They concluded that the expression of *PR* genes may contribute to basal resistance, however, they may not confer resistance in Sumai3 and its derivatives. In addition, Boddu et al. (2007) demonstrated that PR proteins are part of basal resistance and their induction is independent from the effects of DON.

However, Desmond et al. (2008a) and Pritsch (2000) reported that *PR* genes tend to be earlier and stronger expressed in the resistant cultivar than in the susceptible ones. These findings are in contradiction with Li and Yen (2009) but are in agreement with our results. Because, all three pathogenesis related proteins PR1 (TDF432a) and PRB1-2 precuror (TDF347) and thaumatin-like protein (TDF757) were expressed in CM82036 and both DH lines, but not in Remus. These results suggest, that lacking PR-1 and PR-5 expression renders Remus susceptible, whereas expression of PR-1 and PR-5 does not confer resistance to DH2 implying that additional components are required.

In regard to the time-points of upregulation, Pritsch et al. (2000) reported upregulation of *PR* genes such as PR-1, peroxidase, PR-2, and PR-3 in resistant and susceptible genotypes between 6 and 12 hai, which coincides with macroconidia germination and first contact of hyphae with stomata. Steiner et al. (2009) observed chitinases TDF55 and TDF168 to be induced at 12 hai and 48 hai, respectively. TDF347 was induced 24 hai, whereas TDF432a and TDF757, were upregulated 72 hai. Bernardo et al. (2007) reported upregulation of *PR* genes as early as 3 to 6 hai and hypothesised that early induced *PR* genes may act as signals or induce signals that lead to additional activation of defense.

TDF757, a putative thaumatin-like protein shows 100 % sequence similarity to an EST reported from a resistant wheat cultivar challenged with stripe rust (Ta.56897). Pritsch et al. (2000) reported that PR-5 protein was earlier (6 hai) and stronger induced in Sumai3 compared with susceptible cultivar Wheaton (24 hai). Geddes et al. (2008) provided further evidence for upregulation of PR-5 protein in resistant barley cultivars challenged with *Fusarium graminearum* at translational level detecting the protein itself. They reported that several isoforms of

thaumatin-like proteins show distinct behaviour in different pathogen interactions and suggested that this may be owing to different interactions with other PR proteins or different binding capacities to fungal β-1,3-glucans. In addition, a member of the thaumatin like protein family was detected to be significantly differentially expressed between Sumai3 and its susceptible near isogenic lines (Golkari et al. 2009), further indicating that PR-5 may play a crucial role in this plant-pathogen interaction and may even be associated with *Fhb1*. PR-1 and PR-5 proteins both show antifungal activity, however no direct mechanisms have been elucidated yet. Owing to their antifungal activity, they may be capable of slowing down fungal growth and colonisation and may act as signal enhancer.

In additon to PR-1, PR-5 and chitinases, a non-specific lipid transfer protein (TDF308) was found to be exclusively upregulated in CM82036. Interestingly, TDF308 showed similarity to TDF100 and TDFK339 both exclusively upregulated in Remus. According to Lascombe et al. (2008) non-specific lipid transfer proteins may act as signal enhancers and could be involved in long distant signalling. It was demonstrated, that lipid transfer proteins have higher affinity to monoacetylated lipids that derive from the activity of extracellular lipases (Lascombe et al. 2008). FGL1 that acts as a virulence factor (Voigt et al. 2005, 2007) represents such an extracellular lipase.

In additon, Kirubakaran et al. (2008) were able to clone a gene encoding a new antifungal lipid transfer protein (LtpF31) gene from a cDNA library of *Fusarium graminearum* inoculated Sumai3. It was demonstrated that complexes of this lipid transfer protein with JA showed increased binding affinity to the elicitin receptor in comparison with the protein without JA. It was hypothesised that these complexes represent endogenous "indirect PAMPs". This is in the concept of Li and Yen (2008) who reported that spraying JA prior to infection enhanced resistance to *Fusarium graminearum* and even rescued the susceptible line.

5.3.3 Secondary metabolism and physical barriers

With terpene cyclase and cyloartenol found in this experiment, and phenylalanin ammonia lyase (PAL) found by Steiner et al. (2009), three key enzymes of secondary metabolism were found to be upregulated in this experiment. Thereof, terpene cyclase and PAL were exclusively upregulated in the resistant phenotype. While PAL is the key enzyme of the phenylpropanoid pathway leading to lignols and phenolic compounds, terpene cyclaes and cycloartenol are key enzymes in the biosynthesis of terpenes and sterols. Desmond et al. (2008a) reported the induction of cycloartenol synthase in the resistant cultivar at higher levels compared with the susceptible one. Golkari et al. (2009) found significant upregulation of PAL 24 hai in Sumai3 compared with susceptible near isogenic lines.

Formation of physical barriers is one of the significant differences observed between resistant and susceptible cultivars (Pritsch et al. 2000; Kang and Buchenauer 2000). Structural reinforcement requires compounds such as lignols, free phenolic compounds, and callose. Genotype-specific upregulation of PAL and terpene cyclase lend further support to these observations.

In this context a putative actin depolymerising factor (TDF447) was found to be upregulated with quantitative stronger expression in CM82036. The actin cytoskeleton confers preinvasion resistance and guides formation of papillae. Actin depolymerising factors enhance the turnover rate of actin and interact with actin monomers as well as actin filaments (Ferreira et al. 2007). Shimada et al. (2006) had been able to show "extensive reorganisation of actin microfilaments" resulting in large actin bundles towards the site of penetration. Furthermore, Geddes et al. (2008) reported that an actin polymerising factor 3 was found to be upregulated in a resistant barely cultivar challenged with *Fusarium graminearum.*

In addition, several cytochrome P450 and UDP-glycosyltransferases were upregulated that may also be involved in the biosynthesis pathway of secondary metabolites. Members of the large family of cytochromes P450s are reported to be involved in secondary metabolism as oxygenases but also in the process of lignification, antioxidative response, detoxification, and plant hormone homeostasis (Walter et al. 2008; Desmond et al. 2008a).

In our experiment, three TDFs were differentially expressed with homology to different cytochrome P450 reference proteins. TDF787 and TDF788 are pathogen-responsive and upregulated in CM82036 and DH1, whereas TDFK875 is constitutively expressed in all except Remus (Figure 26).

Boddu et al. (2007) identified six cytochrome P450 proteins that were exclusively responsive to DON and are considered to play a role in protection against the effects of DON. Steiner et al.(2009) found a putative cytochrome P450 protein (TDF79) to be quantitatively stronger upregulated in the resistant phenotypes (CM82036 and DH1). Bernardo et al. (2007) found genes with homology to members of the cytochrome P450 gene family that were exclusively upregulated in Sumai3. One gene tag showed homology to a cytochrome P450 subfamily that was reported to be involved in the biosynthesis of DIMBOA, an antifungal compound in maize, wheat, and rye. DIMBOA was shown to inhibit growth of *Fusarium graminearum* in *vitro*.

UDP-glucosyltransferases belong to a mulitgene family with members of diverse and narrow substrate specificity. For example, UDP-glycosyltransferase DOGT1 cloned from *Arabidopsis* was reported to detoxify DON by conversion into 3-O-DON glucoside, and overexpression of DOGT1 in transgenic *Arabidopsis* led to increased tolerance to DON (Poppenberger et al. 2003). In addition, Lemmens et al. (2005) described the activity of an unknown UDP-glucosyltransferase gene that confers resistance to DON by converting it into less toxic 3-O-DON glucoside in wheat. Berthiller et al. (2005) and Lemmens et al. (2005) provided evidence that DON-3-glucoside is the main detoxification product and that this conversion may be the primary resistance mechanism in wheat in association with *Fhb1*. They detected the substrate, the product, and a genomic region associated with, however, the gene has not been cloned yet. Lemmens et al.(2005) hypothesised that either the gene encoding for the UDP-glycosyltransferase or a regulatory factor of the DON detoxifying UDP-glycosyltransferase are associcated with the genomic region on chromosome 3BS. In this experiment, a constitutively expressed transporter (TDFK451) could be identified in association with *Fhb1*. Steiner et al.(2008) identified a UDP-glycosyltransferase (TDF108) that was exclusively expressed in CM82036 and DH1. Characterisation of its ability to detoxify DON in the yeast heterologous expression system is under way.

In respect to the search for UDP-glycosyltransferases, Boddu et al. (2007) narrowed the range of UDP-glycosyltransferases of originally 9 genes expressed in the barley–*Fusarium graminearum* interaction to 3 glycosyltransferases that are exclusively expressed in response to DON accumulation. In this experiment, TDF437 which is strongly induced in all genotypes 72 hai showed homology to a member of the UDP-glycosyltransferase family.

In additon, members of the diverse groups of transporters and transferases were found to be predominantly upregulated in the resistant phenotypes in this experiment. For example, TDFN301 with homology to a major facilitor superfamily protein and TDF400 with homology to a transferase family are exclusively expressed in CM82036. TDF486 with quantitative stronger expression in CM82036 also shows homology to a transferase family. TDF432b is a putative galactosyltransferase and is upregulated in all genotypes except Remus whereas TDFK536 represents a costitutively expressed putative galactosyltransferase in the resistant phenotypes. According to many authors, transporters and transferases are involved in detoxification and transport of DON and other effector molecules. In addition, they may be involved in the transport

and transformation of degradation products. Boddu et al. (2007) found ABC transporters and MATE transporters and Walter et al. (2008) found multi drug resistance proteins to be upregulated in repsonse to DON. Furthermore, Steiner et al. (2009) found a potential calcium transporting ATPase and an ABC transporter upregulated in all genotypes. It can be assumed that members of these groups act in complex networks, in order to remove DON from the cytoplasm or/and to detoxify it, and to maintain cell functions and to promote cell survival.

5.3.4 The potential of ESTs with unkown function

For 14 ESTs, which sequence information was obtained for, no putative function could be assigned. However, all of them showed homology to ESTs reported in the NCBI database. In particular the pathogen-responsive TDF348, TDF444, and TDF786 are exclusively induced in the resistant genotypes, indicating that they may play a role in defence response and resistance. In addition, TDF866 that is early induced after 6 hai and genotype-unspecific may be involved in PAMP triggered immunity. In additon, TDFK601, a constitutive polymorphism between the near isogenic lines corresponding to *Fhb1* could be of major importance. TDFK601 shows homology to a transcribed locus that was also reported in the transcription profiling experiment with Sumai3 by Lazo et al. (2004).

Furthermore, Meyers et al. (2004) suggested that TDFs with no sequence homology to a reference protein may represent splicing variants or RNAs that do not encode proteins. Natural anti-sense transcripts or micro-RNAs may function as regulatory RNAs. Leader et al. (2005) stated that the generation of ESTs is far ahead of functional annotation and suggested that candidate genes with no putative functions might provide completely new insight.

6 CONCLUSION AND PREVIEW

Which gene candidates make a difference in defence response and render plants resistant?

It can be summarized that most pathogen-responsive gene candidates are expressed in resistant and susceptible genotypes and therefore rather contribute to general host defence. Apart from six pathogen-responsive gene candidates exclusively expressed in CM82036, five constitutive and four pathogen-responsive TDFs can be related to the resistant alleles of QTL *Fhb1* and *Qfhs.ifa-5A*. In addition, three and seven constitutively expressed ESTs could be specifically related to *Fhb1 and Qfhs.ifa-5A*, respectively, in the near-isogenic lines. These fifteen ESTs can be considered as the most promising candidates. Three show homology to proteins involved in perception and signalling. Furthermore, two are members of the cytochrome P450 multigene family, a member of the terpene cyclase family, transferases and transporters may be involved in transport and metabolic processes that could also be related to detoxification strategies and regulatory processes in redox homoestasie and hormone balance.

However, it needs to be considered that transcriptional profiling may not represent the level of functional gene products as post-transcriptional and post-translational modifications are reported to play a major regulatory role. In particular regarding to the effects of DON, mRNA levels may not represent the levels of functional gene products available. Therefore, expression profiling should be backed by proteome and metabolome profiling.

In addition, functional annotation based on sequence homology of ESTs as short as 70 to 500 bp can only predict a broad functional category. This is of particular importance in respect to candidate genes that belong to multigene families, such as protein kinases, UDP-glycosyltransferases, and the cytochrome P450s. Regarding the numerous transcription profiling experiments dealing with wheat–*Fusarium* interaction, meta-studies should shed light on similarities and differences in order to narrow the range of gene candidates that belong to multigene families.

