Insight into TaWRKYs transcription factors role during wheat growth and Septoria defense.

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Abstract

Triticum aestivum is the major food source in many parts of the world, providing approximately 20% of calories consumed by humans. The pathogen *Zymoseptoria tritici* that causes *Septoria Tritici Blotch* (STB), is currently the main threat to wheat production, with an average yield loss of 20%. Therefore, understanding the molecular mechanisms that underpin the Septoria-wheat interaction will be crucial for generating new control strategies against STB. WRKY transcription factors are important components of signaling in plants, regulating many molecular mechanisms in response to abiotic and biotic stresses. Published data demonstrate that there are at least 3 wheat WRKYs (TaWRKY) that show altered expression upon Septoria infection. The hormone jasmonic acid (JA) plays key role in biotic stress response, but also in a diverse array of plant processes including development, reproduction, and response to abiotic stress. Most of our understanding of the JA signaling pathway derives from the dicot model plant Arabidopsis thaliana, while corresponding knowledge in wheat is somewhat limited.

Via bioinformatics analysis we identified *TaWRKY10* and *TaWRKY13* genes in wheat and validated the role of two of them during Septoria infection response. Moreover we have been able to demonstrate that *TaWRKY10* is a key component of the JA signalling pathway. We specifically identified its role and downstream targets, as well as 6 putative regulators of its transcription. *TaWRKY10* acting at the JA perception level couples growth and immunity. As growth and immunity are inversely correlated, investigating the molecular basis of their correlation could lead to the discovery of novel breeding tools.

Introduction

World population is rapidly growing, consequently the food demand worldwide is dramatically increasing. The Food and Agriculture organization of the United Nations (FAO) estimates that 70% increment in food production is needed by 2050. This is the new challenge for plant scientists in this era.

This goal can be achieved, from a crop production perspective, either by increasing yield or preventing yield losses. Therefore studying crops, and crop pathology, is gaining importance. In this work we attempted to gain a deeper understanding of hormonal and molecular wheat responses to the pathogen *Zymoseptoria tritici*.

Triticum aestivum (bread wheat) is one of the major food sources in many parts of the world, providing approximately 20% of calories consumed by humans. Bread wheat has a hexaploid genome (AABBDD), which originates from inter species hybridization (Dvorak and Akhunov 2005; Chalupska *et al*., 2008).

In 2013/14 world wheat production was expected to be around 708.5 million tonnes and the average amount of wheat consumed per capita is predicted to be 66.9kg (FAO).

This represents the largest proportion of cereal crops farmed in the world (total predicted cereal production is 2,497.6 million tonnes and forecasted to be 152.4kg per capita consumed). After maize, wheat has the second highest usage for animal feed in 2009, therefore wheat has a double role in food production.

Although the crop is most successful between the latitudes of 30° and 60°N and 27° and 40°S (Nuttonson, 1955), wheat can be grown beyond these limits, from within the Arctic Circle to higher elevations near the equator. Development research by the International Maize and Wheat Improvement Center (CIMMYT) has shown that wheat production in much warmer areas is technologically feasible (Saunders and Hettel, 1994). The crop is grown from sea level to more than 3 000 m in altitude, and it has been reported at 4 570 m in Tibet (Percival, 1921).

The optimum growing temperature is about 25°C, with minimum and maximum growth temperatures of 3° to 4°C and 30° to 32°C, respectively (Briggle, 1980). Wheat is adapted to a broad range of moisture conditions from low to well watered. Although about three-fourths of the land area where wheat is grown receives an average of between 375 and 875 mm of annual precipitation, it can be grown in most locations where precipitation ranges from 250 to 1 750 mm (Leonard and Martin, 1963). Optimal production requires an adequate source of moisture during the growing season; however, too much precipitation can lead to yield losses from disease and root problems. Cultivars of widely differing pedigree are grown under varied conditions of soil and climate and show wide trait variations. Although wheat is being harvested somewhere in the world in any given month, harvest in the temperate zones occurs between April and September in the Northern Hemisphere and between October and January in the Southern Hemisphere (Percival, 1921).Classification into spring or winter wheat is common and traditionally refers to the season during which the crop is grown. For winter wheat, heading is delayed until the plant experiences a period of cold winter temperatures (0° to 5°C). It is planted in the autumn to germinate and develop into young plants that remain in the vegetative phase during the winter and resume growth in early spring. This provides the advantage of using autumn moisture for germination and making effective use of early spring sunshine, warmth and rainfall. Spring wheat, as the name implies, is usually planted in the spring and matures in late summer but can be sown in autumn in countries that experience mild winters, such as in South Asia, North Africa, the Middle East and the lower latitudes. For this study only winter wheat varieties were used. This is due to the fact that winter wheat is more threatened by *Zymoseptoria Tritici* (the model pathogen employed in the study) because of the optimal climate conditions for the pathogen spread. The varieties of choice are KWS *Lili* (*Lili*), as model more resistant cultivar, and KWS *Santiago* (*Santiago*) as more susceptible model cultivar. Eventhough both cultivars allow septoria to colonise and reproduce.

Zymoseptoria Tritici

Zymoseptoria Tritici (also known as *Mychosporella Graminicola*, *Septoria tritici* or commonly known as Septoria) is the causal pathogen of Septoria Tritici Blotch (STB), one of the most threatening wheat foliar diseases across temperate regions, causing up to 40% yield loss if untreated with fungicide (Orton *et al.,* 2011).

It is predicted that around 70% of Europe's fungicide volume is used to treat Septoria (Ponomarenko *et al*., 2011). Wheat is particularly vulnerable when sown upon straw debris and among early sown susceptible varieties. Septoria has become more damaging since the 1960's with the increase in more commercial, early maturing, semi dwarf and higher yielding wheat cultivars that are more susceptible to the fungus (Eyal *et al*., 1987).

At the beginning of the infection Septoria appears as irregular brown chlorotic spots, which expands as the infection progresses, developing into necrotic lesions on either side of the leaf. Developing with the lesions are the pycnidia, small brown raised fruiting bodies produced on the leaf, which go onto release the pycnidiospores (Eyal 1999; Orton *et al*., 2011). A sticky medium that contains sugars and proteins is produced with the spores to protect them from drying out. The fungus spreads its spores by either wind or within rain splashes (Kema *et al*., 1996) infecting the bottom leaves of the wheat plant and potentially going onto infect upper leaves (Eyal 1999). When wheat crops are not present Septoria survives in plant debris as mycelium, in pycnidia and mainly as psuedothecia, which are activated to release the spores when the growth conditions are optimal (Ponomarenko *et al*., 2011). It infects in temperate regions with high rainfall, ideally needing moisture for >24 hours and a temperature of 10-20^{O}C (McMullen and Adhlkarl 2009). 24-48 hours after the spores land on the leaf surface the Septoria hyphae infiltrate the leaf through the stomata, entering the apoplastic space, where it remains throughout its life cycle. During the early symptomless infection, first 12-18 days, the Septoria grows as a biotroph, using nutrients from the apoplastic space to grow its hyphae throughout the mesophyll tissue. It was suggested that the fungus relies more on the breakdown of proteins as opposed to carbohydrate degradation to avoid detection in the biotrophic phase (Goodwin *et al*., 2011). The next stage of growth is the necrotrophic phase where the host's mesophyll cells collapse and die releasing nutrients, leading to leaf surface lesions (Eyal *et al*., 1987; Palmer and Skinner 2002; Ponomarenko *et al*., 2011). The

fungal mycelium rapidly proliferate upon host cell death, with an increase in gene expression of proteins involved in energy production within the fungal cells occurring at the same time as the release of intracellular nutrients (Orton *et al*., 2011). During the necrotrophic feeding stage symptoms start to appear on foliar tissue, in the form of chlorotic lesions that display characteristics of programmed cell death (Keon *et al.,* 2007, Rudd *et al.,* 2008). This phase signals the start of reproductive stage in Septoria. In the reproductive stage, pycnidia (the reproductive organ of the fungus) filled with pycnidiospores, appear as black lesions on leave surfaces. The mature pycnidia release spores that allow Sepotria to colonise the plant canopy, ultimately affecting flag leaf productivity by affecting its photosyntethic potential which ultimately impacts grain yield. (Ponomarenko *et al.,* 2011).

The length of the biotrophic phase could vary depending on Septoria strain, the host variety, environment conditions. But still the key factors (molecular, genetical or environmental) that drive the life cycle shift are not yet identified.

Current methods of controlling the infection include the use of chemical fungicides and resistance varieties (Griton *et al* 2011), nevertheless Septoria has become resistant to commercial fungicides. Maybe this is due to Septoria's very high genome plasticity, in fact the fungus could go through two different types of reproduction: sexual (ascospore) and asexual (conidia) (Orton *et al*., 2011; Ponomarenko *et al*., 2011).

Wheat varieties have been bred to be resistant to different pathogens but these varieties do not yet have resistance to all the economically important diseases so the use of fungicides is still needed to prevent infection (Fraaije *et al*., 2001).

General resistance to fungal pathogens occurs when pathogen-associated molecular patterns (PAMPs) such as eythlene-inducing xylnase (EIX) or chitins are recognised by plant recognition receptors (PRRs) on the cell surface (Ron and Avni 2004; Kaku *et al*., 2006). This recognition trigger a cascade of events that help arm the plant for defence and is known as PAMP-triggered immunity (PTI). Some pathogens have evolved resistance, via suppression or evasion, leading to successive rounds of evolution on both sides that result in specific defences to certain pathogens known as a gene-for-gene relationship (Flor 1942; Zipfel 2008).

Specific resistance to plant pathogens at a species level involves a gene-for-gene interaction (Flor 1942) where a pathogen avirulence protein (Avr) is recognized by a plant resistance protein (R). This leads to something known as an incompatible

infection, which is a defence response that tries to contain the fungus to a small area of the plant (Thomma *et al*., 2005). Susceptible plant species do not have an R gene that corresponds with the pathogen Avr gene and therefore cannot mount a defence, as it does not recognise the pathogen. There has been evidence of a gene-for-gene relationship between resistant varieties of wheat and Septoria. Brading *et al.,* 2002 studied the resistant variety *cv.* Flame and found an R gene, that they designated S*tb6*, recognised a single gene of the *M. graminicola* isolate IPO323. They also showed a strong likelihood that *cv.* Hereward also expresses the same R gene, *Stb6*, through studies of progeny of the two varieties crossed and then studying the susceptibility of the plants to Septoria infection.

Resistance to Septoria is one of the major targets in wheat breeding programmes. Currently 21 distinct genetic loci (*Stb* genes) as well as a large number of quantitative trait loci (QTL) that confer Septoria resistance have been identified in various wheat germplasm screening programs (Brown *et al*., 2015, Kettles and Kanyuka 2016, Saintenac *et al*., 2018). However, the molecular mechanisms underlying wheat resistance to STB is largely unknown.

Jasmonic acid

Jasmonic acid (JA) and its derivatives are fatty acid-derived hormones ubiquitously present in the plant kingdom (Creelman and Mullet, 1997; Wasternack, 2007).

They act as regulatory molecules in many developmental processes that include fertility, sex determination, root elongation and fruit ripening (Mandaokar *et al*., 2006: Yoshida *et al*., 2009). They are also signals activating plant defences against pathogens, herbivory, wounding and abiotic stress (Balbi and Devoto, 2008; Browse and Howe, 2008; Chico *et al*., 2008).

JA play a critical role when plants have to decide ‘to defend’ rather than ‘to grow’, transcriptionally reprogramming the cells to activate defence mechanisms and arrest the cell cycle and growth (Bodenhause *et al*., 2007; Pauwels *et al*., 2008; Zhang *et al*., 2008).

In comparison to Arabidopsis and rice, knowledge of JA biosynthesis and signaling in wheat is limited. JA biosynthesis begins in the chloroplasts where lipoxygenases

encoded by *LOX* genes oxygenate the phospholipids of linolenic acid. Linolenic acid is then liberated from membrane lipids and forms hydroperoxy octadecadienoic acid (HPODE). Under the action of an allene oxide synthase (*AOS*) and an allene oxide cyclase (*AOC*), respectively, encoded by *AOS* and *AOC* genes, HPODE is converted into 12-OPDA (Laudert *et al*., 1996, Stenzel *et al*., 2003) which is subsequently reduced to JAs via the catalysis of a peroxisome-localized enzyme, 12-oxo-phytodienoic acid reductase 3 (*OPR3*), followed by 3 cycles of β-oxidation in the peroxisome (Stintzi *et al*., 2000, Strassner *et al*., 2002).