Candidate genes obtained in this cDNA-AFLP fingerprinting will be confirmed by quantitative real time PCR and compared with results obtained from a microarray experiment using the Affymetrix Wheat Gene Chip.

Because, “Maybes are findings that need experiments to become real scientific answers” (David Schwartz on the Summerschool DNA and Chromosomes in Cargese, 2009), the most promising candidates will be subjected to functional genomic approaches. Mis- and over-expression of genes, reporter gene contructs, and complementation studies may elucidate function, subcellular localisation, and interactions within pathways.

As transformation and mutant screening is reported to be still a major challenge in hexaploid wheat, functional genomics in wheat relies on the transferration of knowledge obtain from heterologous expression systems such as *Saccharomyces cervisae* and plant model systems such as *Arabidopsis thaliana* and *Oryza sativa*. In the near future, the temperate grass species *Brachypodium distachyon* will be available as an additional tool in functional genomics of crops species such as wheat.

Moreover, transcripts of candidate genes can serve for reverse genetic approaches such as targeted induced local lesions in genomes (TILLING) and virus-induced gene silencing (VIGs) that can be directly applied in hexaploid wheat.

Characterisation of the gene candidates obtained from this project may contribute to the elucidation of biochemical and molecular mechanisms of the wheat–*Fusarium graminearum* interaction, so that breeding for FHB resistance can benefit from the improved understanding of this intriguing plant–pathogen interaction in the near future.

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10 SUPPLEMENTARY DATA

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Table I Summary of cDNA-AFLP fingerprinting results

Number of primer combinations of the cDNA-AFLP fingerprinting	**160**
Total number of screened polymorphisms in transcript derived fragments (TDFs)	**953**
Genotype-specific - constitutive TDFs in total	**687**
Polymorphic in the CM-DH1-DH2-Remus, but not in the NILs-experiment	**673**
only in CM	211
only in CM and DH 1	75
in all other lines, except CM	117
only in Remus and in all 4 Nils	98
only in Remus and DH2 and in all 4 Nils	57
in all others, except Remus and in all 4 Nils	61
in Remus and DH1 and in all 4 Nils	28
in CM and DH2	26
Polymorphic in the CM-DH1-DH2-Remus and in the NILs-experiment	**14**
CM, DH1 - NIL1, NIL 3 (Qfhs.ifa-5A), (Nr: K347, K535, K436, K453, K563, K1031, K945)	7
CM, DH1 - NIL1, NIL2 (Fhb1), Nr: K366, K451, K619, K601	4
Remus, DH2 - NIL 2, NIL 4, Nr: K603, K875, K878	3
Pathogen-responsive TDFs - either genotype-specific or -unspecific	**266**
A) Pathogen-responsive and genotype-unspecific	**233**
Confirmed in the CM-DH1-DH2-Remus experiment by 2 replications	124
Confirmed in the CM-DH1-DH2-Remus experiment and the 4 near isogenic lines	109
Timepoint of induction in genotype-unspecific TDFs	
early induction (6 hai)	5
intermediate induction (24 hai)	44
late induction (72 hai)	184
Quantitative differences in response	
similar expression levels	213
quantitative differences in response	20
stronger in CM	18
stronger in CM, Nil1 and Nil 2	2
Repression after mock-inoculation	56
B) Pathogen-responsive and genotype-specific TDFs	**33**
induced only in CM	13
induced in CM and DH1	5
induced in all lines except CM	5
induced only in Remus	2
induced in all lines expect Remus	7
induced in Remus and DH2	1
Timepoint of induction in genotype-specific TDFs	33
early (after 6 h)	0
intermediate (after 24 h)	6
late (after 72 h)	27
Repression after mock-inoculation	1
Summary of all pathogen-responsive TDFs (genotype-specific and -unspecific)	
Response after inoculation in all pathogen-responsive TDFs - summary	266
early (after 6 h)	5
intermediate (after 24 h)	50
late (after 72 h)	211
Repression after mock-inoculation	57

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
Pathogen responsive - genotype specific												
308	late, 72 hai	only in CM	135	gtacgtgaacggtggctcctcgcatcgcatgcaacatatatatagt agtactgcatctgtatggctcgggaatggttgagaagtcccagtcg aaacacgtcctgcgtagccttccggcatcaattcgagttgcag	EX982047.1 TDF100 wheat Fusarium graminearum inoculated, cDNA-AFLP fragment Triticum aestivum cDNA, mRNA sequence 235 235 100% 3e-59 99%	Ta.21646	Transcribed locus, weakly similar to NP_001055844.1 Os05g0477900 [Oryza sativa (japonica cultivar-group)]	Os05g0477900 O. sativa 68.2 132 , Non-specific lipid-transfer protein type 1 , hypothetical protein	**no significant hit**			
TDF 100	intermediate, 48 hai	48hai, fusarium-induced, Remus, Eltern WH1+2	135	agtacgtgaacggtggctcctcgatcgcatgcaacatatatatagt agtactgcatctgtatggctcgggaatggttgagaagtcccagtcg aaacacgtcctgcgtagccttccggcatcaattcgagttgcag	DR740066.1 FGAS084994 Triticum aestivum FGAS: Library 2 Gate 3 Triticum aestivum cDNA, mRNA sequence 242 242 99% 1e-61 100%	Ta.21646	Transcribed locus, weakly similar to NP_001044654.1 [Oryza sativa (japonica cultivar-group)]	A. thaliana ref:NP_568904.1 - nonspecific lipid-transfer protein precursor - like [Arabidopsis thaliana] 48 % / 95 aa	E: 3.6			
K339	constitutive	only in Remus	135	ctgcaactcgaattgatgccggaaggctacgcaggacgtgtttcg actgggacttctcaaccattcccgagccatacagatgcagtacta ctatatatatgttgcatgcgatcgaggagccaccgttcacgtacc	DR740066.1 FGAS084994 Triticum aestivum FGAS: Library 2 Gate 3 Triticum aestivum cDNA, mRNA sequence 242 242 99% 1e-61 100%	Ta.21646	Transcribed locus, weakly similar to NP_001044654.1 [Oryza sativa (japonica cultivar-group)]	A. thaliana ref:NP_568904.1 - nonspecific lipid-transfer protein precursor - like [Arabidopsis thaliana] 48 % / 95 aa	E: 3.6			
348	late, 72 hai	only in CM	146	gaatagtagtaggaattctttcaacctctcctttgggcttcctggcgc agttgaattgcctgaaggaaatgatacacatggggagaatcatac agagcaggatggtgaggttccaaagaaagctcctatgggacgg ctggcacttc	EB515567.1 Ta10c_03b21_R Ta10c_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads Triticum aestivum cDNA clone Ta10c_03b21, mRNA sequence 264 264 100% 6e-68 100%	Ta.47355	transcribed locus, Found only in library 19528: Ta10c_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads	transcribed locus	AP008207.1 Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1 111 111 95% 9e-22 76%			
329	late, 72 h	only in CM	280 -283	gtgatgatgtactgttgtcctcgactgccaaggacgcaactgatga ctttgaggatgtcgggcacagcaccaccgcccgtgcgatgctgg acgagtactatgtcggcgacgtggatacggctacgatacctgccc ggacaaagtacactccgccgaagcaaccacactacaaccaag acaagacccctgagttcatcatcaagatcctccagttcctggtccc cttggccatacttggtctggctgtcgccattaggatgtacaccaagt cggaatcggc	CJ886536.1 CJ886536 Y. Ogihara unpublished cDNA library, whthls Triticum aestivum cDNA clone whthls16l19 3', mRNA sequence 506 506 **100% 2e-140 100%**	Ta.55636	Transcribed locus, strongly similar to NP_001054434.1 Os05g0108800 [Oryza sativa (japonica cultivar-group)]	NP_001054434.1 Os05g0108800 O. sativa 93.2 132 , NP_180831.1 B5 #4 (cytochrome b5 family protein #4); heme binding / transition metal ion binding A. thaliana 81.6 125	AK253135.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf140l01, mRNA sequence 428 428 100% 6e-117 93%	Hv.2182	CDNA clone: FLbaf140l01, mRNA sequence	NP_001054434.1 Os05g0108800 O. sativa 92.5 132 . NP_180831.1 B5 #4 (cytochrome b5 family protein #4); heme binding / transition metal ion binding A. thaliana 81.0 125
N301	late, 72 h	only in CM	191	taatccaaaaaaattctataataataaaggctagtttctttcatactg ctctggcacggccaggaagggtctgaatgtgaggacaagtcca aggagctcgatgatgttcagcagaaagaacaccatctgatcacct ggaaagaagaaagcatgctggcgtttctgcgcccacgagaaca caatgccagcac	CJ688479.1 CJ688479 Y.Ogihara unpublished cDNA library Wh_RDr Triticum aestivum cDNA clone whrd14b21 5', mRNA sequence 345 345 **100% 3e-92 100%**	Ta.57508	Transcribed locus, moderately similar to NP_001066086.1 Os12g0133100 [Oryza sativa (japonica cultivar-group)]	NP_001066086.1 Os12g0133100 O. sativa 88.6 454 ; Major Facilitator Superfamily ; hypothetical protein	AK249921.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf51b07, mRNA sequence 233 233 88% 2e-58 91%	Hv.26664	NP_001066086.1 Os12g0133100 O. sativa 80.6 467, NP_851036.1 ZIFL1 (ZINC INDUCED FACILITATOR-LIKE 1); tetracycline:hydrogen antiporter/ transporter A. thaliana 57.0 476	Major Facilitator Superfamily
444	late, 72 h	only in CM	229	tccgacgaggcgaggctgccggcgctcccacggcacatgtaca ggggccgcacaggatcttgcgtcgtacgggtctttctccgttgaca gcgcaggctccggttgtgttcgctcttcttcggtcagcaccggcaa caccactgtttcttccgagtcgtcctcgaccgcgctgctacgtcattc caaggaatcaagctacgtagttgattcatccagatttgtaattcttga	BM137167.1 WHE2630_C05_E10ZS **Wheat Fusarium graminearum** infected spike cDNA library Triticum aestivum cDNA clone WHE2630_C05_E10, mRNA sequence 401 401 **100% 6e-109 99%**	Ta.19960	trancribed locus	in the Olin Anderson EST library					
400	late, 72 h	only in CM	250 - 270	tagcatcacatcacctcccgtgcagtccaacccatgaataaacgt tttatatgctgggtggacatgtgtgatggagggtggcatacgtgctct gaaggtatctcgtttccacacgggcaatgtggttgggatgagttca ccacatgcaaaatcggctatggatttgataaattggaggatacca aaaccatcagcaatgcaatggcacatgtgaagcccaataacaa atcctccacacttgagttgtgtcac	BU991007.1 HD05L05r HD Hordeum vulgare cDNA clone HD05L05 5-PRIME, mRNA sequence 374 374 100% 9e-101 92% EB514888.1 Ta10b_02c19_R Ta10b_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads Triticum aestivum cDNA clone Ta10b_02c19, mRNA sequence 311 311 100% 9e-82 87%	Zm.34177, only at e - 14	unknown mRNA, NP_001064288.1 Os10g0195600 O. sativa 68.6 483	Transferase; Transferase family , hypothetical protein	EU960820.1 Zea mays clone 228860 transferase family protein mRNA, complete cds 82.4 82.4 56% 9e-13 72%	Zm.34177, only at e - 14	NP_001064288.1 Os10g0195600 O. sativa 68.6 483	Transferase; Transferase family , hypothetical protein

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
848	intermediate 24 hai	CM and DH1	83	gcacattggagaaatggcttccgcagtgcaaaagcgcgatgttacaaaacacctgaatgaaacatggctacattacttgaggt	BM135357.1 WHE0457_E08_J15ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0457_E08_J15, mRNA sequence 150 150 1**00% 5e-34 100%**	Ta.8418	Transcribed locus, weakly similar to NP_001053841.1 Os04g0611800 [Oryza sativa (japonica cultivar-group)]; NP_001053841.1 Os04g0611800 O. sativa 65.5 479	Terpene synthase, metal-binding domain containing protein	AY551436.1 Hordeum vulgare subsp. vulgare ent-kaurene synthase-like protein 1 (KSL1) mRNA, complete cds 73.4 73.4 93% 1e-10 80%	Hv.13581	Ent-kaurene synthase-like protein 1 (KSL1); NP_001053841.1 Os04g0611800 O. sativa 73.8 741 NP_178064.1 GA2 (GA REQUIRING 2); ent-kaurene synthase A. thaliana 51.4 768	Terpene_cyclase_plant_C1
786	late, 72 h	CM and DH1	70	ggcgcttcagggtcttgggcagcacggagatgcggtcaaaacaagcaaactcatagctgccggcattctc	CJ942067.1 CJ942067 Y. Ogihara unpublished cDNA library, whchan Triticum aestivum cDNA clone whchan40b02 3', mRNA sequence 127 127 100% 4e-27 100%	Ta.51942		transcribed locus				
787	late, 72 h	CM and DH1	370	cgggagaacactttggaatatccactgaccacagagagtatagacgccatcttgtttgacctatttgcaggcggtacagaaaccacaggaaacgttttggcgtgggctatgtcggagcttatgcgcagtcctaacattatggctaaagcacaaaaagaagtacacgaactgttgtgtgaaggccgagatctaattactactgggcgacctttctgaactcactatatgaagaaaatcatccaggaagtcctttaagttgcattcggcgggtncncttttattccttnggatttggcccgaggantttgcncgtggggttatgacatccctaaaggtaccgttgtacaca	CJ961698.1 CJ961698 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul27e20 3', mRNA sequence 187 187 94% 4e-44 74%	Ta.62983	Transcribed locus, weakly similar to NP_001058172.1 Os06g0642500 [Oryza sativa (japonica cultivar-group)];	NP_001058172.1 Os06g0642500 O. sativa 55.9 213 ; p450; Cytochrome P450	AB036772.1 Triticum aestivum N-1 mRNA for cytochrome P450, complete cds 187 187 94% 4e-44 74%	Ta.174	N-1 mRNA for cytochrome P450;NP_001046549.1 Os02g0278400 O. sativa 51.6 504	p450; Cytochrome P450
788	late, 72 hai	in CM and DH1	335	ggagccatgtttctccaatgcttcaagaagtggaggatttctttgcaaagtggggagaggaaggcgtagttgatctaaagcttgagttcgagcagctacttatgttgatatcaggccgatgtttgcttggaaaagaggtccgggagaatatgtttgatgaagtctacacacttttttcgcgagatcgaaaagggtgtgaccttgatcagcttcttgttccccatatctccccaactccagcaaaccggccagcgtgatagggcacgtataaggctaacagagatccctctctaatgttgtcgaagtcccgtaaagagctccgggcgagtggaggaggacacg	EB514924.1 Ta10b_02e18_R Ta10b_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads Triticum aestivum cDNA clone Ta10b_02e18, mRNA sequence 567 567 100% 1e-158 98%	Ta.63120	Transcribed locus, strongly similar to NP_054477.1 photosystem II protein D1 [Nicotiana tabacum	NP_114239.1 photosystem II protein D1 T. aestivum 98.7 78 ;Photo_RC; **Photosynthetic reaction centre protein**	DQ680853.1 Avena strigosa cytochrome P450 CYP51H11 (Cyp51H11) mRNA, complete cds 188 188 92% 1e-44 74%	AK240713.1 Oryza sativa Japonica Group 120 120 64% 5e-24 71% :Os.75808	NP_001055558.1 Os05g0416400 O. sativa 64.6 441	p450; Cytochrome P450
N300	late, 72 hai	in all except CM	125	gaacatgtagaggaagatcgtcggaaggataaggtctggatagaagacgagcatgatgaagagtacatggaccgagactgtggtgactgggttttccactggcaaacgtcaccaaaccacttcc	CJ958301.1 CJ958301 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul17a22 3', mRNA sequence 221 221 100% 5e-55 99%	Ta.55468	Transcribed locus, weakly similar to NP_568175.1 C2 domain-containing protein [Arabidopsis thaliana]	NP_001058075.1 Os06g0614000 O. sativa 63.8 631 ; Plant phosphoribosyltransferase C-terminal	EF576046.1 Oryza sativa (indica cultivar-group) clone OSE-577-637-A2 phosphoribosylanthranilate transferase mRNA, partial cds 154 154 98% 7e-35 87%	Os.14843	NP_001059647.1 Os07g0483500 O. sativa **100.0 1010 100 % identity**	Phosphoribosylanthranilate transferase-like protein; Protein kinase C conserved region 2
747	late 72 hai	in all except CM	435	gtcaccgtctccgtctgtgatgtttccatacgtgccgacagggagaagcttatggaaacagtccgagaaacttttcaatagcaagcttgacatactggtgaacaatgcagggcaattgctcttcaagcccactactgaatgtacagtggaggaatactcaaatctgctgaccaccaacttggagtcgagcttccatctcagccagctccgcacaacctctttcttcattacncgctttcttattggccnggaaggaggcnggattcttcacttttcctccatggaagggctcaaancgggtctatgcagggtctccgcaatttagttgctaccacaaaaggnggcaattgaaaccaaacttacaagggagtttgggccaccgagtgggggcctctgacaagattcgtgtgaatgggcatcgccccaggatttgtcacaaccgacatgc	CD873856.1 AZO3.100N16R011123 AZO3 Triticum aestivum cDNA clone AZO3100N16, mRNA sequence 417 417 99% 2e-113 83%	Ta.22286	Transcribed locus, weakly similar to [Arabidopsis thaliana], NP_180490.1 tropinone reductase, putative / tropine dehydrogenase, putative A. thaliana 60.5 253	NP_001068381.1 Os11g0652900 O. sativa 62.2 253 ;	EU958176.1 Zea mays clone 1676974 tropinone reductase 2 mRNA, complete cds 111 111 47% 3e-21 71%	Zm.87915	Clone 1676974 unknown mRNA, NP_001068381.1 Os11g0652900 O. sativa 63.6 260 , NP_180497.1 tropinone reductase, putative / tropine dehydrogenase, putative A. thaliana 55.6 261	fabG; 3-ketoacyl-(acyl-carrier-protein) reductase,Tropinone reductase II
421	intermediate, 24hai	in all except CM	123	taggttagtccccaaaaatgatataaaatagcataataatgcatataaaacgtctaagatggatactataatagcatgaaacaataaaaaattatagatacgttggagacgtatcaaccattc	CJ714268.1 CJ714268 Y.Ogihara unpublished cDNA library Wh_VABA Triticum aestivum cDNA clone whva2a21 5', mRNA sequence 152 152 94% 2e-34 89%	Ta.31625	transcribed locus	transcribed locus		Hv.27452	CDNA clone: FLbaf105n06, mRNA sequence	EST found in barley
407	late 72 hai	in all except CM	85	ccaaccgggaggacgtgctccagcgctttaggatccaccctgctggtacaatggagtgaccgcatacttgctttgttccggcca	CK205814.1 FGAS017359 Triticum aestivum FGAS: Library 5 GATE 7 Triticum aestivum cDNA, mRNA sequence 44.6 44.6 45% 0.050 84%	Ta.8959	Transcribed locus, weakly similar to NP_001058986.1 Os07g0169600 [Oryza sativa (japonica cultivar-group)], NP_001058986.1 Os07g0169600 O. sativa 48.7 276	2OG-Fe(II) oxygenase domain containing protein	NM_001051667.1 Oryza sativa (japonica cultivar-group) Os01g0906200 (Os01g0906200) mRNA, partial cds 37.4 37.4 23% 7.8 100%	Os.54646	NP_001045132.1 Os01g0906200 O. sativa 100.0 407 , NP_564573.3 HIT1 (HEAT-INTOLERANT 1); transporter A. thaliana 74.1 400	

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
791	late 72 hai	in all except CM	116	gttcgacctcgatggcgatggcaaactctcgttcgacgagttccgg gtcatgatgatggcatgatcatcgactcgatctcttcttcgcattcttct agatcacatagaatgttcac	CJ844385.1 CJ844385 Y. Ogihara unpublished cDNA library, whatlal Triticum aestivum cDNA clone whatlal34p14 3', mRNA sequence 154 154 99% 6e-35 90%	Ta.62200	Transcribed locus, weakly similar to NP_001065531.1 Os11g0105000 [Oryza sativa (japonica cultivar-group)]	NP_001065531.1 Os11g0105000 O. sativa 67.4 141 , NP_199053.1 CML37/CML39; calcium ion binding A. thaliana 45.8 142, EFh; EF-hand, calcium binding motif;	AK241127.1 Oryza sativa Japonica Group cDNA, clone: J065096B12, full insert sequence 60.8 60.8 55% 1e-06 80%	Os.83650	NP_001065531.1 Os11g0105000 O. sativa 100.0 148 , Calcium-binding EF-hand domain containing protein	EFh; EF-hand, calcium binding motif;
429	late 72 hai	in all except CM	217	aaggtgcggaatttggaatagaaatgcgagaaatctggttggcag gataagtcgtatacgagtacggccgggaagagtccgaaaaacc cccagcgccacgcaagaaaatagtactcctgttcatacataaca cttcgccagaatctttgagactggtggtcgtttcgatctcgaaaata gttgctttccctttggttccgttggcaaaccagctacc	CJ896203.1 CJ896203 Y. Ogihara unpublished cDNA library, whthls Triticum aestivum cDNA clone whthls9a16 3', mRNA sequence 356 356 100% 2e-95 96%	Ta.9392	Transcribed locus, moderately similar to NP_001063833.1 Os09g0544900 [Oryza sativa (japonica cultivar-group)]NP_001063833.1 Os09g0544900 O. sativa 89.2 313 , NP_177742.2	MaoC-like dehydratase domain containing protein maoC-like dehydratase domain-containing protein A. thaliana 67.3 308	AK252578.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf160c07, mRNA sequence 315 315 100% 7e-83 92%	Hv.6190	NP_001063833.1 Os09g0544900 O. sativa 89.8 312 , NP_177742.2 maoC-like dehydratase domain-containing protein A. thaliana 66.1 298	MaoC-like dehydratase domain containing protein
757	late, 72 hai	in all except Remus	115	aagcagtacacctttttatttatatggccgtatatctacgtacgccttta ttgattcgcggtgcgacgtatagaggcttcatggacagaaggtgat ctggtagttattattgcca	GH456152.1 XZ9104_CY32_C39 SSH cDNA library for wheat adult plant resistance to stripe rust Triticum aestivum cDNA 5' similar to putative acidic pr5 precursor, mRNA sequence 208 208 100% 3e-51 100%	Ta.56897	Thaumatin-like protein, NP_001105702.1 pathogenesis related protein-5 Z. mays 71.8 169	pathogenesis related protein-5 , Thaumatin; Thaumatin family	X58394.1 T.aestivum mRNA for a thaumatin-like protein 208 208 100% 3e-51 100%	Ta.56897	Thaumatin-like protein, NP_001105702.1 pathogenesis related protein-5 Z. mays 71.8 169	pathogenesis related protein-5 , Thaumatin; Thaumatin family
N304=503	late, 72 hai	in all except Remus	107	gtccgtgatcaaagtgtactcttcacatcctgtcattccccacacca gatcaccgggcttgtaatcctggtgcccggattccaccaccttgctt acgcccaaagtgg	CJ643011.1 CJ643011 Y.Ogihara unpublished cDNA library Wh_EMI Triticum aestivum cDNA clone whei11h21 5', mRNA sequence 194 194 100% 6e-47 100%	Ta.55935	Transcribed locus, moderately similar to NP_001066434.1 Os12g0227400 [Oryza sativa (japonica cultivar-group)]	NP_001066434.1 Os12g0227400 O. sativa 79.8 327 , NP_186958.1 NADP-dependent oxidoreductase, putative A. thaliana 72.8 334 , Allyl alcohol dehydrogenase, COG2130; Putative NADP-dependent oxidoreductases	AK251937.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf136n04, mRNA sequence 176 176 100% 2e-41 96%	Hv.24708	NP_001066432.1 Os12g0226700 O. sativa 74.8 344 , NP_186958.1 NADP-dependent oxidoreductase, putative A. thaliana 65.5 341	Putative NADP-dependent oxidoreductases [General function prediction only]
432/a	late, 72 hai	in all except Remus	97	tccttccgaggcgcagaatacgccgcaggactacgtttcaccgc acaacgcagcccgcgccaccgtcggcgtgggcgcggtgagct ggagcacgaag	CJ561501.1 CJ561501 Y.Ogihara unpublished cDNA library Wh_KMV Triticum aestivum cDNA clone rwhkv15p23 3', mRNA sequence 176 176 100% 1e-41 100%	Ta.278	Pathogenesis-related protein 1	NP_001043091.1 Os01g0382400 O. sativa 79.0 166, NP_179068.1 PR1 (PATHOGENESIS-RELATED GENE 1) A. thaliana 58.5 151	Z26321.1 H.vulgare mRNA for pathogenesis-related protein pbr1-3 134 134 96% 5e-29 91%	Hv.26591	Hv-PR-1b mRNA for a basic PR-1-type pathogenesis related protein	NP_001043091.1 Os01g0382400 O. sativa 73.0 166 NP_179068.1 PR1 (PATHOGENESIS-RELATED GENE 1) A. thaliana 54.6 , Pathogenesis-related protein PRB1-2 precursor
432/b	late, 72 hai	in all except Remus	97	ctccaatcaaaggatgccacgcagatattgccagcttgccctttcc actcgacaatctggtggagttccacagcacatgttcctttcatcaat atga	CJ786332.1 CJ786332 Y. Ogihara unpublished cDNA library, whatl Triticum aestivum cDNA clone whatl14b17 3', mRNA sequence 167 167 100% 7e-39 98%	Ta.29456	Transcribed locus, strongly similar to NP_001047174.1 Os02g0566800 [Oryza sativa (japonica cultivar-group)]	NP_001047174.1 Os02g0566800 O. sativa 93.3 387 ,NP_974164.2 galactosyltransferase family protein A. thaliana 80.8 373 , Galactosyl_T; Galactosyltransferase	NM_001137846.1 Zea mays hypothetical protein LOC100192632 (LOC100192632), mRNA >gb|EU966359.1| Zea mays clone 293872 avr9 elicitor response protein mRNA, complete cds 136 136 100% 1e-29 91%	Zm.10824	Hypothetical protein LOC100192632 (LOC100192632), mRNA, NP_001131318.1 hypothetical protein LOC100192632 Z. mays 100.0 397 NP_974164.2 galactosyltransferase family protein A. thaliana 83.4 373	Galactosyl_T; Galactosyltransferase