Subsequently, JA-Ile, the JA bioactive form of JA, is formed through a conjugation of JA and isoleucine (Ile) under the action of the GH3 family amido synthetase Jasmonate resistant 1 (*JAR1*) (Staswick *et al*., 2001).

The core of JA perception and signaling is currently defined by the SCF^{COI1}/JAZ/MYC2 module.

COI1 is an F-box component of an SCF (SKIP–CULLIN–F-box) complex. These complexes are multiprotein E3 ubiquitin ligases catalysing the ubiquitination of proteins and their subsequent degradation. The F-box is the component conferring specificity for the substrate. *JAZ*s are the repressors of the transduction whereas *MYC2* is the transcriptional activator of JA response.

In the absence of JA JAZs perform its repression via binding and sequestering MYC2, therefore preventing MYC2 association with the promoters of its targets. In presence of JA, COI1 recognize the JAZ proteins, and targets them to 26S proteasome degradation (Thines *et al*., 2007, Chini *et al*., 2007). Once degraded, JAZ repressors release MYC2, a bHLH transcription factor that binds the G-box (CACGTG) or the T/Gbox (AACGTG) in the promoters of JA-regulated genes, triggering their activation (Yan et al., 2007).

In comparison to Arabidopsis and other monocots such as rice and maize, knowledge on JA signaling and biosynthesis in wheat is limited and fragmented. However, JA-dependent responses to diseases, biotic and abiotic stresses have been increasingly investigated in wheat during the last 2 decades. The PR genes *PR1.1* and *PR1.2* were highly induced in 3 weeks post-emergence wheat by JA application (Hongwuei et al., 2016), the same pattern of expression was detected on wheat infected with the fungal pathogens *Tilletia tritici* and *Tilletia laevis* (Lu et al., 2006).

Fusarium pseudograminearum infection, the causative agent of wheat crown rot

disease, leads to induction of 6 different PR genes (Desmond et al., 2006).

Similarly, using a transcriptome-based method, it was revealed that JA biosynthesis genes such as *LOX*, *AOS*, *AOC* and *OPR3* and JA signaling transduction genes, including *COI1*, *JAZ*, *MYC2*, were induced in a fusarium head blight resistant wheat variety (Xiao et al., 2013). These evidences strongly suggest the involvement of JA in wheat defense against pathogens.

Moreover functions which are unrelated to defense have been recently proposed for some genes involved in the JA signaling pathway. For example, overexpression of *TaAOC1* enhanced salinity tolerance in wheat via a JA pathway-dependent manner (Zhao et al., 2014).

Recently transcriptomic studies established that JA biosynthetic genes are upregulated upon Septoria infection (Rudd *et al*., 2015) Nevertheless the mechanism underpinning the signal perception and transduction remains yet unclear.

WRKY transcription factors

WRKYs are one of the largest families of plant specific transcriptional regulators, with 72 representatives in Arabidopsis, more than 100 members in rice and 45 in barley (Eulgem et al., 2007, Mangelsen et al., 2008, Rushton et al., 2010, Agarwal et al., 2011); in this work 135 WRKYs have been identified in wheat.

WRKYs can act as transcriptional activators or repressors, in various homo- and heterodimer combinations. WRKY transcription factors (TFs) are important components of a plant signaling web which regulate many plant processes such as biotic and abiotic stress response, but also in response to stimulus that triggers developmental processes, and include additional DNA-binding and non-DNA-binding proteins interactors (Ulker *et al*., 3004; Rushton *et al*., 2010; Agarwal *et al*., 2011; Bakshii and Oelmuller 2014).

The WRKY members are characterized by displaying one or two WRKY domains.

This domain is 60 amino acid long, and it contains a highly conserved heptapeptide motif WRKYGQK at the N-terminus and a novel zinc-finger-like motif at the C-terminus.

The WRKY domain consists of a 4-stranded β-sheet, with the zinc coordinating Cys/His residues forming a zinc-binding pocket. Both heptapeptide sequence and zinc-finger-like motif are required for the high binding affinity of WRKY TFs to the consensus *cis*-acting elements termed W box (TTGACT/C).

Even though the presence of a highly conserved W box in the DNA sequence, WRKYs binding affinity could varies. This is mainly due to different peculiarieties among zinc-finger-like motifs. A classification based on this feature has been made, dividing WRKYs transcription factors in 3 different groups (Eulgem et al., 2006).

Into group I we can find proteins with 2 WRKY domains, whereas protein with only 1 WRKY domain belongs to group II. Group I and group II share the same potential zinc ligand pattern: C–X4–5–C–X22–23–H–X1–H, denominated C_2-H_2 pattern.

Proteins with only 1 WRKY domain, but displaying different patterns of zinc finger motifs are classified into group III. Instead of a C_2-H_2 zinc finger motif, group III domain contains a C_2-HC pattern (C–X7–C–X23–H–X1–C).

The WRKYs can act up or downstream of hormones, are involved in the antagonistic functions of salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET), control developmental processes via auxins, cytokinins, and brassinosteroids (Guo *et al*., 2005; Nillson *et al*., 2010; Rushton *et al*., 2010; Antoni *et al*., 2011).

From a pathogen response point of view the main hormones involved are SA and JA, the first is associated with resistance to biotrophic pathogens, while the second is associated to the response against necrotrophic pathogens (Penninckx *et al*, 1997; Thomma *et al*., 2011). In this context the above-mentioned phytohormones have opposite effects on plant defence mechanisms that need to be integrated and coordinated by TFs activity, for example *AtWRKY70* act as a repressor of JA-associated genes transcription and simultaneously as activator of SA-elicited genes (Li *et al*., 2004).

Rapid pathogen-induced *WRKY33* expression does not require SA signaling, but is dependent on *PAD4*, a key regulator upstream of SA (Lippock et al., 2007). WRKY33 is activated in the case of *Botrytis cinerea* infection, a necrotrophic pathogen, and its essential to coordinate SA and JA equilibrium during the infection (Birkenbihl *et al*., 2012). *wrky33* loss of function mutant show abnormal activation of SA pathway and downregulation of JA associated

response during *Botrytis cinerea* infection, increasing the plant susceptibility to the necrotrophic pathogen. This effect is due to over-activation of JA-ZIM domain repressor proteins (JAZ) (Birkenbihl *et al*., 2012).

The expression profile of *VvWRKY11* from grapevine in response to treatment with SA or the pathogen *Plasmopara viticola* is rapid and transient (Liu *et al*., 2011). Transgenic *Arabidopsis* overexpressing *VvWRKY11* showed higher resistance to drought stress compared to their controls. These results demonstrated that *VvWRKY11* is involved in the response to dehydration stress, as well as pathogen response. This case demonstrates again how WRKYs have an important role in coordinating plant response to external stimuli.

It has been demonstrated that double knock-out mutation of *AtWRKY18* and *AtWRKY40* increase *Arabidopsis thaliana* resistance to the biotrophic powdery mildew fungus *Golovinomyces orontii* (Schon *et al*., 2013). Resistance in *wrky18 wrky40* double mutant plants is accompanied by massive transcriptional reprogramming, imbalance in SA and JA signaling, altered *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) expression, and accumulation of the phytoalexin camalexin. Genetic analyses identified SA biosynthesis and *EDS1* signaling as well as biosynthesis of the indole-glucosinolate 4MI3G as essential components required for increased resistance toward *G. orontii* in the above mentioned mutant background. The analysis of *wrky18 wrky40 pad3* mutant plants impaired in camalexin biosynthesis revealed an uncoupling of pre – from post-invasive resistance against *G. orontii*. Interestingly, WRKY18 and WRKY40 act as positive regulators in effector-triggered immunity, as the *wrky18 wrky40* double mutant was found to be strongly susceptible toward the bacterial pathogen *P. syringae* DC3000 expressing the effector AvrRPS4 but not against other tested Pseudomonas strains. It appears that *G. orontii* depends on the function of WRKY18 and WRKY40 to successfully infect *Arabidopsis* wild-type plants while, in the interaction with *P. syringae* AvrRPS4, they are required to mediate effector-triggered immunity (Schon *et al*., 2013).

Knock- down of the rice *OsWRKY45* severely reduces SA/benzothiadiazole (BTH)-induced resistance to the fungal pathogen *Magnaporthe oryzae* and the bacterial pathogen *Xanthomonas oryzae*. Conversely, overexpression of *WRKY45* induces extremely strong resistance to both of these pathogens (Nakayama *et al*., 2013). A detailed analysis of the transgenic rice plants uncovered a central role of WRKY45 in

BTH-induced disease resistance. Furthermore, the SA-activated WRKY45 protein induces the accumulation of its own mRNA (Nakayama *et al*., 2013).

Panicle blast 1 (*Pb1*) is a panicle blast resistance gene derived from the indica rice cultivar "Modan." *Pb1* encodes a coiled- coil-nucleotide-binding site-leucine-rich repeat (CC-NB-LRR) protein and confers durable, broad-spectrum resistance to *M. oryzae* races. The Pb1 protein interacts with WRKY45. Pb1- mediated panicle blast resistance is largely compromised when *WRKY45* was knocked down in a Pb1-containing rice cultivar. Leaf-blast resistance by Pb1 overexpression was also compromised in *WRKY45* knockdown/*Pb1* overexpressor rice. Blast infection induced higher accumulation of WRKY45 in the Pb1 overexpressor than in control Nipponbare rice.

The involvement of WRKYs in JA defense pathway is still poorly understood in crops. Overexpression of *VvWRKY1* in grapevines induces expression of JA pathway-related genes and confers higher tolerance to the downy mildew (Marchive et al., 2013). On the other hand, it has been reported that dehydration-induced *WRKY* genes from tobacco and soybean respond to JA treatments in cell culture (Rabara et al., 2013). To fully understand the role of WRKYs and their involvement in hormonal pathways in crops, more investigations are required, maybe amplifying the spectrum of pathogens and cultivars analyzed.

Published data demonstrate that there are at least 3 WRKYs that shows altered expression upon Septoria infection: 39, 53 and 68 (Lee *et al*., 2015) other microarray data collected from field samples strongly support the involvement of TaWRKY TFs in the wheat response to STB.

We have identified a susbset of these WRKYs transcription factors which are responsive to Septoria infection in wheat. We would like to determine whether any of these identified TaWRKYs affect wheat immunity against STB.

Virus-Induced Gene Silencing (VIGS)

Genetics and molecular biology studies on wheat are made harder due to the wheat's large hexaploid genome (16,000Mb) (Flavell *et al*., 1974). The hexaploid genome is made up from three genomes designated A, B and D that are all closely related. For nearly all wheat genes there are three or n*three copies. This causes a problem with functional redundancy as many of these homeologous genes are expressed, therefore a mutation must be made across each of the three genomes to allow the study of the gene function. (Mochida *et al*., 2003; Travella *et al*., 2006).

Reverse genetics is a very useful tool in molecular biology, it permits the study of protein function by modifying their coding gene expression or the gene itself. Those alteration in transcriptional profile leads to phenotypical changes, directly linked to the function of the target. Those results could be obtained by insertional mutations in the coding region of the gene (eg. T-DNA insertion), chemical mutagenesis (using ethyl nitrosurea or ethylmethane sulphonate) or posttranscriptional gene silencing (eg. Virus induced gene silencing).

Virus-induced gene silencing (VIGS) is a particularly useful tool for plant functional genomics and reverse genetics because it permits knockdown of genes of interest and observation of elicited phenotypes within 2 to 3 weeks. VIGS avoids production of knockout mutants or stable RNA interference (RNAi) and can also be performed on species that are difficult to transform (Robertson 2004; Scofield and Nelson 2009). The VIGS principle is based on antiviral responses that target RNAs for degradation and is triggered by accumulation of double-stranded RNAs (dsRNA) appearing in the infection cycle (Baulcombe 1999). By inserting sequence fragments derived from ‘‘genes-of-interest’’ into VIGS vectors, the corresponding mRNAs are selectively degraded during virus infection to result in transient silencing of the targeted gene.