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
541 = TDF 59	intermediate, 24 hai	in all except Remus	169	tgcagactacatacgaatatatatagacgtactttcgagtgtagatt cactcattttgctccgtatgtaatctatagtggaatgtttagaaagact tatatttaggaacggatgaggtagtaaagcttgcagtgcactaatg ctcttgtagatgacaagatgctactgc	EX982044.1 **TDF59 wheat Fusarium** graminearum inoculated, cDNA-AFLP fragment Triticum aestivum cDNA, mRNA sequence 297 297 97% 1e-77 100%	Ta.11718, unigene at E- 34	Transcribed locus, moderately similar to NP_001042520.1 Os01g0235200 [Oryza sativa (japonica cultivar-group	Chromosome segregation ATPases	EU878778.1 Hordeum vulgare subsp. vulgare Adf2 (Rpg4), RGA1, NBS-LRR-S/TPK stem rust resistance protein (Rpg5), and ADF3 genes, complete cds 147 369 71% 2e-32 87%	very weak, Hv.15744	NP_001063251.1 Os09g0434200 O. sativa 82.0 217 , NP_197591.1 zinc finger (C3HC4-type RING finger) family protein A. thaliana 45.9 205	Zn-finger, RING domain containing protein
TDF59	intermediate,12 hai	12hai, fusarium-induced, CN, DH1, DH2, Eltern WH1+2	164	gcagtagcatcttgtcatctacaagagcattagtgcactgcaagctt tactacctcatccgttcctaaatataagtctttctaaacattccactat agattacatacggagcaaaatgagtgaatctacactcgaaagta cgtctatatatattcgtatgtagt	CJ595070.1 CJ595070 Y.Ogihara unpublished cDNA library Wh_SHDr Triticum aestivum cDNA clone rwhsd3b01 3', mRNA sequence 150 208 70% 8e-34 88%	Ta.11718	Transcribed locus, moderately similar to NP_001042520.1 [Oryza sativa (japonica cultivar-group)]	hypothetical protein F2K15.110 - Arabidopsis thaliana	AY821692.1 Australopyrum retrofractum beta amylase gene, partial cds; and Stowaway MITE, complete sequence 138 193 69% 6e-30 86%		P5CDH delta-1-pyrroline-5-carboxylate dehydrogenase [Hordeum vulgare]	
442	late, 72 hai	in all except Remus	114	tctacttgaagaagtatgggaggtcgagcacgtagaggaggaag taggcccaggtggcaccggagacacaccgaagaagaagcctc cgggtgaactgcgacacccttcagcggt	BEB513481.1 Ta08_07g16_R Ta08_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads Triticum aestivum cDNA clone Ta08_07g16, mRNA sequence 185 185 100% 4e-44 97% , CJ966857.1 CJ966857 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul5i10 3', mRNA sequence 185 185 100% 4e-44 97%	Ta.53952	Transcribed locus, strongly similar to NP_001066659.1 Os12g0420400 [Oryza sativa (japonica cultivar-group)]	NP_193016.1 PSAL (photosystem I subunit L) A. thaliana 80.7 217 , NP_001066659.1 Os12g0420400 O. sativa 95.2 207	AK252471.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf161o03, mRNA sequence 154 154 100% 6e-35 91%	Hv.23268	Photosystem I hydrophobic protein, NP_001066659.1 Os12g0420400 O. sativa 94.2 207 NP_193016.1 PSAL (photosystem I subunit L) A. thaliana 77.4 216	PsaL; Photosystem I reaction centre subunit XI
372 aligns with K2412	late, 72 hai	in all except Remus	207 - 211	ggaacactgccccaccgccggatgcaacagcggcaaccactg cttcgagcactccgtccccggcggcaactgccacggcgtcaccg agatcaagatcgtctactacaacccgtagatcgatcgacccgtcg cccgcctaggtagctgctgcaactgcttgtagtgttgaataatgttgc gggtaggtcgatctcagctctgttgtcgg	CJ539704.1 CJ539704 Y.Ogihara unpublished cDNA library Wh_EM Triticum aestivum cDNA clone rwhem11c12 3', mRNA sequence 324 324 98% 1e-85 96% , BM137901.1 WHE0472_B08_C16ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0472_B08_C16, mRNA sequence 282 282 85% 4e-73 96%	Ta.39538	Transcribed locus, weakly similar to NP_001067071.1 Os12g0569200 [Oryza sativa (japonica cultivar-group)]	NP_001067071.1 Os12g0569200 O. sativa 60.4 163 , hypothetical protein	AK251628.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf119f15, mRNA sequence 237 237 91% 1e-59 88%	Hv.8216	CDNA clone: FLbaf119f15, mRNA sequence, NP_001067071.1 Os12g0569200 O. sativa 59.8 163	hypothetical protein
347	intermediate, 24 hai	in all except Remus, starts intermediate	547	gtgttcgcccagagctacgccaaccagaggatcaacgactgca agctccagcactcgggcgggccgtacggggagaacatcttctgg gggtcggccggcgcggactggaaggcggcggacgcggtgaac gcgtgggtgggcgagaagcaggactacaactatggctccaaca cctgcgcggcggggaaggtgtgcgggcactacacgcaggtggt atggcgcgcgtccaccancatcngctgcgctcgcgtcgtctgcaa caacaacctcggcgtcttcatcacctgcaactacgagccccgcg ggaatatcattggacagaaaccatactagtagctgtgatgcatgtc ttcgcagtacatacatgcatacatagtcctctcgtccttacaaacat gcattcgtatacacacatgcaacggttcatgcaccttcctattccta actagctagagggancgangcgagtaaatataaggaatgccgt agtgagccatgtaattccgtcctgggactggtataaataatgagctt ctctatggcagtc	CJ668712.1 CJ668712 Y.Ogihara unpublished cDNA library Wh_KMV Triticum aestivum cDNA clone whkv15p23 5', mRNA sequence 886 886 98% 0.0 96%	Ta.278	NP_001043091.1 Os01g0382400 O. sativa 79.0 166 ,NP_179068.1 PR1 (PATHOGENESIS-RELATED GENE 1) A. thaliana 58.5 Pathogenesis-related protein 1	151 , Pathogenesis-related protein PRB1-2 precursor	AF384143.1 Triticum aestivum pathogenesis-related protein 1 mRNA, complete cds 702 702 99% 0.0 88% * strange E-value 00.0?????	Ta.278	NP_001043091.1 Os01g0382400 O. sativa 79.0 166 ,NP_179068.1 PR1 (PATHOGENESIS-RELATED GENE 1) A. thaliana 58.5 Pathogenesis-related protein 1	151 , Pathogenesis-related protein PRB1-2 precursor
409	late, 72 hai	only in DH2 and Remus	62 - 67	ccaagttattgtcgagcacgttacaatcctagcacctttgaattcgc caaacaccgacggca	CD868995.1 AZO2.110G08F001124 AZO2 Triticum aestivum cDNA clone AZO2110G08, mRNA sequence 113 113 100% 7e-23 100%	Ta.4469	NP_567126.1 glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein A. thaliana 71.2 445	Glyco_hydro_28; Glycosyl hydrolases family 28 , Glycoside hydrolase, family 28 protein	EU970881.1 Zea mays clone 352977 polygalacturonase mRNA, complete cds 84.2 84.2 98% 3e-14 90%	Zm.26648	NP_001051812.1 Os03g0833800 O. sativa 88.0 468 , NP_567126.1 glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein A. thaliana 66.3 466	Glycoside hydrolase, family 28 protein