The first VIGS vectors were derived from Tobacco mosaic virus (TMV) (Kumagai *et al*., 1995), Potato virus X (PVX) (Himber *et al*., 2003) and Tobacco rattle virus (TRV) (Ratcliff *et al*., 2001), and these vectors were initially used for *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) gene silencing. Recently the use of VIGS has extended to multiple plant species, and 30 viruses have shown potential application as VIGS vectors (Yuan *et al*., 2011). Examples include Apple latent spherical virus (ALSV) (Igarashi *et al*., 2009), Bean pod mottle virus (BPMV) (Zhang *et al*., 2010), Brome mosaic virus (BMV) (Ding et al., 2006), Pea early browning

virus (PEBV) (Constantin *et al*., 2004), Rice tungro bacilliform virus (Purkayastha *et al*., 2010).

Barley stripe mosaic virus (BSMV) vectors suitable for cereal VIGS have also been described (Holzberg *et al*., 2002; Tai *et al*., 2005). BSMV is a positive-sense RNA virus with a broad host range (Jackson et al., 2009). The tripartite genome consists of RNAs α, β and γ, and each of the genomic (g) RNAs has a methylated 59 cap and a 39 polyadenylate sequence followed by a tyrosine accepting tRNA-like structure. RNAα of the BSMV ND18 strain encodes the methyltransferase/ helicase subunit of the RNA-dependent RNA polymerase (RdRp). RNAβ specifies the coat protein (CP) and three major triple gene block (TGB) proteins (TGB1, TGB2 and TGB3) that are essential for cell-to-cell movement of the virus (Jackson et al., 2009). RNAγ encodes the polymerase (GDD) subunit of the RdRp and the γb protein, which has significant roles in viral pathogenesis, long distance movement and suppression of host RNA silencing defenses.

BSMV was first modified as a VIGS vector (Holzberg *et al*., 2002) for use in barley (*Hordeum vulgare*), and subsequently was used to down regulate expression of wheat (*Triticum aestivum*) genes (Scofield *et al*., 2005). Applications of BSMV-based VIGS include functional genomics research in wheat (Zhou *et al*., 2007; Campbell *et al*., 2010), barley (Hu *et al*., 2009; Meng *et al*., 2009), *Brachypodium distachyon* (Demircan *et al*., 2010), a model organism for cereals. In these studies, fragments from genes-of-interest were initially inserted into RNAγ either downstream or upstream of the γb gene and shown to determine various phenotypes, or to function in morphogenesis or disease responses (Cakir *et al*., 2010). For example, BSMV VIGS has been used to disrupt several wheat resistance pathways, including Lr1-, Lr10- and Lr21-mediated leaf rust resistance (Scofield *et al*., 2005), stripe rust resistance (Zhou *et al*., 2007), functional alleles in the Pm3 powdery mildew resistance locus (Buhllar *et al*., 2009), and Stpk-V, a key member of the Pm21 powdery mildew resistance gene complex (Cao *et al*., 2011). Barley studies have focused on powdery mildew Mla13-mediated resistance (Hein *et al*., 2005), stem rust Rpg5 R-gene regulation (Bruggerman *et al*., 2008) and the role of a susceptibility factor, *HvBI-1*, that modulates cell wall-associated defenses (Eichmann *et al*., 2010). In a novel approach that has been called ‘‘host-induced gene silencing’’ (HIGS), the possibility of down regulating pathogen genes with BSMV VIGS has been shown to reduce transcripts of a wheat powdery mildew (*Blumeria graminis* f. sp. tritici) or rust fungi (*Puccinia*

striiformis f. sp. tritici) gene and to result in effective interference with infection of wheat (Nowara *et al*., 2010; Yin *et al*., 2011). BSMV VIGS also has potential for determining aphid defence gene functions in wheat (van Eck *et al*., 2010). In this case *TaWKRY53* transcription factor and an inducible phenylalanine ammonia-lyase (PAL) were shown to have key roles in resistance responses to aphid (*Diuraphis noxia*) infestations. In addition BSMV- VIGS has been adapted for studies of morphogenesis and development in crop plants (Wang *et al*., 2011).

The first generation BSMV VIGS systems were under the control of the T7 promoter. Those viral vectors were requiring in-vitro RNA transcription to bulk them up prior plant inoculation. This limitation was resulting in a time consuming and expensive process because capped in vitro transcripts from the α, β and γ cDNA clones are all required for a successful infection (Cakir *et al*., 2010). The problem has been circumvented by engineering plasmids containing each of the cDNAs, and incorporating a double Cauliflower mosaic virus (CaMV) 35S promoter immediately upstream of the cDNAs and a Hepatitis delta virus (HDV) ribozyme immediately downstream of the cDNAs (Hu et al., 2009; Meng et al., 2009). Inoculation via biolistic introduction of these plasmids into barley leaves resulted in replication of BSMV VIGS products containing candidate genes predicted to affect powdery mildew resistance, and sap from the systemically infected leaves was suitable for secondary inoculations to other cereals. However, a limitation of this method is the potential instability of gene inserts that can occur during systemic invasion of the primary inoculated plants (Lawrence and Jackson 2001). To permit more effective use of BSMV VIGS for functional genomics experiments, it has been developed an approach with *Agrobacterium tumefaciens* strains harboring the BSMV α, β, and γ cDNAs in Ti plasmids for initiation of BSMV infections upon infiltration of N. benthamiana leaves. The Agrobacterium mediated BSMV VIGS vectors have been engineered by inserting BSMV cDNAs between the double 35S promoter and a ribozyme sequence (Rz) from Tobacco ringspot virus (TRSV) satellite RNA (Annamalai *et al*., 2005). In addition, it has been inserted a ligation independent cloning (LIC) site similar to that used for TRV VIGS (Dong *et al*., 2007) into BSMV to facilitate efficient cloning of desired gene fragments. Infiltrated N. benthamiana leaves accumulate high levels of BSMV and provided excellent sources for secondary infections to elicit VIGS in wheat, barley, and the model grass, B. distachyon (Vogel and Bragg 2009).

For this study we adopted the above mentioned technique, inoculating 10-15 days old wheat seedlings. In our conditions we are able to maintain a solid silencing throughout all the wheat growth stages, we have been able to asses silencing up to the flag leaf. The disadvantage of VIGS is that being transient, it gives rise to a mosaic silencing, different for every single cell.

The main aims of the PhD programme

WRKYs have been identified as major player in plants stress responses, in this work we will demonstrate how two of them are major player during wheat response to *Septoria tritici* infection. We divided our work in 3 main sections:

1. Use bioinformatic tools to identify the WRKYs gene family in wheat.
2. Identify WRKY transcription factors that are important in defense against the hemibiotrophic pathogen *Zymoseptoria tritici*.
3. Identify factors that act downstream and upstream of the defence related WRKY transcription factors.

Isolating novel components involved in wheat immune response

Chapter 1: WRKYs identificiation.

1.1 Wheat WRKY gene family identification

As a first step to discover the role of WRKYS in Septoria defence we decided to define the WRKY gene family in bread wheat.

DNA sequences corresponding to the conserved domain in the WRKY gene family have been used as query to interrogate the PLANT ENSEMBL genomic database for wheat. The decision of utilizing DNA sequences rather than protein sequences was made because to date the nucleotide information for wheat genome was more complete then the proteome data. A total of 135 individual DNA sequences was retrieved from the wheat PLANT ENSEMBL genomic database via this method. Not all of the DNA sequences were complete and fully annotated, some were partial, and others did not encode WRKYs after in silico translation. In order to refine the dataset obtained each sequence was translated in silico using the freely available on line protein translation software ExPASy translate (Gaisteger *et al*., 2003).

Each translated DNA sequence was further analysed to ensure that I isolated only targets with complete coding sequences, from START (ATG) to STOP(TAA), as well as all the conserved domains that define WRKYs transcription factors (Bahksi and Oelmuller, 2014)(WRKY domain and Zinc finger domain). This in-silico screen

defined a subset of sequences designated as *TaWRKYs* with 71 complete sequences (fig 1.1).

In fig 1.2 the conserved WRKY motifs in the sequences identified was created via GLAM2 (Bailey *et al*., 2009), which is an algorithm belonging to the MEME (Multiple EM for Motif Elicitation) Suite web server (Bailey *et al*., 2006).

MEME is a web-based tool that is freely available that enables the discovery of novel signals (motifs) in proteins or DNA sequences. The input data for MEME includes multiple sequences that share a conserved motif. MEME software permits the discovery of novel motifs via an algorithm that performs a local, gapless and multiple sequence alignment, searching for statistically significant similarities among the input sequences.

GLAM2 is an upgraded version of MEME that enables alignments with gaps, enhancing the discovery power (Bailey *et al*., 2009). In our study the software was used in a slightly different way to validate the presence of at least a WRKY domain and a Zinc-finger motif in our subset of 71 complete WRKY encoding DNA sequences.

As shown in fig 1.2 all the sequences that was used as input for the algorithm display both a WRKY domain (WRKYGQK) and one of the two possible Zinc-finger domains (C_2-H_2 or C_2-HC).

1.2 Prioritization of WRKYs Tfs

In order to prioritize candidates for further analysis a preliminary bioinformatics analysis to ascertain WRKYs that respond to environmental stress was performed.

This procedure was carried out in order as there was no transcriptomic analysis available at the start of the study for wheat WRKYs. Furthermore expression analysis via PCR on all the 71 complete sequences would be time and resource consuming.

Initially I interrogated published data in Arabidopsis and rice on WRKYs involved in biotic stress response that showed protein sequence homology to the 71 complete wheat WRKYs.

From published literature I identified 21 Arabidopsis WRKYs and 8 rice WRKYs genes with a well-established role in defense (Table 1.1). I used these sequences as a query to interrogate NCBI-BLAST (Altschul *et al*., 1990) database, to identify putative homologs in wheat. Thirty wheat DNA sequences recognised as encoding WRKYs Tfs were selected for *in planta* RNA expression analysis.
A phylogenetic tree representing the chosen WRKYs is displayed in fig 1.3. The publically available CLUSTAL-OMEGAsoftware (Sievers *et al*., 2011) was used to generate the tree.

1.3 Expression Analysis of wheat WRKY genes.

A gene expression evaluation was carried out to identify putative targets suitable for further investigation in any putative role in the defence response in wheat.
The screen was perfrmed by semi-quantitative RT-PCR. Gene specific primers for each WRKY displayed in fig 1.3 was designed and optimized for the best amplification efficiency, the transcript abundance of the targets was quantified via the software ImageJ evaluating relative band intensity against the housekeeping genes; *Elongation-factor-1* (*TaEDF1*). The RNA samples used for this analysis are from the wheat cultivar *Avalon*. This cultivar was chosen because it was extensively phenotyped in Septoria pathology studies previously in the laboratory (Lee *et al*., 2015; Millyard *et al*., 2016). Relative transcript abundance of our targets was compared between non-infected and infected leaf tissues, the template for the PCR was obtained by transcribing total RNA from *Avalon* leaves to cDNA. Only one critical time-point of Septoria infection was analyzed at this stage. The time-point chosen corresponded to 12 days post Septoria inoculation. This time point is exactly prior to Septoria switching to its necrotrophic life-cyle phase, or onset of necrotrophy (Palma-Guerrero et al., 2016). It has been previously demonstrated that perturbating specific gene expression can delay the appearance of necrotic symptoms on wheat leaf surface, resulting in altered pathogen sporulation capability (Derbyshire *et al*., 2015; Lee *et al*., 2015; Millyard *et al*., 2016). Therefore due to the importance of this

time point we chose it for transcriptional analysis of putative WRKY genes with a potential role in defence in our initial screen.

1.4 *TaWRKY10* transcription decreases during Septoria infection in cv. *Avalon*.

The first positive WRKY target with a transcriptional response to Septoria in wheat bearing homology to the 30 WRKYs published to have a role in defence in other plants was *TaWRKY10*.

Semi-quantitative RT-PCR was performed comparing gene expression at 12 days post infection with non-infected tissue. The PCR amplification was performed for 28 cycles. The housekeeping gene *TaEDF1* was used as loading control as previously described. Fig 1.4 displays the relative transcript abundance quantification. Fig 1.4a indicates the band intensity quantification, obtained via ImageJ software (Schneider *et al*., 2012). The intensity of the band corresponding to *TaWRKY10* transcript was normalized against *TaEDF1* band intensity, averaged between the two technical replicates and then plotted to compare the intensity of the bands corresponding to the infected tissue with the non-infected tissue. Fig 1.3b shows the electrophoresis gel corresponding to the above mentioned PCR. The PCR experiment was replicated with 3 different biological samples. At this stage of infection *TaWRKY10* transcript level is downregulated about 10 fold in infected tissue compared to non-infected tissue.