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
Pathogen responsive -quantitative differences												
486	late, 72 hai	stronger in CM	469	gtgacacaactaaaatgtggaggatttgttattgggcttcacatgtgt cattgcattgctgatggttttggtattcttcaatttatcaaaactatagc cgattttggatgtggtgaacttatcccaaccactttgcccgtgtggaa aagagatatcttcacagcacgcatgccaccatccattgcacatgtt tacccggcatataaaccatttcttcatgggttagagtgcacgggag atgacgtgatgctatcaactccgcagaatgcatggaagtgcaata tttattctttgggccaaatgagatagatattttgagaagccatgtccc agaacacctctctaaatctactacaacattttgagttgattacggctg tcatgtggcggtgccgcacattgggcactgggctatgaatctactc aaagtcgtgtcctgtttaccctaataccgtgggagaagc	EB514888.1 Ta10b_02c19_R Ta10b_AAFC_ECORC_Fusarium_graminearum_inculated_wheat_heads Triticum aestivum cDNA clone Ta10b_02c19, mRNA sequence 520 520 92% 2e-144 87% , DV519293.1 ZM_BFb0203K12.r ZM_BFb Zea mays cDNA 5', mRNA sequence 91.5 91.5 30% 3e-15 74%	Zm.34177, with value 3e-15	NP_001064288.1 Os10g0195600 O. sativa 68.6 483	Transferase; Transferase family	AP008213.1 Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7 150 150 89% 4e-33 67%	Os.52173 with e-17	NP_001066733.1 Os12g0458100 O. sativa 100.0 445	Transferase; Transferase family
489	late, 72 hai	quant stronger CM	95/85 2 allele?	tgcctagcctcgtacgctacgagcatccacatatctaccaatccgc acaccccggacctttcccggcctcggccggtctcacttcatcccatc cac	CJ849264.1 CJ849264 Y. Ogihara unpublished cDNA library, whsctal Triticum aestivum cDNA clone whsctal11l24 5', mRNA sequence 95.1 95.1 100% 4e-17 82%	Ta.33180	Transcribed locus, weakly similar to NP_001052935.1 Os04g0450000 [Oryza sativa (japonica cultivar-group)]	NP_001052935.1 Os04g0450000 O. sativa 65.5 171 , FYVE/PHD zinc finger domain containing protein	**no real hit**			
529	intermediate, 24 hai	quant stronger CM	88	ggtgggcttgtcgtcctcttgatggtgtatgtattgtcgaggctgcatt gacatgtagtcgtacaccaacatcagctcacctttccgc	FL864312.1 CCGI8368.b1 CCGI Panicum virgatum etiolated seedlings (H) Panicum virgatum cDNA clone CCGI8368 5', mRNA sequence 62.6 62.6 70% 2e-07 84% , CA108745.1 SCSGHR1066F11.b HR1 Saccharum officinarum cDNA clone SCSGHR1066F11 3', mRNA sequence 62.6 62.6 81% 2e-07 81% EE174142.2 ZM_BFc0152L01.f ZM_BFc Zea mays cDNA clone ZM_BFc0152L01 3', mRNA sequence 60.8 60.8 62% 7e-07 84%	Sof.6709, Zm.13043	Transcribed locus, moderately similar to NP_001053397.1 Os04g0531500 [Oryza sativa (japonica cultivar-group)],	(RECEPTOR LECTIN KINASE); kinase A. thaliana 66.9 586 NP_001053397.1 Os04g0531500 O. sativa 88.4 539 , NP_181307.1 kinase A. thaliana 61.8 516 , Protein kinase family protein	CT827958.1 Oryza sativa genomic DNA, chromosome 4, BAC clone: OSIGBa0125M19, complete sequence 53.6 96.3 67% 1e-04 80%	Os.97307	NP_001053397.1 Os04g0531500 O. sativa 100.0 735 , NP_181307.1 RLK (RECEPTOR LECTIN KINASE); kinase A. thaliana 64.4 648	Protein kinase family protein , Serine/Threonine protein kinases
321	early, 6 hai	quant stronger CM	169	ccgacgacgcaagccatttatggcagactcacacagaaatcaa atcaccaaacagcaaacccgataaacaaactctatggagatcg gcttagaagcgtggacttgatgcactccgtcttgggctaggccggg cttggcgaggaagcggcgacgcgcttgcggtggtcgc	DV487417.1 5531_D06_H11RZ_042 Brachypodium distachyon root EST library Brachypodium distachyon cDNA clone 5531_D06_H11, mRNA sequence 69.8 69.8 28% 3e-09 92%	Os.55628	NP_001053955.1 Os04g0627900 O. sativa 100.0 243 , NP_175831.1 eukaryotic translation initiation factor SUI1, putative A. thaliana 68.9 89	Translation initiation factor SUI1 family protein	NM_001060490.1 Oryza sativa (japonica cultivar-group) Os04g0627900 (Os04g0627900) mRNA, partial cds 59.0 59.0 63% 6e-06 74%	Os.55628	NP_001053955.1 Os04g0627900 O. sativa 100.0 243 , NP_175831.1 eukaryotic translation initiation factor SUI1, putative A. thaliana 68.9 89	Translation initiation factor SUI1 family protein
536	late, 72 hai	quant stronger CM	265	ctacagatgatgctgcaaccgggatgacaggcacaccatggtgc tggcagaaagcttgatgcctttgtgagatgaatccacagtgctgat ctttatgttcacactgttggcagacatcagggtcgtaacttgtaaatg attgaggttgtttgtacacatcagttgctacgtttgctgagatgagctt ctgaagagggcattgtgtcgatggttccacaagcgggatgacgtt gatgacgcggtgcctgtaatagatctccgtcact	DT693918.1 s13dFA37D05HS044_437450 Tall fescue, Festuca arundinacea Schreb, Heat-stressed shoots Festuca arundinacea cDNA, mRNA sequence 311 311 99% 1e-81 86% , CJ954878.1 CJ954878 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul5o20 5', mRNA sequence 260 431 91% 2e-66 99%	Hv.21610	Transcribed locus, weakly similar to NP_001105659.1 Ser/Thr receptor-like kinase1 Z. mays 59.8 224 , Ser/Thr receptor-like kinase1 [Zea mays]	NP_001105659.1 Ser/Thr receptor-like kinase1 Z. mays 59.8 224 , Ser/Thr receptor-like kinase1	NM_001048367.1 Oryza sativa (japonica cultivar-group) Os01g0114700 (Os01g0114700) mRNA, complete cds 98.7 152 76% 1e-17 78%	Os.80698	NP_001041832.1 Os01g0114700 O. sativa 100.0 560	TyrKc; Tyrosine kinase, catalytic domain; Phosphotransferases.
866	early, 6 hai	early -genotype unspecific	101	ccccccatcagtgatgataccagtcagctctgcacagtatgcctgta tctgaagtctgaagtgttgacgatatgattcgtatgtgtgggacatga ttgtggt	CJ528578.1 CJ528578 Y.Ogihara unpublished cDNA library Wh_DPA20 Triticum aestivum cDNA clone rwhdp7p04 3', mRNA sequence 183 183 100% 1e-43 100%	Ta.41092	transcribed locus	no reference gene available	no significant hit			

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
487	early, 6 hai	early -genotype unspecific	256	gtcttctcgggggtcgacgtctgcgacgacgccacccaagtgtcc acgggctggtcggcgtgccgcgtggtgtcgacgaagcccgaaat gtggcgctgcgcggatataaatcaccgttcacgtggactgcggtg gacctgcaagaagtaactgaatcggttcattgccgggcatgatat gttccgcctaaaataaaaataaaaagctcaggcgaaatgagca ggtcatcgttgctattgcgtgtgtgtggccaa	CJ956132.1 CJ956132 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul9n22 5', mRNA sequence 401 401 99% 1e-108 86%	Ta.54143	NP_001057442.1 Os06g0298400 O. sativa 72.2 759 , NP_187930.2 protein binding A. thaliana 59.9 182	WW/Rsp5/WWP domain containing protein	L11882.1 Triticum aestivum protein of unknown function (wali5) mRNA, complete cds 349 349 81% 7e-93 87%	Ta.56885	Wali5 protein,	Triticum aestivum protein of unknown function (wali5)
865	early, 6 hai	early -genotype unspecific	186	ccccaccgccgccgccagcatgaaggtcgtgatcgacgtcgtcc cgagttcctcctcaccgtcgtcacccatgccggctgccggtccgg acgcgagcaacccgccgccaccctcctctaccgcatcctctgtc ggggccacagcaggatttggccttgtcgtcctactggtcgccagtc tcatggt	CJ849214.1 CJ849214 Y. Ogihara unpublished cDNA library, whsctal Triticum aestivum cDNA clone whsctal11b18 5', mRNA sequence 336 336 100% 2e-89 100% , BQ161880.1 WHE0484_a04_a08zT Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0484_a04_a08, mRNA sequence 333 333 100% 2e-88 99%	Ta.5654	NP_001064000.1 Os09g0572700 O. sativa 61.3 168 ,	Cu_bind_like; Plastocyanin-like domain	AK251999.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf139m03, mRNA sequence 176 176 99% 3e-41 84%	Hv.22283	NP_001064000.1 Os09g0572700 O. sativa 59.8 169	Plastocyanin-like domain containing protein
513 aligns with 401	early, 6 hai	early -genotype unspecific	165	caatataaaagctctgtatgtgcgcgcggacagctgatcaatatat gcgttcaagccatgagaccggccgccagtagggcgacgaggc caaatcctgctgtggcaccgactggaggtagcggcgttggangg cggcgccgggggagatgttgctcgcgccaggac	CN012594.1 WHE3898_E06_J12ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE3898_E06_J12, mRNA sequence 259 259 100% 3e-66 97%	Ta.57084	Transcribed locus, weakly similar to NP_001064000.1 Os09g0572700 [Oryza sativa (japonica cultivar-group)]	NP_001064000.1 Os09g0572700 O. sativa 59.4 169 , NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 46.8 171 ,Plastocyanin-like domain containing protein	AF031195.1 Triticum aestivum blue copper-binding protein homolog (S85) mRNA, complete cds 230 230 100% 2e-57 92%	Ta.55631	Blue copper-binding protein homolog, NP_001064000.1 Os09g0572700 O. sativa 60.5 167 NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 45.6 171	Plastocyanin-like domain containing protein

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
Pathogen responsive - genotype unspecific												
316	late, 72 hai	unspecific	149_151	ctgcatgagccctgtttccttcaagattggtgtaaaccttgtcttgactggaaaggtaactttctccccaccctccagatgggagaagctccttggacaaaagaaattcacacgcttttctgattgctaggctatttacatacgtcctcc	CJ890105.1 CJ890105 Y. Ogihara unpublished cDNA library, whthls Triticum aestivum cDNA clone whthls27n13 3', mRNA sequence 271 271 100% 4e-70 100%	Ta.31805	Transcribed locus, moderately similar to NP_001045848.1 Os02g0139700 [Oryza sativa (japonica cultivar-group)]	NP_001045848.1 Os02g0139700 O. sativa 72.6 755 , Cycloartenol synthase	AB181246.1 Lotus japonicus OSC5 mRNA for cycloartenol synthase, complete cds 109 109 82% 3e-21 79%	Lja.3486	OSC5 mRNA for cycloartenol synthase	NP_178722.1 CAS1 (CYCLOARTENOL SYNTHASE 1) A. thaliana 82.6 754
437	late, 72 hai	unspecific	248_252	tcccgtgtgacaccgtgtgcatagtcgccggtcttgggcaggagtacatcccgaaccaactcgtcgaaattgcggtgggacgaaccgccaggcttggccgccatgatagccttctcctgccactcctccgccctcttcctcatctgcttgcccttctccccgtccatgagctccgcgatgaggcccgtgacggcatcgcgctggacgttgctatcgatctccatgccgacgccccactctttgcactggtac	CJ887274.1 CJ887274 Y. Ogihara unpublished cDNA library, whthls Triticum aestivum cDNA clone whthls19e18 3', mRNA sequence 401 401 100% 7e-109 95%	Ta.51888	Transcribed locus, weakly similar to NP_564170.1 ATUGT85A5 (UDP-GLUCOSYL TRANSFERASE 85A5); transferase, transferring glycosyl groups [Arabidopsis thaliana]	NP_564170.1 ATUGT85A5 (UDP-GLUCOSYL TRANSFERASE 85A5); transferase, transferring glycosyl groups A. thaliana 64.7 249	NM_001054700.1 Oryza sativa (japonica cultivar-group) Os02g0755900 (Os02g0755900) mRNA, complete cds 251 251 86% 8e-64 85%	Os.53614	Os02g0755900	NP_173653.1 ATUGT85A2 (UDP-GLUCOSYL TRANSFERASE 85A2); UDP-glycosyltransferase/glucuronosyltransferase/transferase, transferring glycosyl groups A. thaliana 61.5 480 ; NP_001048165.1 Os02g0755900 O. sativa 100.0 484
401 aligns with 513	late, 72 hai	genotype unspecific	165	taacaaaagataaagtcgtatgtgcgcgcggcagctgatcaatatatgcgttcaagccatgagaccggccgccaggagggcgacgaggccaaatcctgctgtggcaccgacggaggtagcggcgttggagggcggcgccgggggagagttgctcgcgccaggac	CJ967954.1 CJ967954 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul9d16 3', mRNA sequence 297 297 100% 1e-77 100%	Ta.57084	Transcribed locus, weakly similar to NP_001064000.1 Os09g0572700 [Oryza sativa (japonica cultivar-group)]	NP_001064000.1 Os09g0572700 O. sativa 59.4 169 ; NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 46.8 171	AF031195.1 Triticum aestivum blue copper-binding protein homolog (S85) mRNA, complete cds 246 246 100% 2e-62 93%	Ta.55631	Blue copper-binding protein homolog (S85)	NP_001064000.1 Os09g0572700 O. sativa 60.5 167 ; NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 45.6 171
447	late, 72 hai	genotype unspecific	180	tccgctgccactctctcagctccgcctatacatcacacggcacaatctcaaaccacggataatttttctaaatcaaagcccaaaccctcgtcaggttgcatcgaggctgccggcagagcacacccaaccagccccaatcagcgtccgtccgattatagtacagcggcgatcgtcgcgcagaa	CJ968041.1 CJ968041 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul9i13 3', mRNA sequence 327 327 100% 8e-87 100%	Ta.54277	Transcribed locus, strongly similar to NP_001049525.1 Os03g0243100 [Oryza sativa (japonica cultivar-group)]	NP_001049525.1 Os03g0243100 O. sativa 95.1 142 ; NP_565390.1 ADF5 (ACTIN DEPOLYMERIZING FACTOR 5); actin binding A. thaliana 77.6 142	AK252510.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf159k01, mRNA sequence 125 125 96% 5e-26 77%	Hv.3860	CDNA clone: FLbaf67j14	NP_001049525.1 Os03g0243100 O. sativa 95.1 142 ; NP_565390.1 ADF5 (ACTIN DEPOLYMERIZING FACTOR 5); actin binding A. thaliana 79.0 142
N304	late, 72 hai	genotype unspecific	107	gtccgtgatcaaagtgtactcttcacatcctgtcattccccacaccagatcaccgggcttgtaatcctggtgcccggattccaccaccttgcttacgcccaaagtgg	CJ643011.1 CJ643011 Y.Ogihara unpublished cDNA library Wh_EMI Triticum aestivum cDNA clone whei11h21 5', mRNA sequence 194 194 100% 6e-47 100% ; BM138472.1 WHE0492_B03_C06ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0492_B03_C06, mRNA sequence 194 194 100% 6e-47 100%	Ta.55935/ Ta.55242	Transcribed locus, moderately similar to NP_197200.2 NADP-dependent oxidoreductase, putative [Arabidopsis thaliana]	NP_001066432.1 Os12g0226700 O. sativa 76.8 344 ; NP_197200.2 NADP-dependent oxidoreductase, putative A. thaliana 74.9 226	AK251937.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf136n04, mRNA sequence 176 176 100% 2e-41 96% ; AY904340.1 Hordeum vulgare subsp. vulgare 2-alkenal reductase (ALH) gene, complete cds 176 176 100% 2e-41 96%	Hv.24708	CDNA clone: FLbaf136n04, mRNA sequence	NP_001066432.1 Os12g0226700 O. sativa 74.8 344 ; NP_186958.1 NADP-dependent oxidoreductase, putative A. thaliana 65.5 341

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
420	late, 72 hai	genotype unspecific	151	tatcctagaagacgcgcagaaatgatggaacgaaaaaacacc catctaaatccacatacacacacctgttttgacacgaaatgaaca agaatcgcaatgcatatccgggggatcaaatcggtcgagaaac cctgcgggtaggcgacctctc	CJ786340.1 CJ786340 Y. Ogihara unpublished cDNA library, whatl Triticum aestivum cDNA clone whatl14c01 3', mRNA sequence 273 273 100% 1e-70 100% ; BQ162142.1 WHE0498_A03_B06ZT Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0498_A03_B06, mRNA sequence 206 206 99% 2e-50 90%	Ta.58105	Transcribed locus, weakly similar to NP_001061936.1 Os08g0448100 [Oryza sativa (japonica cultivar-group)]	NP_001061936.1 Os08g0448100 O. sativa 59.0 160	NM_001068471.1 Oryza sativa (japonica cultivar-group) Os08g0448100 (Os08g0448100) mRNA, complete cds 71.6 71.6 33% 8e-10 92%	Os.55850	Os08g0448100	NP_001061936.1 Os08g0448100 O. sativa 100.0 163 , hypothetical protein
490	late, 72 hai	unspecific	110	tggcggcgatgaccttgcttgtccttcatacccgcaagatgttggat agcgcccgaaatacctacggccatgtatagctcaggagcaatca ccttgcctgtttgacccac	CD459889.2 Fg09_06l08_A Fg09_AAFC_ECORC_Fusarium_graminearum_simple_substrate Gibberella zeae cDNA clone Fg09_06l08, mRNA sequence 190 190 100% 8e-46 99%				XM_382102.1 Gibberella zeae PH-1 hypothetical protein partial mRNA 190 190 100% 8e-46 99%	G		FixB; Electron transfer flavoprotein, alpha subunit
N302 aligns with 401, 513	late, 72 hai	genotype unspecific	164	taacaaaagataaagtcgtatgtgcgcgcggcagctgatcaatat atgcgttcaagccatgagaccggccgccaggagggcgacgag gccaaatcctgctgtggcaccgacggaggtagcggcgttggagg gcggcgccgggggagagttgctcgcgccaggac	CJ967954.1 CJ967954 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul9d16 3', mRNA sequence 303 303 100% 1e-79 100%	Ta.57084	Transcribed locus, weakly similar to NP_001064000.1 Os09g0572700 [Oryza sativa (japonica cultivar-group)]	NP_001064000.1 Os09g0572700 O. sativa 59.4 169 ; NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 46.8 171	AF031195.1 Triticum aestivum blue copper-binding protein homolog (S85) mRNA, complete cds 246 246 100% 2e-62 93%	Ta.55631	Blue copper-binding protein homolog (S85)	NP_001064000.1 Os09g0572700 O. sativa 60.5 167 ; NP_566810.1 plastocyanin-like domain-containing protein A. thaliana 45.6 171
785	late, 72 hai	genotype unspecific	53	tgaagtgatgagaaaaaagaaataactggtcagttgcacaacaa tttgaactc	Accession Description Max score Total score Query coverage E value Max ident Links CJ560992.1 CJ560992 Y.Ogihara unpublished cDNA library Wh_KMV Triticum aestivum cDNA clone rwhkv14b18 3', mRNA sequence 96.9 96.9 100% 4e-18 100%	Ta.36036	Transcribed locus, moderately similar to NP_001068433.1 Os11g0672300 [Oryza sativa (japonica cultivar-group)]	NP_001068433.1 Os11g0672300 O. sativa 83.6 213 ; hypothetical protein	AK248703.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf2f01, mRNA sequence 57.2 57.2 100% 3e-06 84%	Hv.1891	CDNA clone: FLbaf138a04, mRNA sequence	NP_001068433.1 Os11g0672300 O. sativa 81.7 197
408	late, 72 hai	genotype unspecific	66	ccaacttgtggcattcatctccaccggcgaaagattacaaggcga gcaccatgcatagtggtccca	CJ780074.1 CJ780074 Y. Ogihara unpublished cDNA library, whatl Triticum aestivum cDNA clone whatl30o18 5', mRNA sequence 120 120 100% 5e-25 100% ; BM140325.1 WHE0474_d04_h08zS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0474_d04_h08, mRNA sequence 120 120 100% 5e-25 100%	Ta.8323	Transcribed locus, weakly similar to NP_001046954.1 Os02g0515200 [Oryza sativa (japonica cultivar-group)]	NP_001046954.1 Os02g0515200 O. sativa 63.6 349 ; hypothetical protein, BAR; BAR domain	EU974836.1 Zea mays clone 459517 hypothetical protein mRNA, complete cds 53.6 53.6 93% 7e-05 79%	Zm.9760	Clone 459517 unknown mRNA	NP_001052655.1 Os04g0394700 O. sativa 81.1 615 , NP_180907.2 hydroxyproline-rich glycoprotein family protein A. thaliana 47.9 599
528	late, 72 hai	pathogen responsive - genotype unspecific	392	cccctggcaacctccagattatagtttccgtagccatcgatttctact ccttgtggattgaagcgtgcaagggtgttcttcatgcctacaaggtg gcgcatcacgccctcgcgcgacagcgtaccctcttggtcatggttg ccgagcacagcagcccagggaagtttcatggcaatggccggag cgatcgccgcatccatcgacttggccgcatcggtttgaatcctcgc caaaaatgttatcaccggtgaagacgacgaggtcggggtcctcg gcgcggaggaggcggtagaggaaggcggtggtgttgaggtcgg agcaggcggggcgctgcgcgggcagcacgtccctgcactgtgt gctccgcccgtccgcgtagtgcatgtccgcac	CJ670654.1 CJ670654 Y.Ogihara unpublished cDNA library Wh_KMV Triticum aestivum cDNA clone whkv3c17 5', mRNA sequence 672 672 99% 0.0 98%	Ta.43343	Transcribed locus, moderately similar to NP_001063768.1 Os09g0533300 [Oryza sativa (japonica cultivar-group)]	NP_001063768.1 Os09g0533300 O. sativa 80.0 384 ; NP_201119.1 ATPAP29/PAP29 (purple acid phosphatase 29); acid phosphatase/ protein serine/threonine phosphatase A. thaliana 63.0 341	NM_001070303.1 Oryza sativa (japonica cultivar-group) Os09g0533300 (Os09g0533300) mRNA, complete cds 444 444 97% 1e-121 85% ;	Os.12157	Os09g0533300	NP_001063768.1 Os09g0533300 O. sativa 100.0 397 ; NP_201119.1 ATPAP29/PAP29 (purple acid phosphatase 29); acid phosphatase/ protein serine/threonine phosphatase A. thaliana 66.3 341 ; Phosphoesterase At2g46880 family protein

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
Constitutive - genotype specific NIL polymorph												
K601	constitutive	Nils polymorphic	204 - 207	taattgtgtggccagtgtacacatccagcgtagctttatagaccgcg acctatttgtgttctttctgaactaatttacactcaattgaaccgtcgatt gagacatagcaaaaccgaacaaacacacatacagtgtcggct caaagagaaaaagaactagtaccactgtagcaagtcaaacag actgtagtaaacgtcgaaatgt	BM138011.1 WHE0479_F12_K23ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0479_F12_K23, mRNA sequence 304 304 83% 1e-79 99%	Ta.12795	transcribed locus	Found only in library 9984: Wheat Fusarium graminearum infected spike cDNA library, Mühlbauer project	**no hit, mouse hit**			
K453	constitutive	in CM, DH1, Nils 1u 3 qtl 5A	142	tcggggaagcgcttggcatgaatgtccaaccggccgacactcga gctcagcaaggtaactgtactgattcaccctcaaagtacgacata tttagatggttagctgtgtcagcaattcgtgtttctttaccttgggcgtgt a	no real hit				**no real hit**		closest Zea mays, general regulatory...	
K875	constitutive	Remus, DH2, Nils 2 and 4	315	gtgatgatgtactgttgtcctcgactgccaaggacgcaactgatga ctttgaggatgtcgggcacagcaccaccgcccgtgcgatgctgg acgagtactatgtcggcgacgtggatacggccacgatacctgcc cggacaaagtacactccgccgaagcaaccacactacaaccaa gacaagacccctgagttcatcatcaagatcctccagttccttggtc cccttgggcattacttggntctgggcttggtcggccattaaggaatg tacacccaagtcggaatcggt	CV781282.1 FGAS075693 Triticum aestivum FGAS: Library 2 Gate 3 Triticum aestivum cDNA, mRNA sequence 441 441 99% 9e-121 95%	Ta.3111	Transcribed locus, moderately similar to NP_001054434.1 Os05g0108800 [Oryza sativa (japonica cultivar-group)]	NP_001054434.1 Os05g0108800 O. sativa 88.7 132 ; NP_180831.1 B5 #4 (cytochrome b5 family protein #4); heme binding / transition metal ion binding A. thaliana 78.4 125 ; Cyt-b5; Cytochrome b5-like Heme/Steroid binding domain	AK253135.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf140l01, mRNA sequence 369 369 99% 5e-99 90%	Hv.2182	NP_001054434.1 Os05g0108800 O. sativa 92.5 132 , NP_180831.1 B5 #4 (cytochrome b5 family protein #4); heme binding / transition metal ion binding A. thaliana 81.0 125	Cyt-b5; Cytochrome b5-like Heme/Steroid binding domain
K878	constitutive	in Remus, DH2 and Nils 2, 4	119	gtgggcggaaaattatcattctacataaaaactaaataagcttgca aaaaaccactcatcatactagtaaagcaggagatgttatatagga gtagaaatcaacctacattctctaaagc	GH727863.1 G608.107M06F010907 G608 Triticum aestivum cDNA clone G608107M06, mRNA sequence 215 215 100% 2e-53 100% , CJ698796.1 CJ698796 Y.Ogihara unpublished cDNA library Wh_SHDr Triticum aestivum cDNA clone whsd14f19 5', mRNA sequence 136 136 100% 2e-29 86%	Ta.2917	Transcribed locus, moderately similar to NP_001058248.1 Os06g0655100 [Oryza sativa (japonica cultivar-group)]	NP_001058248.1 Os06g0655100 O. sativa 85.8 330 ; NP_195146.1 EDA9 (embryo sac development arrest 9); NAD binding / amino acid binding / binding / catalytic/ cofactor binding / oxidoreductase, acting on the CH-OH group of donors, NAD or NADP as acceptor / phosphoglycerate dehydrogenase A. thaliana 76.5 314 ; D-3-phosphoglycerate dehydrogenase family protein	**no real hit**			
K535	constitutive	in NIL 1, NIL 3, CM, DH1	188	tcacacaaaaaactaagcacatactcccaactctgtacctcgttc gctccacatctttctttacttcatcacttcttctttctgtctcttatgtatata ctggccagctagatacttggacgccatcatcgccacttgctgagct cgagtgtcggccggttggacattcatgccaagcgcttccccga	EX586396.1 HDP07M01w HDP Hordeum vulgare subsp. vulgare cDNA clone HDP07M01, mRNA sequence 129 129 65% 5e-27 83%	Hv.16854	Transcribed locus, weakly similar to NP_001068075.1 Os11g0550500 [Oryza sativa (japonica cultivar-group)]	NP_001068075.1 Os11g0550500 O. sativa 55.1 912 , Leucine-rich repeat (LRR) protein	**no real hit**			