The downregulation of WRKY10 transcript level is appreciable not only from the analysis plot but as well from a clear difference in the band intensity reported in fig 1.4b. It can be speculated at this stage that *TaWRKY10* transcription could be actively downregulated by wheat during pathogen infection, therefore TaWRKY10 could play a role as a repressor of wheat immunity, or its downregulation could be linked to Septoria activity, where *TaWRKY10* could be a Septoria effector target. A deeper investigation into *TaWRKY10* activity is going to be presented in the next chapter.

TaWRKY10 at the protein sequence level is most similar to *AtWRKY50* or *AtWRKY51*. *AtWRKY50* and *AtWRKY51* are involved in Jasmonic acid and Salicylic acid related pathways in Arabidopsis (Gao *et al*., 2011). *TaWRKY10* belongs to WRKY sub-group 2, defined by a single WRKY domain and a C_2-H_2 Zinc finger domain.

1.5 *TaWRKY13* is upregulated upon Septoria infection in cv. *Avalon*

TaWRKY13 is the best blast hit in wheat proteome when I used *AtWRKY46* seqeunce as a query. *AtWRKY46* has been reported to be able to boost resistance against the pathogen *P. syringae pv. Tabaci* in *N. benthamiana* when transiently overexpressed, as well as being a target for MAPKs activity (Sheikh *et al*., 2016). *TaWRKY13* is also well similar to *AtWRKY70*, which is directly involved in the regulation of the cross-talk between Salycilic acid and Jasmonic acid (Sung Shim *et al*., 2013; Li *et al*., 2013).

The procedure to generate figures 1.4a and 1.4b are the same that was used to generate figure 1.3. The transcript level of *TaWRKY13* is 8-fold higher in infected tissue compared to non-infected tissue at 12 days post infection. This evidence suggested that *TaWRKY13* has a role in immunity against Septoria in wheat. *TaWRKY13* protein carries a single WRKY domain and a C_2-HC Zinc- finger domain, positioning it in WRKY group 3.

1.6 *TaWRKY13* shows expression variability among different elite varieties.

Since TaWRKY13 showed altered expression upon Septoria infection, therefore I wanted to ascertain if this could be a source of variation in defence responses in different elite cultivars displaying different degrees of susceptibility to Septoria in the fields. Eight different varieties with well established in-field defence phenotypes were chosen: KWS *Santiago*, KWS *Lili*, , *JB Diego*, *Gallant*, *Relay*, *Dunston* and *Sundance*. The phenotypic evaluations and relative disease scores from field trials corresponding to the varieties of choice is deposited in the AHDB recommended list for cereals and oilseed 2017/18.

The expression analysis was carried out under controlled conditions, using mRNA from 2-weeks old seedlings as template for qRT-PCR. As shown in fig 1.6 *Lili* and *Relay* are showing the highest gene expression, whereas *Santiago* and *Sundance* the lowest. The fold difference of gene expression between the varieties showing the

greatest changes in WRKY13 gene expression is 3.1-fold. Interestingly TaWRKY13 gene expression correlated with resistance against Septoria.

1.7 *TaWRKY13* expression increase upon Septoria infection in elite cultivars.

In order to have a complete picture of *TaWRKY13* expression profile during Septoria infection a time-course experiment was carried out.

Twelve different time points of infection was sampled to compare infected to non-infected tissue, harvesting samples every 2 days after Septoria strain IPO323 (Arraiano and Brown, 2006) infection, as performed by Lee *et al*., 2015. Total RNA was extracted from the tissue, reverse-transcribed to cDNA and then analysed via qRT-PCR. For this experiment 2 different cultivars were chosen: KWS *Lili* (*Lili*) and KWS *Santiago* (*Santiago*). Both varieties display high yield performance in field conditions, but *Santiago* is particularly susceptible to Septoria infection, *Lili* instead display a higher degree of resistance (AHDB recommended list for cereals and oilseed 2014/15, and recently AHDB recommended list for cereals and oilseed 2017/18).

Fig 1.7 shows the expression profile of *TaWRKY13* in *Lili*.

No difference of transcription can be noticed till 12 days post infection between infected tissue and the non-infected control, whereas a statistically significant 3 fold-change upregulation appears at 14 days post infection for the infected samples. The upregulation reaches its peak at 16 days post infection, displaying a 4 fold-change increase. At 18 days post infection the transcript level decreases to a 2.5 fold-change upregulation compared to its own control and then returning to control levels at 20 days post infection. The expression of *TaWRKY13* shows no change throughout the time course in the non-infected control.

The expression time-course of cultivar *Santiago* is shown in fig 1.8.

The transcript level of WRKY13 does not change throughout the time-course in the non-infected controls. Upon Septoria infection *TaWRKY13* expression level begin to increase significantly at 10 days post infection to 2 fold-change higher compared to

the control. The 12 days post infection time is to the peak of *TaWRKY13* gene expression upregulation, with 4 fold-change increase. At 14 days post infection the transcript level starts to decrease, showing a 3 fold-change upregulation compared to its control, only 2 fold-change increment at 16 days post infection and finally back down to control levels at 18 days post infection. At 20 days post infection the infected tissue displays an even lower *TaWRKY13* transcript level compare to the control, but the expression went back up to control levels at 22 days post infection.

1.8 *TaWRKY13* silencing leads to increase susceptibility to Septoria

The upregulation of *TaWRKY13* transcription upon Septoria infection led us to speculate that it could be involved in wheat immunity response.

In order to test this hypothesis post-transcriptional gene silencing (PTSG) technology using a virus based vector in wheat was employed (Baulcombe, 1999, refs), and subsequently the silenced plants were phenotyped for septoria infection.

Virus Induced Gene Silencing (VIGS)(Watson *et al*., 2005) was employed to silence *TaWRKY13*. Barley Stripe Mosaic Virus (BSMV) has previously been modified to effectively silence many wheat genes (Matthew, 2004; Watson *et al*., 2005). Gene silencing vectors based on BSMV (Ratcliff *et al*., 2001; Burch-Smith et al., 2004; Wang & Metzlaff, 2005; Lee *et al*., 2012), carrying the 5'-UTR and 3'-UTR of *TaWRKY13* mRNA, were used to induce sequence-specific degradation of the endogenous *TaWRKY10* mRNA and therefore a knock down in its gene expression in the wheat varieties *Lili* and *Santiago*. BLAST analyses confirmed that both DNA fragments (*BSMV:TaWRKY13_*1 and *BSMV:TaWRKY13_2*) were unique to *TaWRKY13*. The siRNA finder software si-fi predicted *TaWRKY13* specific silencing (Millyard *et al*., 2015, Lee *et al*., 2014). The effectiveness of gene silencing was confirmed by qRT-PCR on mRNA from emerging leaves of plants 14 days after *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* inoculation (Fig1.9). This data indicated that *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* yielded 60% and 65% silencing of *TaWRKY13 gene expression* in *Lilli* and 66% and 68% silencing in *Santiago* respectively compared to empty vector controls .

Fourteen days after BSMV inoculation the emerging 3rd-5th leaves were infected with a Septoria spores solution (7.5 x 10^6 spores per ml), and necrotic symptoms appearance was recorded every day throughout the infection cycle with white light pictures. At the end of Septoria life cycle picnidia production and sporulation was quantified.

Fig1.10 show the development of necrotic symptoms on infected leaves, comparing BSMV:00 inoculated empty vector control and *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* silenced plants. In *Lili* there are no visible differences between the treatment up to 14 days post infection, when necrotic symptoms start to appear in both silenced lines, 2 days prior to controls. The leaves appear completely blotched at 18 days post infection for the silenced lines.

In *Santiago* the same pattern is observed, but two days earlier than Lilli, due to its higher overall susceptibility. The first symptoms start to appear at 11 to 12 days post infection for the silenced lines, while no necrosis can be observed before 13 days post infection for the empty vector controls inoculated plants.

After 30 days of infection picnidia start to appear on leaves surface, and spores get produced within them. Quantifying picnidia and spore production is the optimal way to assess the resistance of a wheat plant, because Septoria spread in the field is tightly correlated to its reproduction capability. Picnidia and spores wash experiments were performed as indicated in Lee *et al*., 2015.

Fig 1.11a show the picnidia counted on leaves of *Lili* and *Santiago* 30 days post Septoria infection. For both varieties silencing *TaWRKY13* leads to a slight but statistically significant increase in picnidia production. For the controls an average of 45.1 and 51.25 picnidia per leaf centimeter? were counted respectively for *Lili* and *Santiago*.

TaWRKY13 silenced *Lili* an average production of 58.3 picnidia per leaf centimeter (5.85% increase compared to the control) and 62.2 picnidia per leaf centimeter (7.74% increase compared to the control) picnidia was observed in *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* silenced plants.

Santiago silenced plants produced 65.25 picnidia per leaf centimeter, corresponding to a 7.1% increase, in *BSMV:TaWRKY13_1* inoculated samples, and 70.3 picnidia per leaf centimeter in *BSMV:TaWRKY13_2* inoculated samples, reaching a 9.7% increased picnidia production.

Spore production showed the same pattern as picnidia production, as shown in figure

1.11b. Silenced lines in both cultivars exhibit an increase in spores production.
Lili mock silenced produced an average of 122.25 spores/µl, while the TaWRKY13 silenced lines*; BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* gave rise to 155.75 spores/µl and 153.87 spores/µl, corresponding to an increase of 25% and 24% respectively.
TaWRKY13 Santiago silenced lines showed an increase of 18% and 16% respective for *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* . *Santiago* BSMV:00 empty vector control produced on average 222.25 spores/µl , *BSMV:TaWRKY13_1* 262.5 spores/µl and *BSMV:TaWRKY13_* 258.89 spores/µl.

1.9 Discussion.

WRKYs transcription factors are ubiquitously present in plant species, and they are part of the intricate signalling web developed to respond to both internal (developmental) and external (stress response) stimului (Bakshi and Oelmuller , 2014). The involvement of WRKYs in crop immune response has been demonstrated in barley (Dey *et al*., 2014) and rice (Cheng *et al*., 2015). WRKYs role in abiotic stress response in non-model plants has been established in *Triticum durum* (Yousfi et al., 2017) and sheepgrass (Ma *et al*., 2014). Investigations on WRKYs role in wheat are not extensive to date, but still both Ding *et al*., 2014 and Lee *et al*., 2015 stated a role in immune response for WRKYs, in particular *TaWRKY68* (Ding *et al*., 2014, Lee *et al*., 2015), *TaWRKY53* and *TaWRKY39* (Lee *et al*., 2015).
Starting from there I decided to perform a deeper investigation of WRKYs in Septoria defense, in order to establish breeding targets for increased defence against pathogens. The first step was to identify WRKYs in wheat genome. Due to the lack of genomic data, we speculate that only a fraction of them have been identified and validated as WRKYs (71). We started with sequence based homology search with conserved WRKY and Zinc finger domains (Zhang *et al*., 1997) against the available wheat genome and proteome, and I was able to find complete DNA sequences as well as partial sequences and proceeded with complete sequences in order to have the highest possible confidence of establish WRKY genes in wheat. These sequences were

validated utilizing the online software GLAM2 (Bailey*et al*., 2009), the software showed that all our selected sequences contain at least one WRKY domain and at least one Zinc finger domain.The presence of partially annotated sequences allows us to speculate that the WRKYs gene family in wheat could be more than 71.

In order to quickly screen for target WRKYs that show altered transcription upon Septoria infection, I decided to prioritise the 71 WRKYs. I identified in published literature 29 WRKYs showing a well established role in immunity, both in Arabidopsis and rice. The protein sequences of these WRKYs were aligned (Clustal Omega, Sievers *et al*., 2011) against our WRKYs database, in order to find putative homologous WRKYs.

TaWRKY13 is similar to *AtWRKY46* and *AtWRKY70*, their roles are: defence against Pseudomonas and regulation of the SA-JA crosstalk respectively (Sheikh *et al*., 2016, Sung Shim *et al*., 2013; Li *et al*., 2013). These similarities in protein sequence led me to speculate a role for *TaWRKY13* in Septoria defence. Moreover *AtWRKY46* and *AtWRKY70* have been both identified as regulators of brassinosteroid pathway in the same study (Chen *et al*., 2017), connecting their activity to development.