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
K347	constitutive	Polymorphic in NIL 1 and NIL 3	345	aagatgttcagcaccttcaccgcaactgggaatgaagggaagatggttgaggcagtgaacggttcagatgttgttactacttacgaagacaaggatggagactggatgcttgtcggagacgtcccatgggagatgtttgctgcttcatgcaaacgcttgaggatcatgaaggggcccgaagccatcggtcttgcaccaagggcccaaggacaagtacaagaaacaagagctnaaacatggggcagtaaagaggaagactatgatgccataaaggatgggttgttcttggcagtgttgcttattgggtttccnttanatttataatgttttcatttattagctgga	DR741616.1 FGAS030669 Triticum aestivum FGAS: Library 2 Gate 3 Triticum aestivum cDNA, mRNA sequence 497 497 100% 2e-137 94%	Ta.24229	IAA1 protein	NP_001051230.1 Os03g0742900 O. sativa 86.9 235 ; NP_187124.1 IAA16 (indoleacetic acid-induced protein 16); transcription factor A. thaliana 68.3 229	AJ575098.1 Triticum aestivum partial mRNA for IAA1 protein 482 482 100% 4e-133 93%	Ta.24229	IAA1 protein	NP_001051230.1 Os03g0742900 O. sativa 86.9 235 ; **NP_187124.1 IAA16 (indoleacetic acid-induced protein 16); transcription** factor A. thaliana 68.3 229
K436 aligns with K563	constitutive	constitutive polymorphic in CM, DH1, NIL1and 3	76 aligns with K563!!	tttgtagatttccctggaggaaagctttcctttgttggtgaaatgaatttcctgccagagtcgcaaatggaaagga	BM137364.1 WHE2632_H11_P22ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE2632_H11_P22, mRNA sequence 138 138 100% 2e-30 100%	Ta.55259	Transcribed locus, strongly similar to NP_001066324.1 Os12g0183100 [Oryza sativa (japonica cultivar-group)]	NP_001066324.1 Os12g0183100 O. sativa 90.9 472 ; NP_974756.1 2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative / branched-chain alpha-keto acid dehydrogenase E1 alpha subunit, putative A. thaliana 82.9 396 ; AcoA; Pyruvate/2-oxoglutarate dehydrogenase complex	AK249348.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf31e08, mRNA sequence 129 129 97% 1e-27 98%	Hv.9500	CDNA clone: FLbaf31e08, mRNA sequence	NP_001066324.1 Os12g0183100 O. sativa 86.9 472 ; NP_974756.1 2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative / branched-chain alpha-keto acid dehydrogenase E1 alpha subunit, putative A. thaliana 79.8 395
K563	constitutive	constitutive polymorphic in CM, DH1, NIL1and 3	77/74 aligns with K436	tcctttccatttgcgactctggcaggaaattcatttcaccaacaaaggaaagctttcctccagggaaatctacaaa	BM137364.1 WHE2632_H11_P22ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE2632_H11_P22, mRNA sequence 138 138 100% 2e-30 100%	Ta.55259	Transcribed locus, strongly similar to NP_001066324.1 Os12g0183100 [Oryza sativa (japonica cultivar-group)]	NP_001066324.1 Os12g0183100 O. sativa 90.9 472 ; NP_974756.1 2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase, putative	AK249348.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf31e08, mRNA sequence 129 129 97% 1e-27 98%	Hv.9500	CDNA clone: FLbaf31e08, mRNA sequence	NP_001066324.1 Os12g0183100 O. sativa 86.9 472 ; NP_974756.1 2-oxoisovalerate dehydrogenase, putative / 3-methyl-2-oxobutanoate dehydrogenase,
K2412	constitutive	constitutive polymorphic in NIL1 and 3, time dep	207 - 219	ggacactgcccaccgcggatgcaacagcggcaacactggcttcgagcactccggtccccggcggcaactgcacgggcgtcacgagatcaagatcgtctactacaacccggtagatcgatcgacccggtcgcccgcctaggtagctgctgcaactggcttggtagtgttgaataatggttgcggtaggtcgatctcagctctgttgtcgg	CJ539704.1 CJ539704 Y.Ogihara unpublished cDNA library Wh_EM Triticum aestivum cDNA clone rwhem11c12 3', mRNA sequence 275 275 100% 5e-71 92% , BM137901.1 WHE0472_B08_C16ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE0472_B08_C16, mRNA sequence 232 232 86% 6e-58 92%	Ta.39538	Transcribed locus, weakly similar to NP_001067071.1 Os12g0569200 [Oryza sativa (japonica cultivar-group)],	NP_001067071.1 Os12g0569200 O. sativa 60.4 163 , hypothetical protein	AK251628.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf119f15, mRNA sequence 188 188 92% 6e-45 84%	Hv.8216	CDNA clone: FLbaf119f15, mRNA sequence	NP_001067071.1 Os12g0569200 O. sativa 59.8 163 ; hypothetical protein
K366	constitutive	constitutive, polmorphic NIL 1, NIL3, CM, DH1	251	ccaacactcattgagctcacagcgagtccgaggcaattagaccgttccagaggccaccaggcaaacaggtggggatgcgggccatgccgcacacgccgaacacggaataacgccatcggcggcattgctggcgacaaggccaccagccagcatgaaggcaaagcagagacagtcggcggcactctgcatccctggattcgagggcagcaacggttcttgtcggacgggcttgtccttcccggtttgaagt	CN011790.1 WHE3888_G10_M20ZS Wheat Fusarium graminearum infected spike cDNA library Triticum aestivum cDNA clone WHE3888_G10_M20, mRNA sequence 408 408 100% 5e-111 97%	Ta.30925	Transcribed locus		no real hit			
K451	constitutive	constitutive, polmorphic NIL 1, NIL2, CM, DH1 FHB1	232	tagaagcgctaaatgaaggtaaggcatgctacatcatgcggctagttgcgtcgatgaagggaaactatcctcactcctcagagaagcagcttgggtgagtgagaaagagagtcagacatcggcgaagatatggtatgtcttcgagtcgcgggcgatgagcctgagcgcggcgatggcggcggtgatggagagaacggtgaaaaagacgaatgttgacccagtgcactgactt	CJ595209.1 CJ595209 Y.Ogihara unpublished cDNA library Wh_SHDr Triticum aestivum cDNA clone rwhsd3j23 3', mRNA sequence 381 381 99% 6e-103 97%	Ta.53888	Transcribed locus, moderately similar to NP_001058673.1 Os07g0100800 [Oryza sativa (japonica cultivar-group)]	NP_001058673.1 Os07g0100800 O. sativa 77.8 404 , NP_974444.1 ProT2 (PROLINE TRANSPORTER 2) A. thaliana 68.4 382	AB073084.1 Hordeum vulgare HvProT mRNA for proline transporter, complete cds 215 215 99% 5e-53 80%	Hv.20641	HvProT mRNA for proline transporter	NP_001058673.1 Os07g0100800 O. sativa 80.7 424 , NP_001078025.1 ProT1 (PROLINE TRANSPORTER 1) A. thaliana 68.3 428

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
				Constitutive - genotype specific								
K1108	constitutive	only in CM	185	ctccatactaatgtgtgctgaccaataacatgtttccgcaatcaacaaccgaattgaacaatgttgctccaacagtgcattcaacattcaggcatgtcgcggcccatatatcatgaagtaaacctgttccgaactatctcatagataacccgagggacgctcttggacgattcatcttcaggtaa	CJ603889.1 CJ603889 Y.Ogihara unpublished cDNA library Wh_V483 Triticum aestivum cDNA clone rwhv3n17n22 3', mRNA sequence 325 325 100% 3e-86 98%	Ta.48338	Transcribed locus, moderately similar to NP_001067588.1 Os11g0242100 [Oryza sativa (japonica cultivar-group)]	NP_001067588.1 Os11g0242100 O. sativa 86.7 944 , NP_199801.2 catalytic A. thaliana 66.3 939 , hypothetical proteine; Trehalase; Trehalase	weak hit,NM_001074120.1 Oryza sativa (japonica cultivar-group) Os11g0242100 (Os11g0242100) mRNA, complete cds 71.6 71.6 45% 1e-09 78%	Os.87864	NP_001067588.1 Os11g0242100 O. sativa 100.0 949 , NP_199801.2 catalytic A. thaliana 67.5 939	trehalase
K936	constitutive	only in CM	139	ggtcgaacattccggggcgggctgtgttgacggggccgacggtggcctgcttgtagaggctgtagaggcagagcatgctctcgttggtggtggtgtcgggcagcgtcttggccttctccgcgtactcctcaaactcctc	15598.1 PT0133c.A04.BR PT013 Puccinia triticina cDNA clone PT0133c.A04.BR 5'-end, mRNA sequence 251 251 100% 4e-64 100% CJ917868.1 CJ917868 Y. Ogihara unpublished cDNA library, whthkles Triticum aestivum cDNA clone whthkles3j22 3', mRNA sequence 251 251 100% 4e-64 100%	Ta.54135	Transcribed locus, moderately similar to NP_001056611.1 Os06g0115300 [Oryza sativa (japonica cultivar-group)]	NP_001056611.1 Os06g0115300 O. sativa 81.3 90 , NP_174462.1 ACBP (ACYL-COA-BINDING PROTEIN); acyl-CoA binding A. thaliana 69.6 91	AK251185.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf114i23, mRNA sequence 210 210 100% 1e-51 93%	Hv.4526	CDNA clone: FLbaf114i23, mRNA sequence	NP_001056611.1 Os06g0115300 O. sativa 79.1 90 , NP_174462.1 ACBP (ACYL-COA-BINDING PROTEIN); acyl-CoA binding A. thaliana 68.5 91 , Acyl-CoA-binding protein
K348	constitutive	only in CM	141	aagggtatagaattcttggaaaccttcaagtcatcgaagaatgcacagttcagagatgggaaacataacgggcaatgactgaaaatatactactgtgaataagcgtggggttgtacaattgcgcaaaccagttatgcggc	GH729995.1 G750.110M23R010802 G750 Triticum aestivum cDNA clone G750110M23, mRNA sequence 253 253 99% 1e-64 100% CV065300.1 WNEL20g5 Wheat EST endosperm library Triticum aestivum cDNA clone WNEL20g5 5' similar to Oryza sativa, mRNA sequence 253 253 99% 1e-64 100%	Ta.31181	Transcribed locus, strongly similar to NP_001052278.1 Os04g0224900 [Oryza sativa (japonica cultivar-group)]	NP_001052278.1 Os04g0224900 O. sativa 96.3 377 ; NP_187648.1 glycerol-3-phosphate dehydrogenase, putative A. thaliana 82.9 397 , GlpA; Glycerol-3-phosphate dehydrogenase	AK104305.1 Oryza sativa Japonica Group cDNA clone:001-012-D11, full insert sequence 129 129 85% 3e-27 85%	Os.21792	Os04g0224900,	NP_001052278.1 Os04g0224900 O. sativa 100.0 377 , NP_187648.1 glycerol-3-phosphate dehydrogenase, putative A. thaliana 84.3 375
K617	constitutive	CM and DH1	286	acccagattcagacgacagttacggcattgacctggacatgacgctgcctcaggcgttgaagaaatggcaggcctttgaggacagcagccatttcgacggcgcgcacctcttcgttcctcgcgagcctggataacacccagaccagcatcccgacgcggcctccagggtttgctgattccttcacctccgccgacggaagattaaccgcgctcgaaagccagaagcaagatgatcattctgttcattcattcctttagcctcttcacccccctccctccctgcctt	CJ932435.1 CJ932435 Y. Ogihara unpublished cDNA library, whchan Triticum aestivum cDNA clone whchan11f19 3', mRNA sequence 509 509 100% 2e-141 99%	Ta.29629	Transcribed locus, moderately similar to NP_001051079.1 Os03g0717000 [Oryza sativa (japonica cultivar-group)]	NP_001051079.1 Os03g0717000 O. sativa 84.2 848 , NP_178291.1 leucine-rich repeat protein kinase, putative A. thaliana 63.9 844 , S_TKc; Serine/Threonine protein kinases	AC091532.14 Oryza sativa chromosome 3 BAC OSJNBa0078A17 genomic sequence, complete sequence 219 219 69% 5e-54 84%	Os.4149	Os03g0717000	NP_001051079.1 Os03g0717000 O. sativa 100.0 961 , NP_176789.1 TMK1 (TRANSMEMBRANE KINASE 1) A. thaliana 69.8 914 , _TKc; Serine/Threonine protein kinases
K651 aligns with K486, dif	constitutive	CM and DH1	174	gtgattgaggcaaagtctgctttggaggcaatgaccataaaaaggatggagcggttactgcttgctcatttattgactacttccttcgcaacttcaatcatcatgacgcaccctccatgctcgcattcacccgcacgaccgactccatcccgagcacagctaaaggagcgagtg	CJ955294.1 CJ955294 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul7g05 5', mRNA sequence 315 315 100% 5e-83 100%	Ta.47374	Transcribed locus, weakly similar to NP_001105048.1 D-type cyclin [Zea mays]	NP_001105048.1 D-type cyclin Z. mays 45.3 232, CYCLIN; Cyclin box fold	AF512432.1 Triticum aestivum cyclin D2 (CycD2) mRNA, complete cds 176 176 78% 3e-41 88%	Ta.21873	Cyclin D2 (CycD2)	CYCLIN; Cyclin box fold, NP_001105048.1 D-type cyclin Z. mays 76.8 357, NP_201345.1 CYCD4;1 (CYCLIN D4;1); cyclin-dependent protein, kinase regulator A. thaliana 50.0 282 ,