Comparing *TaWRKY13* expression among 7 different wheat varieties in control conditions, I detected a small but clear difference between them. The highest expressing variety is *Riley* while the lowest is *Santiago*, with a maximum 3 fold expression change between them. The expression level matches the field trial detected resistance to Septoria, in an inversely correlated fashion (field trial score source: AHDB recommended list for cereals and oilseed 2017/18). The variation of expression among elite varieties makes *TaWRKY13* a suitable target for breeding for Septoria resistance, as this variation could be introgressed from a variety to another, providing that the single nucleotide polymorphism (SNP) associated with this molecular trait can be identified.

The importance of *TaWRKY13* in immunity was demonstrated in 2 ways: by its expression pattern, the transcript increase upon Septoria infection for 3 different varieties analyzed (*Avalon*, *Lili* and *Santiago*). The increase in transcription is variety-specific: in *Avalon* we detected 7 fold change increase, in *Lili* and *Santiago* 3.5 and 4 fold change increase respectively. We can speculate that the plant's capability to upregulate *TaWRKY13* expression upon Septoria has been bred out during the programs, as *Avalon* is an older variety.

In order to precisely correlate *TaWRKY13* to Septoria defense reverse genetics and phenotyping are needed. VIGS-silenced WRKY13 lines were developed, and their defence phenotype compared with their controls. The silenced lines show an earlier onset of necrotic symptoms, accompanied by a slight but significant increase in Septoria sporulation. Thus I demonstrated that *TaWRKY13* plays a positive role in immunity in 2 elite varieties: *Lili* and *Santiago*.

The Plant Homeodomain (PHD) *TaR1*, studied by Lee *et al*., 2015, shows a really similar expression pattern to *TaWRKY13*, but contrarily *TaR1* knock-down leads to increased resistance (diminished sporulation), whereas *TaWRKY13* silencing leads to increased susceptibility to Septoria (via increased sporulation), even though silencing the two genes give rise to the same leaf phenotype: earlier onset of Septoria necrotrophic growth (Lee *et al*., 2015).

In Millyard *et al*., 2016, the role of the ubiquitin conjugating E2 TaU4 during Septoria infection was studied. *Tau4* transcript level decreases during the crucial moment of Septoria switch to necrotrophic and reproductive life stage. In this case silencing the target give rise to a phenotype characterised by a delayed onset of necrosis symptoms and diminished sporulation.

It is important to note that the same leaves phenotype could be connected to completely opposite Septoria fitness capability. In other words the same earlier onset of necrosis on leaves surface could be associate with increased or decreased spore production. Therefore, in order to precisely evaluate the resistance of a certain variety, the spore counts is the main quantitative data needed.

Even though the gene silencing had been consistent and successful in downregulating the *TaWRKY13* transcript at the 20% to 30% of the control level, the sporulation phenotype gave back a small difference between the controls and the silenced lines. We speculate that maybe a gene redundant to *TaWRKY13* may be present in wheat genome I could not identify yet. The new wheat genome assembly released by IWGSC (Appels *et al*., 2018) could be the resource needed to clarify this issue.

So far we have been able to isolate, identify and localize 71 WRKYs transcription factors in wheat genome. This resource is really important for future work.

1.10 Figures chapter 1

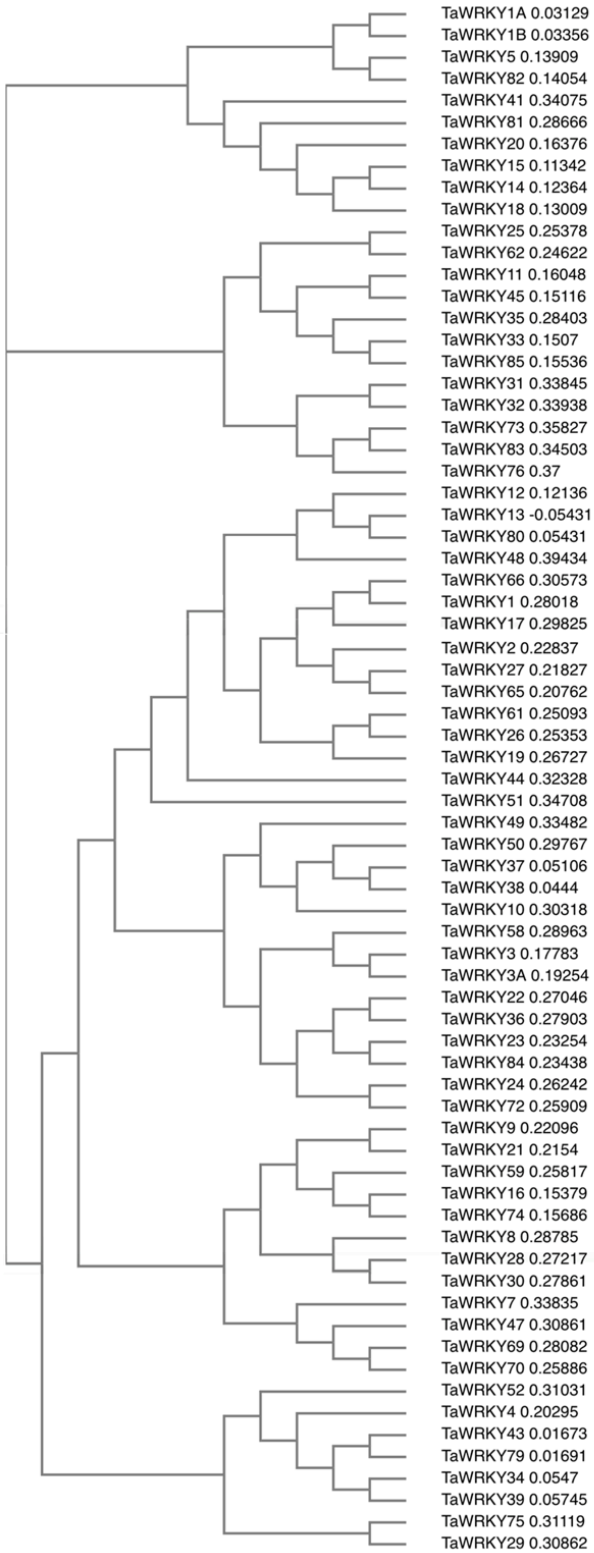


Figure 1.1

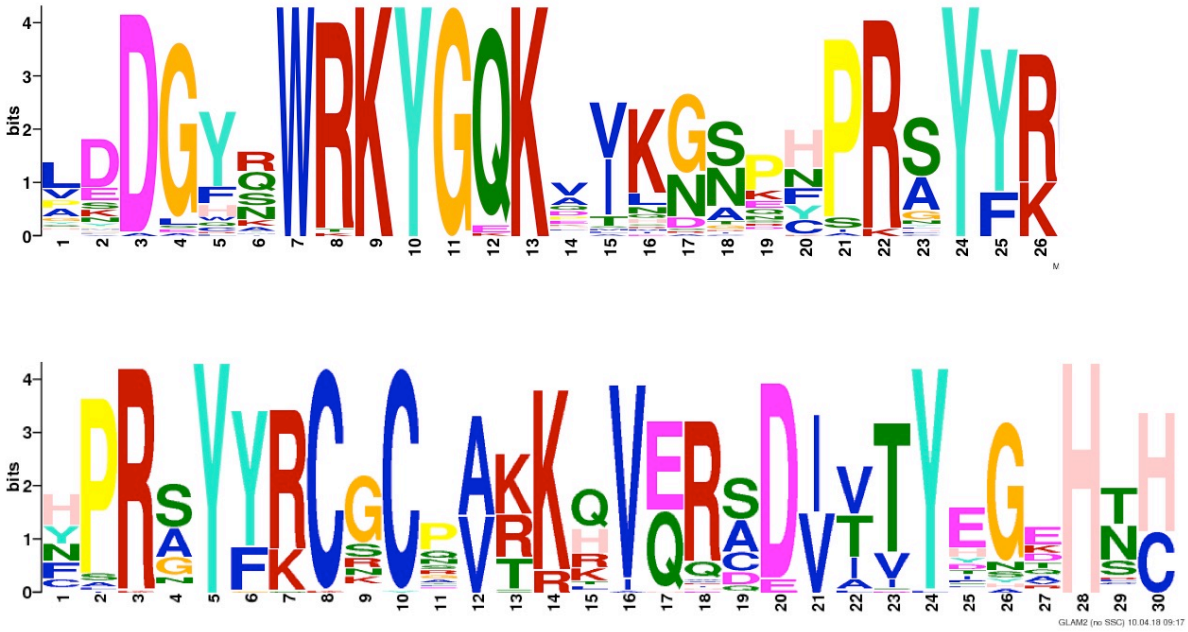
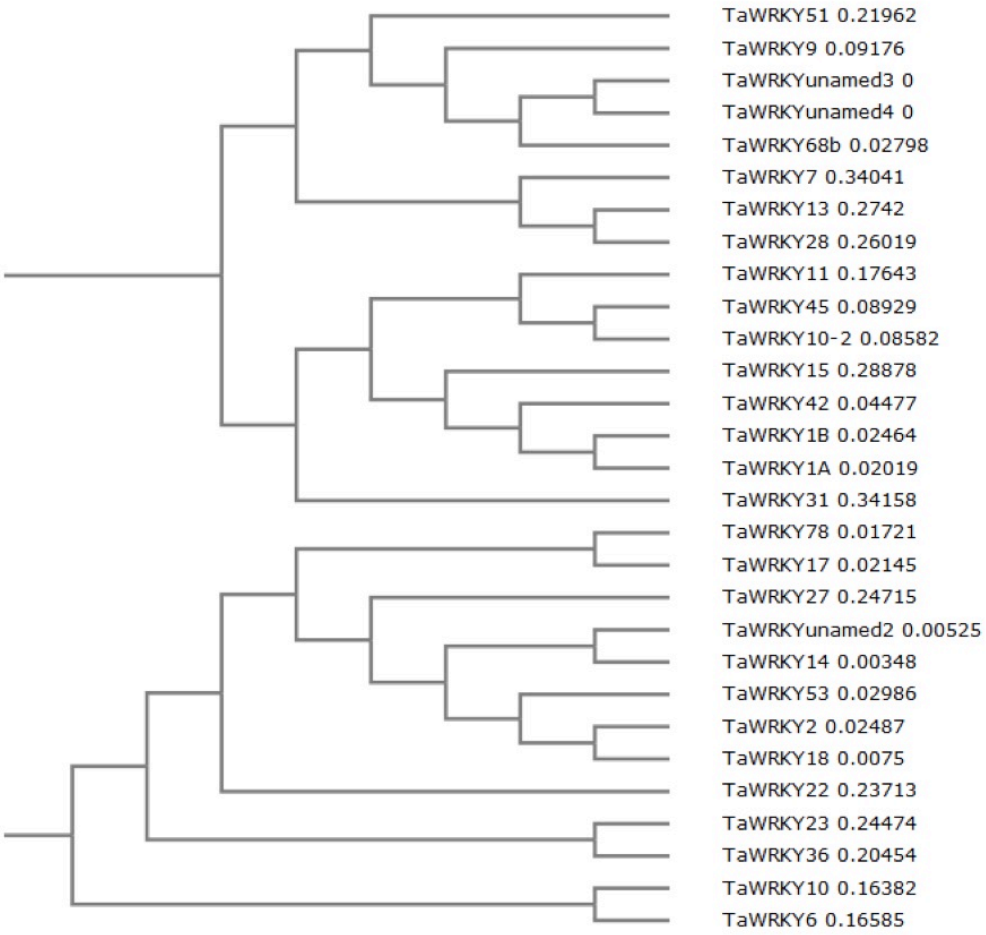


Figure 1.2

Gene	Reference	Role
AtWRKY6	Ma *et al*., 2014	Interaction with *Piriformospora indica*
AtWRKY7	Kim *et al*., 2006	Negative regulator *Pseudomonas syringae* defense
AtWRKY8	Chen *et al*., 2018	Positive regulation defense against *Botrytis cinerea*
AtWRKY11	Catalino *et al*., 2006	Negative regulator *Pseudomonas syringae* defense
AtWRKY17	Catalino *et al*., 2006	Negative regulator *Pseudomonas syringae* defense
AtWRKY18	Birkenbihl *et al*., 2017	Negative regulator *Pseudomonas syringae* defense
AtWRKY23	Levee *et al*., 2009	Resistance to rust
AtWRKY25	Li *et al*., 2009	Induced by SA
AtWRKY27	Mukhtar *et al*., 2008	Negative influences onset of bacterial wilt symptoms
AtWRKY33	Verma *et al*., 2017	Positive regulator *Botrytis cinerea* defense
AtWRKY38	Kim *et al*., 2008	Induced by *Pseudomonas syringae*
AtWRKY40	Birkenbihl *et al*., 2017	Negative regulator *Pseudomonas syringae* defense
AtWRKY41	Higashi *et al*., 2008	FLG induced
AtWRKY46	Hu *et al*., 2012	positive regulator *Pseudomonas syringae* basal resistance
AtWRKY48	Xing *et al*., 2008	Negative regulator of basal defense
AtWRKY50	Gao *et al*., 2011	Repressor of JA
AtWRKY51	Gao *et al*., 2011	Repressor of JA
AtWRKY53	Hu *et al*., 2012	Positive regulator *Pseudomonas syringae* basal resistance
AtWRKY60	Xu *et al*., 20016	Negative regulator *Pseudomonas syringae* defense
AtWRKY62	Kim et al., 2008	Induced by *Pseudomonas syringae*
AtWRKY70	Hu *et al*., 2012	Positive regulator *Pseudomonas syringae* basal resistance
OsWRKY13	Qui *et al.*, 2009	Enhances SA prodctuion and suppresses JA production
OsWRKY28	Chujo et al., 2013	Negative regulator of defense against Magnaporthe oryzae
OsWRKY31	Zhang *et al*., 2008	AUX response and defense response balancing
OsWRKY45	Huangfu *et al*., 2016	JA induced - SA induced
OsWRKY53	Chujo et al., 2014	Early suppressor of induced defensed, resistance to blast
OsWRKY62	Peng et al., 2008	Negative regulator of defense against *Xanthomonas oryzae*
OsWRKY71	Liu *et al*., 2007	JA induced - SA induced
OsWRKY89	Whang *et al*., 2007	JA induced

Table 1.1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TaWRKY9 0.09176
TaWRKYunamed3 0
TaWRKYunamed4 0
TaWRKY68b 0.02798
TaWRKY7 0.34041
TaWRKY13 0.2742
TaWRKY28 0.26019
TaWRKY11 0.17643
TaWRKY45 0.08929
TaWRKY10-2 0.08582
TaWRKY15 0.28878
TaWRKY42 0.04477
TaWRKY1B 0.02464
TaWRKY1A 0.02019
TaWRKY31 0.34158
TaWRKY78 0.01721
TaWRKY17 0.02145
TaWRKY27 0.24715
TaWRKYunamed2 0.00525
TaWRKY14 0.00348
TaWRKY53 0.02986
TaWRKY2 0.02487
TaWRKY18 0.0075
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Figure 1.3

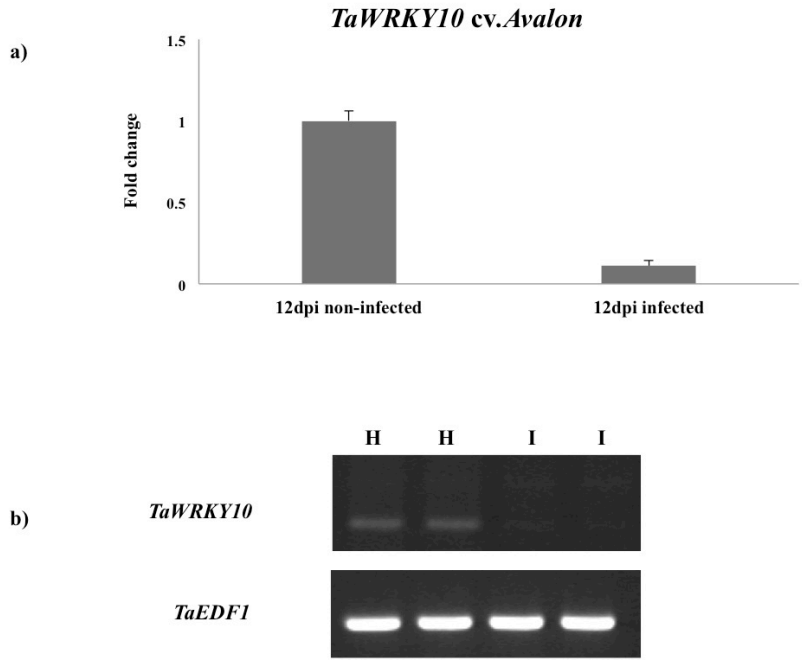


Figure 1.4

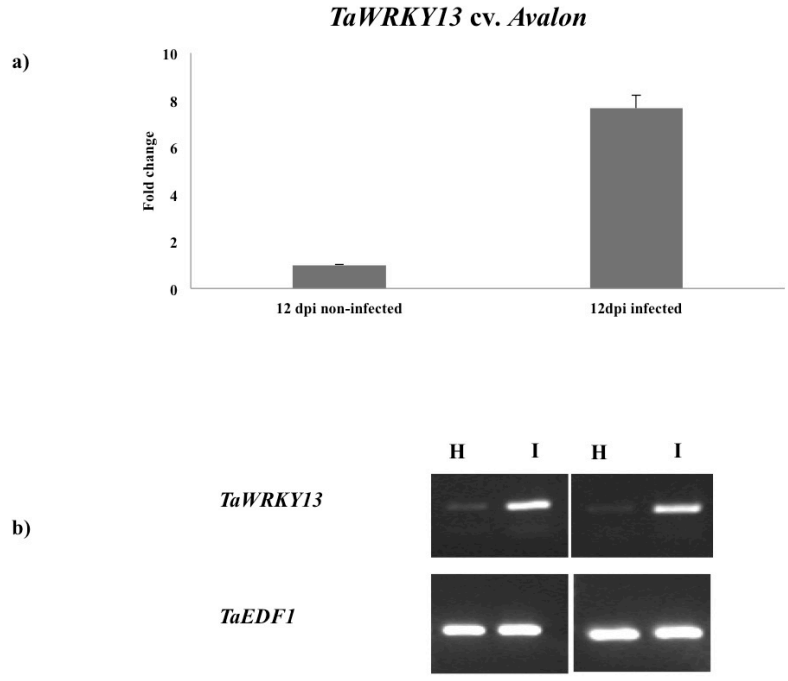


Figure 1.5

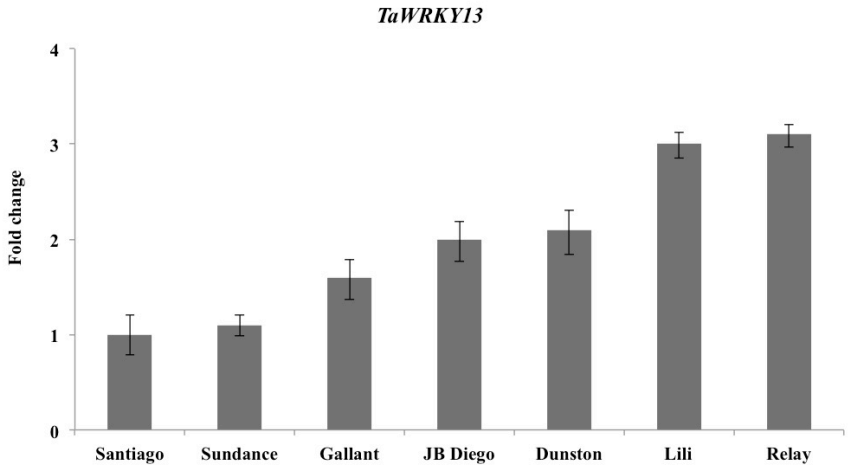

TaWRKY13
Fold change
4
3
2
1
0
Santiago
Sundance
Gallant
JB Diego
Dunston
Lili
Relay