Table II Summary of candidate genes

TDF no.	time of induction	banding pattern	Size in bp	Sequence	BlastN_est_others best hit	uni gene	unigene info	reference protein	BlastN_nr best hit	uni gene	unigene info	reference protein
K536	constitutive	CM and DH1	96	tcatattgatgaaaggaacatgtgctgtggaactccaccagattgtgagtggaaagggcaagctggcaatatctgtgtggcatcctttgattggag	CJ964615.1 CJ964615 Y. Ogihara unpublished cDNA library, whchul Triticum aestivum cDNA clone whchul36c23 3', mRNA sequence 174 174 100% 5e-41 100% CA736666.1 wpi1s.pk008.m3 wpi1s Triticum aestivum cDNA clone wpi1s.pk008.m3 5' end, mRNA sequence 174 174 100% 5e-41 100%	Ta.29456	Transcribed locus, strongly similar to NP_001047174.1 Os02g0566800 [Oryza sativa (japonica cultivar-group)]	NP_001047174.1 Os02g0566800 O. sativa 93.3 387 , NP_974164.2 galactosyltransferase family protein A. thaliana 80.8 373 , Galactosyl_T; Galactosyltransferase	EU966359.1 Zea mays clone 293872 avr9 elicitor response protein mRNA, complete cds 147 147 100% 7e-33 93%	Zm.10824	Hypothetical protein LOC100192632 (LOC100192632), mRNA	NP_001131318.1 hypothetical protein LOC100192632 Z. mays 100.0 397 , NP_974164.2 galactosyltransferase family protein A. thaliana 83.4 373
K561	constitutive	CM and DH1	268	ttgatgtgttggatcacgtttggtttcatggcggagtcatggaaaaaggggactgattcggaattagggaggtacatataagaggaggaactcgttctgcttctttttttggcaagttgccacagatgcatggaaggacagcaacatgtgtgtgtcgttggtagtatgatgatgtattttggtggcaattggatggacagccaaactgaactcgtctatatcggcgtatgtctatttgctactgttgtcgttgacttgcattatgtatga	CD927480.1 GR45.102E05F010315 GR45 Triticum aestivum cDNA clone GR45102E05, mRNA sequence 430 430 100% 1e-117 96%	Ta.35185	Transcribed locus, weakly similar to NP_001055282.1 Os05g0353400 [Oryza sativa (japonica cultivar-group)]	NP_001055282.1 Os05g0353400 O. sativa 45.3 139 , KAP95; Karyopherin (importin) beta [Intracellular trafficking and secretion] hypothetical protein	**no real hit**			
K487	constitutive	in all except Remus	126	taactgggagacggcctggttcggcatgtttgatgcacccatcagacccatgacagtccacaggagatccgcggatgagctgtacgctacgctggtgggagtgtagctctttcccgttgtagtcac	CJ569550.1 CJ569550 Y.Ogihara unpublished cDNA library Wh_MS Triticum aestivum cDNA clone rwhms5b01 3', mRNA sequence 132 132 100% 2e-28 82%	Ta.4590	Transcribed locus, moderately similar to NP_001051253.1 Os03g0746800 [Oryza sativa (japonica cultivar-group)]	NP_001051253.1 Os03g0746800 O. sativa 82.6 385 , NP_563675.1 transducin family protein / WD-40 repeat family protein A. thaliana 52.7 376 , WD40; WD40 domain, hypothetical protein	EU965388.1 Zea mays clone 285705 WD-repeat protein 4 mRNA, complete cds 89.7 89.7 96% 2e-15 75%	Zm.5357	Clone 285705 unknown mRNA	NP_001051253.1 Os03g0746800 O. sativa 83.0 434 NP_563675.1 transducin family protein / WD-40 repeat family protein A. thaliana 56.9 422
K673	constitutive	all except Remus	154	gtctagccacgcaacttcagggatgaagtcagataggtttggcaatccgacggtcacgtttagctctgcgagcatggccttgaagtccgaaggcttgacatggcggtcatcgaggacggccaactcagttgagaagatggtggaggagagcagg	FF352940.1 BG02026B1F04.r1 BG02 - primary and normalized libraries Pseudoroegneria spicata cDNA clone BG02026B1F04.r1, mRNA sequence 237 237 100% 9e-60 94%	no			AK250090.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf61j11, mRNA sequence 210 210 100% 1e-51 90%	Hv.15358	CDNA clone: FLbaf61j11, mRNA sequence	NP_001049554.1 Os03g0248200 O. sativa 67.7 494 , NP_182079.1 CYP76C4 (cytochrome P450, family 76, subfamily C, polypeptide 4); oxygen binding A. thaliana 47.7 493
K381	constitutive	DH2 and Remus	60	gacaacacaaagccaggactagaaactcttcgtcgtcgtcatcatcagcggcagttcc	CJ937301.1 CJ937301 Y. Ogihara unpublished cDNA library, whchan Triticum aestivum cDNA clone whchan26e15 3', mRNA sequence 111 111 100% 2e-22 100%	Ta.63412	Transcribed locus		EU569763.1 Triticum aestivum clone TaAM SSR marker 89.7 89.7 93% 8e-16 93%	Ta.55306	Clone TaAM SSR marker	NP_001063967.1 Os09g0568000 O. sativa 83.5 138
K989	constitutive	constitutive only in Remus	125	accaggaggtctaaggttctgccggccaggatcaacgcgacgacagctagggcaactgcgactccgatgaagatgtactgccgcgttctcctttgaacttgttacgcaggggtgcgggcatggg	Accession Description Max score Total score Query coverage E value Max ident Links CJ940087.1 CJ940087 Y. Ogihara unpublished cDNA library, whchan Triticum aestivum cDNA clone whchan34i08 3', mRNA sequence 219 219 100% 2e-54 99%	Ta.10027	Transcribed locus, weakly similar to NP_001062226.1 Os08g0513600 [Oryza sativa (japonica cultivar-group)]	NP_001062226.1 Os08g0513600 O. sativa 58.2 606 , PRK03918; chromosome segregation protein	EU973253.1 Zea mays clone 395563 hypothetical protein mRNA, complete cds 59.0 59.0 84% 4e-06 72%	Zm.10282	Clone 293761 mRNA sequence	NP_001062226.1 Os08g0513600 O. sativa 55.5 422 , PRK03918; chromosome segregation protein
K990	constitutive	constitutive only in Remus	95	acccgggggatcatcattattcagtcgtcggtcagaagaaattgacctgccggatgggcgccggcgccagcgaccgcttctccatgaggttagg	CJ818518.1 CJ818518 Y. Ogihara unpublished cDNA library, whsct Triticum aestivum cDNA clone whsct34j03 3', mRNA sequence 172 172 100% 2e-40 100%	Ta.6845	Transcribed locus, moderately similar to NP_001051886.1 Os03g0847600 [Oryza sativa (japonica cultivar-group)]	NP_001051886.1 Os03g0847600 O. sativa 85.4 416 , NP_001118974.1 MHK A. thaliana 66.3 380 , S_TKc; Serine/Threonine protein kinases, catalytic domain. Phosphotransferases of the serine or threonine-specific kinase subfamily	AK249307.1 Hordeum vulgare subsp. vulgare cDNA clone: FLbaf30m14, mRNA sequence 102 102 91% 3e-19 86%	Hv.18405	CDNA clone: FLbaf30m14, mRNA sequence	NP_001048331.1 Os02g0785900 O. sativa 85.1 206 , NP_193884.3 nucleotide binding A. thaliana 79.3 82,WD40; WD40 domain

ZUSAMMENFASSUNG

In diesem Projekt wurde die Fragestellung behandelt warum Weizenlinien anfällig gegen das Pilzpathogen *Fusarium graminearum* sind, und welche Gene zur Resistenz gegen *Fusarium graminearum* beitragen.

Ziel dieser Diplomarbeit war es Genkandidaten zu identifizieren, die zwischen anfälligen und resistenten Weizenlinien unterschiedlich exprimiert werden.

Das Pilzpathogen *Fusarium graminearum* befällt die Blühorgane des Weizens während diese für die Bestäubung geöffnet sind. *Fusarium graminearum* verursacht in anfälligen Linien *Fusarium* Head Blight (FHB) — Ährenfusariose. Befallene Blütchen zeigen zu Beginn der Infektion wässrige dunkelbraune Lesionen und mit fortschreitendem Befall breiten sich die Symptome auf die ganze Ähre aus und führen zu Bleiche und Frühreife der Ähren mit geschrumpften und tauben Körnern. Zusätzlich produziert *Fusarium graminearum* das Mycotoxin Deoxynivalenol (DON), das zur Mycotoxinbelastung des Erntegutes führt und daher eine ernstzunehmende Bedrohung für die globale Lebensmittelsicherheit darstellt.

Zahlreiche quantitative trait loci (QTL) mit stärkerem und schwächerem Einfluß auf die Resistenz gegen Ährenfusariose wurden mittels QTL-Kartierung in den letzten zwanzig Jahren identifiziert. In der Kartierungspopulation CM82036/Remus wurden zwei QTL mit bedeutendem Einfluss auf die Resistenz gefunden. Während *Qfhs.ifa-5A* auf Chromosome 5A mit Resistenz gegen die Primär-Infektion in Verbindung steht, hat QTL *Fhb1* auf Chromosome 3BS Einfluss auf die Ausbreitung des Pathogens vom primär infizierten Blütchen auf die ganze Ähre. Zusätzlich hat QTL *Fhb1* Einfluß auf die Fähigkeit der Pflanzen das Pilzgift zu detoxifizieren.

In diesem Projekt wurden die Effekte der beiden QTL auf Genexpression-Level im frühen Stadium der Pathogen-Weizen Interaktion untersucht, um mögliche Kandidatengene für QTL *Qfhs.ifa-5A* und *Fhb1* zu identifizieren. Der anfällige und der resistente Elternteil, sowie zwei engverwandte Geschwisterlinien aus der Kartierungspopulation CM82036/Remus mit überdurchschnittlich großer genetischer Ähnlichkeit wurden einer Punktinokulation mit *Fusarium graminearum*-Macrokonidien und Wasser (Positivkontrolle) unterzogen. Unterschiede in Genexpression zwischen den Inokulationsvarianten und zwischen den Genotypen wurden in drei Zeitpunkten über den Zeitraum von 72 Stunden nach Inokulation mittels cDNA-AFLP fingerprinting analysiert.

CM82036 und Schwesterlinie DH1 representieren den resistenten Phänotyp und besitzen die resistenten Allele in beiden QTL auf Chromosome 5A und 3BS. Remus und Schwesterlinie DH2 representieren den anfälligen Phänotyp und besitzen die anfälligen Allele in beiden QTL. Um die Effekte der beiden Resistenz-QTL im anfälligen Genotyp von Remus zu testen, wurden nah-isogene Linien (near isogenic lines – NILs) entwickelt und in das Genexpressions-Experiment einbezogen.

Transcript derived fragments (TDFs), welche nach *Fusarium graminearum* Inokulation unterschiedlich exprimiert wurden und Unterschiede zwischen den Genotypen zeigten, wurden geklont und sequenziert. Unter den siebzig Gen-Kandidaten mit annotierter putativer Genfunktion befanden sich Rezeptoren, Protein-Kinasen und Transkriptionsfaktoren involviert in Erkennung und Signal-Transduktion. Weiters wurden Schlüsselproteine der Pathogenesis wie zum Beispiel PR-1 Protein und PR-5 Protein sowie Schlüsselenzyme im Sekundärmetabolismus identifiziert.

Genkandidaten aus diesem c-DNA-AFLP Experiment bilden die Basis für weitere Experimente in Functional Genomics und Feinkartierungs-Projekten.