Figure 1.6

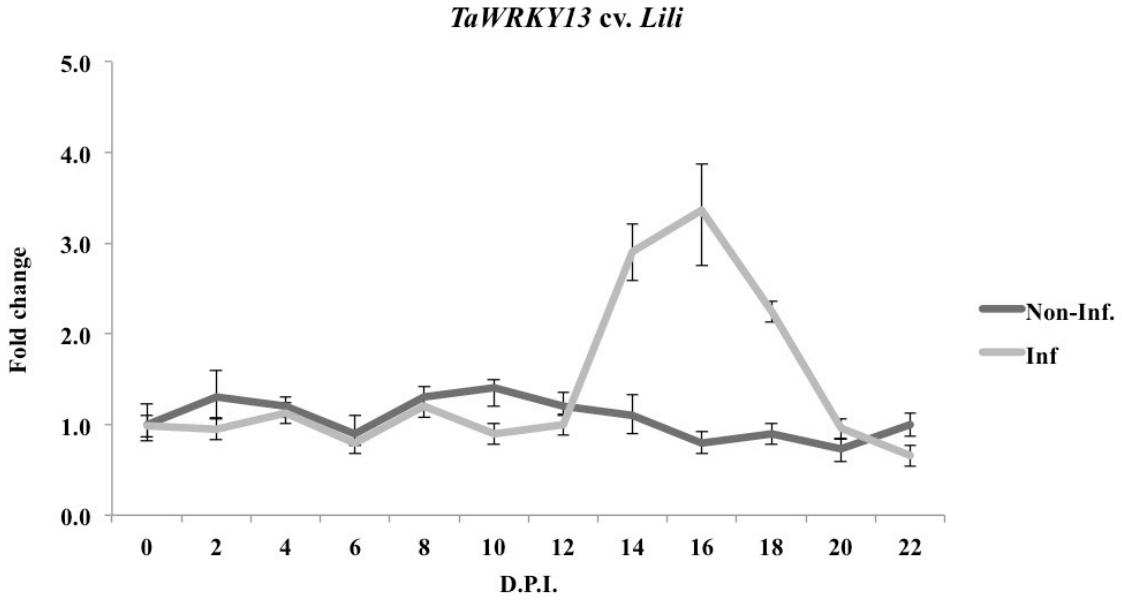


Figure 1.7

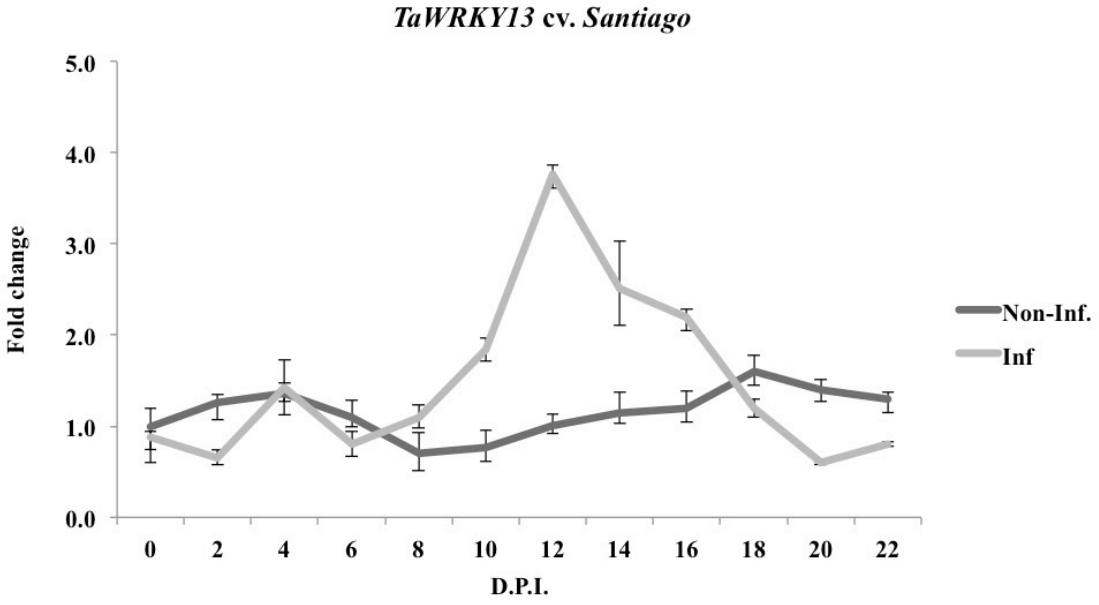


Figure 1.8

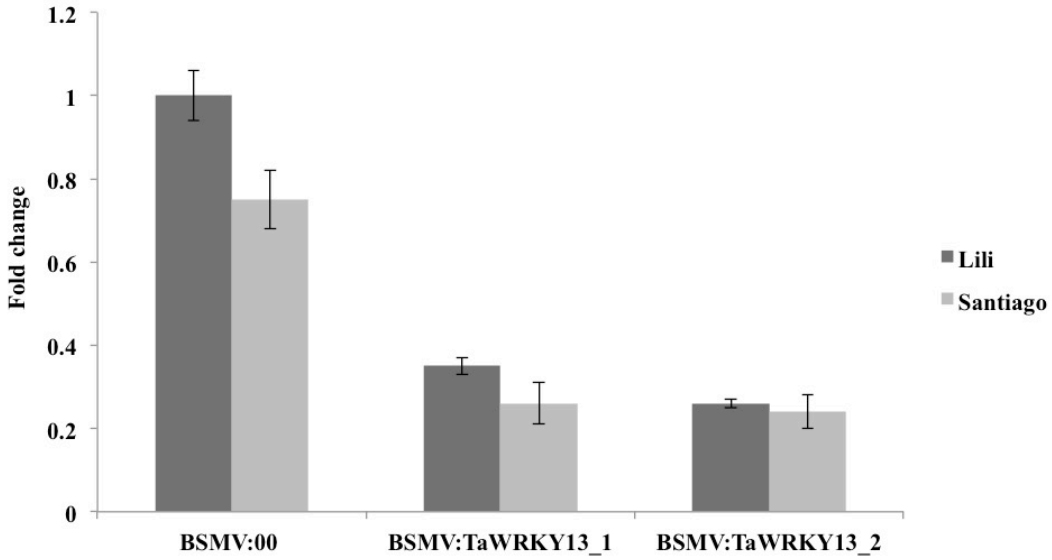

1.2
1
0.8
0.6
0.4
0.2
0
Fold change
BSMV:00
BSMV:TaWRKY13_1
BSMV:TaWRKY13_2
Lili
Santiago

Figure 1.9

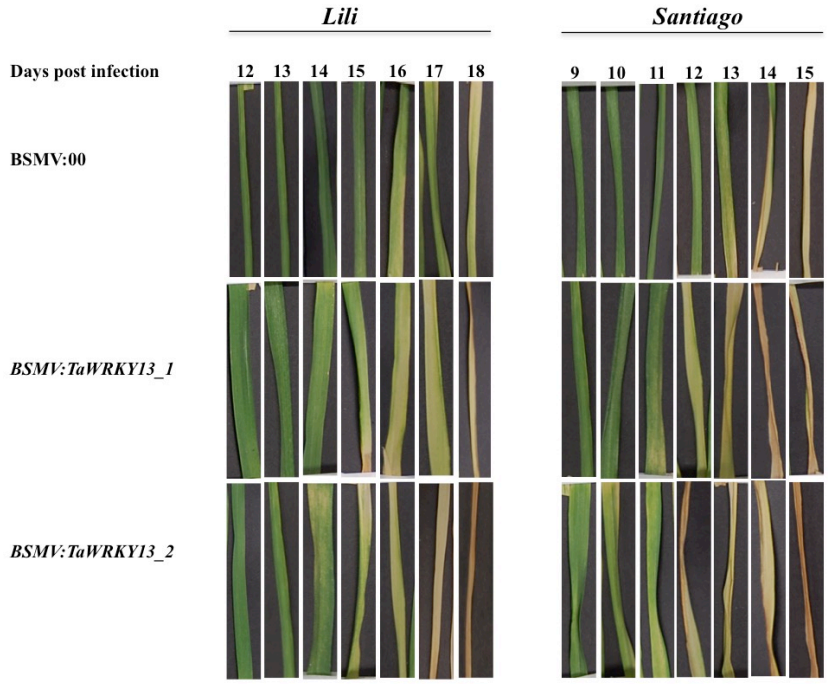


Figure 1.10

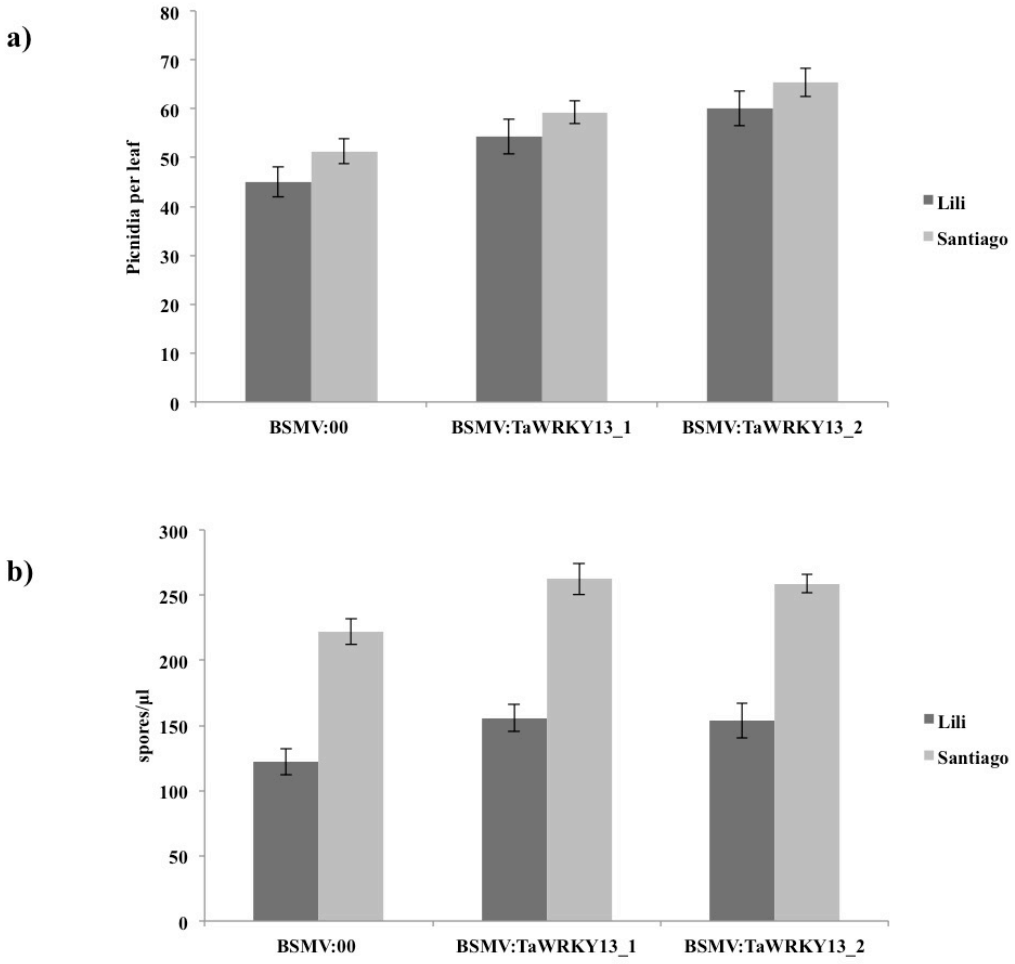


Figure 1.11

1.11 Figures legend chapter 1.

Fig 1.1 Wheat WRKYs phylogenetic tree.

Phylogenetic tree of all the WRKYs transcription factors mapped in the wheat genome. The tree was generated with ClustalOmega (Sievers *et al*., 2011).

Fig 1.2 GLAM2 alignment verify that the sequences isolated all belonging to WRKY family of transcription factors.

Peptide sequence logo plot of the WRKY sequences isolated from the wheat genome. The logo was made by constructing position-weighted Kullback Leibler logos of the multiple alignment of the cluster peptides, using the GLAM2 webserver. Height corresponds to the amount of information contained in a specific position. Large symbols represent frequently observed amino acids. The GLAM2 default amino acid colour coding is used.

Table 1.1 Arabidopsis and rice gene references.

Literature search was carried out to identify 21 Arabidopsis and 8 rice WRKYs with a role during biotic stress response.

Fig 1.3 Phylogenetic tree of the putative targets.

The amino acid sequences belonging to reference genes from Arabidopsis and rice have been used as a query to interrogate NCBI-BLAST database in order to find putative homologs genes in *Triticum aestivum*. The sequences isolated from wheat are represented as a phylogenetic tree generated via CLUSTAL-OMEGA.

Fig 1.4 *TaWRKY10* expression is down regulated upon Septoria infection.

The level of gene expression of *TAWRKY10* at 12 days post Septoria infection on cultivar *Avalon*. The relative level of expression was detected by semi-qRT-PCR.

A 10-fold-change down regulation of expression was detected for this gene during Septoria infection. a) Relative fold-change differential calculated evaluating PCR band intensity with ImageJ software. b) electrophoresis DNA gel of the amplicons obtained by PCR of *TaWRKY10* and housekeeping gene *TaEF1.*

Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates. In the figure 'H' indicates helthy tissue, whereas 'I' indicates infected tissue.

Fig 1.5 Septoria infection leads to increasing *TaWRKY13* transcript level.

The level of gene expression of *TAWRKY13* at 12 days post Septoria infection on cultivar *Avalon*. The relative level of gene expression was detected by Semi-qRT-PCR.

TaWRKY13 is 6 fold-change upregulated upon Septoria infection, compared to non-infected sample. a) Relative fold-change differential calculated evaluating PCR band intensity with ImageJ software. b) electrophoresis DNA gel of the amplicons obtained by PCR of *TaWRKY10* and housekeeping gene *TaEF1.*

Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 1.6 *TaWRKY13* gene expression is variable among different elite wheat varieties.

qRT-PCR performed on leaves of 2 weeks old wheat seedlings from different varieties (KWS *Lili*, KWS *Santiago* , *JB Diego*, *Gallant*, *Relay*, *Dunston Sundance*). The data here demonstrate that *TaWRKY13* transcript level varies between cultivars. *Santiago* shows the lowest expression of the gene, while *Relay* show the highest, with a 3 fold-change increase. *TaEF1* was used as a housekeeping gene.

Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 1.7 *TaWRKY13* expression upregulated upon Septoria infection in *Lili*.

A 22-day time-course experiment was performed in order to investigate the role of *TAWRKY13* throughout Septoria infection in elite variety Lili. The transcript level starts to increase 12 days post infection, reaching its peak at 16 days post infection (3 fold-change higher compared to its control). At 18 days post infection the expression starts to decrease, reaching back control level at 20 days post infection.

TaEF1 has been used as a housekeeping gene.

Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 1.8 *TaWRKY13* expression upregulated upon Septoria infection in *Santiago.*

The same procedure adopted to analyze *TaWRKY13* expression in *Lili* was used for *Santiago*, obtaining similar results. In *Santiago* TaWRKY13 expression starts to increase at 8 days post infection, reaching a 3.9 fold-change induction at 12 days post infection. The transcript level then decreases to non-infected control level at 18 days post infection. In Santiago a decrease below control level has been observed at 20 days post infection.*TaEF1* was used as housekeeping gene.

Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 1.9 *TaWRKY13* silencing in *Lili* and *Santiago*.

qRT-PCR data shows the expression of *TaWRKY13* is reduced in virus-induced gene silencing (VIGS) treated plants silenced by *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2,* compared to BSMV:00 empty vector control in both *Lili* and *Santiago*. The gene expression silencing observed is among 60% to 75% compared to non-silenced controls.

The relative gene expression were all normalized to BSMV:00 treated KWS *Lili*.

Error bars, ±SE of the mean of raw data.

Fig 1.10 *TaWRKY13* silencing in *Lili* and *Santiago* leads to earlier onset of blotching symptoms.

White-light image of a single leaf of BSMV:00 mock silenced, *BSMV:TaWRKY13_1* and *BSMV:TaWRKY13_2* silenced plants for both *Lili* and *Santiago* from day 12 to day 18 and from day 9 to 15 after Septoria inoculation, respectively. The symptoms, namely necrotic lesions, in the silenced lines start to be visible 2 days earlier compared to the mock silenced controls, for both the varieties analyzed.

Fig 1.11 *TaWRKY13* silenced lines are more susceptible to Septoria infection compared to their controls.

In order to address phenotypic change attributed to silencing *TaWRKY13*; picnidia and spores quantification analysis was performed comparing *TaWRKY13* silenced lines and their control. Silencing *TaWRKY13* leads to increase susceptibility against Septoria.

a) The number of picnidia produced on the leaves of *TaWRKY13* silenced plants shows 15% to 30 % increase compared to mock silenced plants, for both the varieties examined (*Lili BSMV:TaWRKY13_1* 15%, *Lili BSMV:TaWRKY13_2* 30%, *Santiago BSMV:TaWRKY13_1* 15, *Santiago BSMV:TaWRKY13_2* 27%) . Error bars, ±standard error (SE) of the mean of raw data. Student's T-test show significance difference of the silenced lines compared to their own control (*Lili BSMV:TaWRKY13_1* $P=2.12x10^{-4}$, *Lili BSMV:TaWRKY13_2* $P=8.14x10^{-3}$; *Santiago BSMV:TaWRKY13_1* $P=3.05x10^{-6}$ *Santiago BSMV:TaWRKY13_2* $P=5.34x10^{-5}$) (b) Spore washes performed 32 days after Zymoseptoria infection show 16% to 25% increase in spores produced on *TaWRKY13* silenced plants (*Lili BSMV:TaWRKY13_1* 25%, *Lili BSMV:TaWRKY13_2* 24%, *Santiago BSMV:TaWRKY13_1* 18%, *Santiago BSMV:TaWRKY13_2* 16%) . Error bars, ±standard error (SE) of the mean of raw data. Student's T-test show significance difference of the silenced lines compared to their own control (*Lili BSMV:TaWRKY13_1* $P=4.76x10^{-4}$, *Lili BSMV:TaWRKY13_2* $P=6.34x10^{-4}$; *Santiago BSMV:TaWRKY13_1* $P=5.42x10^{-6}$ *Santiago BSMV:TaWRKY13_2* $P=3.78x10^{-5}$).

Chapter 2: *TaWRKY10* is a novel component of wheat JA signalling.

2.1 *TaWRKY10* is a *Pooideae*-specific gene that shows differential gene expression across wheat cultivars that exhibit varying susceptibilities to Septoria.

As first step to characterize *TaWRKY10* its protein sequence was used as a query to interrogate PLANT-ENSEMBL database in order to find its closest homologs in different species, and with the data an alignment and a phylogenetic tree was created (Fig 2.1a and b).

This evolutionary tree indicated that *TaWRKY10* (Fig 2.1b), is a *Pooideae*-specific WRKY transcription factor with no known homologs in other lineages except in *Hordeum Vulgare* (*HvWRKY10*). As *TaWRKY10* showed altered gene expression during Septoria infection we wanted to determine if its gene expression could be a source of variation in susceptibility to *Septoria* observed in wheat varieties. Seven different varieties (KWS *Santiago*, KWS *Lili*, , *JB Diego*, *Gallant*, *Relay*, *Dunston* and *Sundance*) with known variation in susceptibility to Septoria under field conditions (AHDB recommended list for cereals and oilseed 2017/18) were chosen for transcriptional analysis to determine *TaWRKY10* expression. Under control conditions messenger RNA from 2-weeks old seedlings was used as a template for qRT-PCR analysis. As indicated in Fig 2.1c, KWS *Lili* (*Lili*) (less susceptible) showed the least *TaWRKY* expression and KWS *Santiago* (*Santiago*) (highly susceptible) showed the highest expression with the latter displaying 2 fold higher

TaWRKY10 transcription indicating that *TaWRKY10* gene expression correlates to Septoria susceptibility.

2.2 Wheat varieties *Lili* and *Santiago* show differential susceptibilities to Septoria that correlates with *TaWRKY10* gene expression.

The AHDB Septoria susceptibility scores reflect yield losses and foliar damage at adult plant stage. However we observed differential *TaWRKY10* gene expression in 4 week old growth stage and therefore to reconcile these differences and to ascertain if we can observe the same susceptibility phenotypes in 4-6 week old plants as observed in field conditions we decided to perform Septoria infection assays in wheat varieties under controlled conditions in the laboratory. Since the greatest amplitude of variation in *TaWRKY10* transcription among the different wheat varieties was observed in *Santiago* and *Lilli* we used these varieties to quantify the levels of susceptibility to Septoria in controlled conditions in the laboratory.

Both varieties were infected with virulent Septoria isolate, IPO323 (Arraiano and Brown, 2006). White light pictures were taken daily with pycnidia and spores count performed after 30 days of infection as previously described (Lee *et al.,* 2015).

Lili leaves start to show the first symptoms of Septoria mediated necrotic lesions after 14 days post infection (DPI), while for *Santiago* these necrotic lesions were observed earlier at 12 DPI (Fig 2.2). To provide a better quantification of STB symptoms, the percentage of blotched area in the leaves was determined from the white light pictures using ImageJ software with the data analyzed by exploiting the "Area Under Disease Progession Curve (AUDPC)" method (Madden *et al*., 2007). The AUDPC quantification shown in fig 2.3a,b indicate a significant difference in disease severity and progression between *Lili* and *Santiago* post infection. The AUDPC was calculated from 11 to 17 DPI comparing the two varieties, giving a value of 251.84 for *Lili* and 413.25 for *Santiago*. This data indicated that the spread of the lesions is slower in *Lili* compared to *Santiago.* It has been established that Septoria sporulates within necrotic lesions containing pycnidia on the surface of leaves (Keon *et al*., 2007). Therefore we proceeded to quantify Septoria sporulation by performing pycnidia and spore counts via the spore wash technique (Lee *et al.,* 2014). Septoria infection in *Santiago* produced up to 40 pycnidia per leaf and 250 spores/ μ l, while on *Lili*, Septoria only produced on average 30 pycnidia per leaf and 200 spores/ μ l

(fig 2.4 a,b). The quantification data demonstrate that *Lili* produces significantly less pycnidia and spores per leaf indicating that Septoria is less effective in causing STB in *Lili* compared *Santiago* (Fig 2.4 a,b).

A Septoria infection time-course experiment indicated that *TaWRKY10* gene expression is downregulated in both varieties, 2 days prior to the switch to the symptomatic necrotrophic phase (fig 2.5 a,b). Taken together with the differential susceptibility to Septoria in *Lili* and *Santiago* out data strongly suggests a novel role for *TaWRKY10* in wheat defence against Septoria.

2.3 Silencing *TaWRKY10 gene expression* leads to increased resistance to STB.

Since *Santiago* showed the greatest expression of *TaWRKY10*, and was the most susceptible, we wanted to ascertain if *TaWRKY10* gene activity could contribute to the differences in susceptibities between *Lili* and *Santiago*. Furthermore we have also established that field observed differential susceptibility to Septoria can be recapitulated in younger plants in controlled laboratory conditions therefore providing a tractable means to ascertain the role *TaWRKY10* in STB.

Due to the large hexaploid wheat genome isolating single gene knockouts are currently not feasible. Therefore, Virus Induced Gene Silencing (VIGS)(Watson *et al*., 2005) was employed to silence *TaWRKY10*. Barley Stripe Mosaic Virus (BSMV) has previously been modified to effectively silence many wheat gene expression (Matthew, 2004; Watson *et al*., 2005). Gene silencing vectors based on BSMV (Ratcliff *et al*., 2001; Burch-Smith et al., 2004; Wang & Metzlaff, 2005; Lee *et al*., 2012), carrying the 5'-UTR and 3'-UTR of *TaWRKY10* mRNA, were used to induce sequence-specific degradation of the endogenous *TaWRKY10* mRNA and therefore a knock down in its gene expression in the wheat varieties *Lili* and *Santiago*. BLAST analyses confirmed that both DNA fragments (*BSMV:TaWRKY10*_1 and *BSMV:TaWRKY10_2*) were unique to *TaWRKY10*. The siRNA finder software si-fi predicted *TaWRKY10* specific silencing (Millyard *et al*., 2015, Lee *et al*., 2014). The effectiveness of gene silencing was confirmed by qRT-PCR on mRNA from emerging leaves of plants 14 days after *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2*

inoculation (fig 2.6 a,b). This data indicated that *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2* yielded 70% and 65% silencing of *TaWRKY10* in *Lilli* and 68% and 60% silencing in *Santiago* respectively compared to empty vector controls .

Fourteen days after silencing treatment the 3rd– 5th wheat leaves were subjected to Septoria infection (7.5 x 10^6 spores per ml) and symptom development was recorded for 28 days (fig 2.7) before pycnidia and spore counts were performed to measure fungal development (fig 2.8 a,b). White light pictures of disease progression were taken daily. As shown in fig 2.7 silencing *TaWRKY10* leads to an earlier onset of disease symptoms compared to the controls, for both varieties. In *Lili* no difference in symptom development was observed till 15 DPI, after which the *TaWRKY10* silenced lines begin to show necrotic lesions however these appear 2 days later for the empty vector inoculated control *Lili* plants. For *Santiago* the same pattern of earlier onset of symptoms was observed. The empty vector control leaves start to show blotching phenotype at 14 DPI, while in the *TaWRKY10* silenced plants Septoria symptoms appear at 11 DPI. After 30 days Septoria in the infected leaves start to sporulate through pycnidia production. The pycnidia production across multiple plants over a 2 cm leaf sections was quantified. Figure 2.8 a indicates that in *TaWRKY10* silenced leaves Septoria pycnidia formation was significantly reduced, with 45% reduction in pycnidia on *BSMV:TaWRKY10_1* and a 43% reduction on *BSMV:TaWRKY10_2* infected plants compared to empty vector BSMV:00 control. As expected from the reduced number of fruiting bodies there is also a significantly reduced number of spores produced in the *TaWRKY10* silenced wheat leaves (fig 2.8 b) with a 50% reduction on *BSMV:TaWRKY10_1* and a 46% reduction on *BSMV:TaWRKY10_2* infected plants when compared to the BSMV:00 control. *BSMV:TaWRKY10_1* is more efficient at silencing TaWRKY10 than *BSMV:TaWRKY10_2*. Strikingly the spore production from Septoria in the *BSMV:TaWRKY10* silenced *Santiago* lines was equivalent to spore produced from BSMV:00 control *Lili* plants (Fig 2.8 b). This data indicates that *TaWRKY10* is a major factor contributing to the difference in susceptibility between *Lili* and *Santiago*. Furthermore silencing *TaWRKY10* can further enhance resistance to *Lili* to Septoria.

2.4 Silencing *TaWRKY10* leads to root growth inhibition.

To ascertain whether *TaWRKY10* silencing leads to any changes in morphology we ascertained plant height and root length in the silenced lines and compared it to empty vector controls. Plants from both varieties were pre-germinated on filter paper for 3 days and subsequently moved to sand filled pots, and grown for 10 days before BSMV inoculation . Seven weeks after germination the sand was washed away, the roots and the shoots were photographed and the length was quantified via ImageJ software. No significant difference was observed in plant height in 7-week old *TaWRKY10* silenced plants compared to controls (fig 2.9 a,c). However in *Lili* we observed a root growth inhibition of 25% (fig 2.9 a,b) compared to empty vector controls. This phenotype was also observed in *Santiago* (fig 2.9 c,d) . This evidence suggests a role for *TaWRKY10* in regulating root growth as well as immunity against Septoria.

The sessile nature of plants dictates that growth must be integrated with changes in the natural environment. Modulation of hormone signalling pathways plays a key role in this process. Our data suggests that *TaWRKY10* may play a role in regulating hormone responses that play a role in growth and immunity.

2.5 *Lili* is more sensitive to JA compared to *Santiago*.

It is well established that JA is a growth regulator as well as a key hormone in defence signaling (Chini *et al*., 2016, Shinegaga *et al*., 2016, Goossens *et al*., 2015). JA is well known to inhibit root growth. Indeed, this growth inhibitory effect of JA was exploited in many genetic screens for plants with altered JA sensitivity (Staswick *et al*., 1992).

To ascertain if the JA response was affected in the varieties that showed the greatest differential *TaWRKY10* expression, we germinated seeds of both *Lili* and *Santiago* varieties on MS agar plates then transferred 2 day old seedlings to plates that were supplemented with 10μM JA to monitor root growth daily. As shown in fig 2.10 a,b seedlings of *Lili* show root growth inhibition after 24 hours (percentage root growth

inhibition 40%) compared Santiago. Indeed under these conditions *Lili* roots barely elongate at all for the first 24 hours of JA treatment indicating dramatic JA-mediated root growth inhibition response . At 48 hours after JA treatment there was similar root growth inhibition in both varieties. Whereas we observed that *Santiago* roots remain unresponsive to JA and elongate at the rates comparable to untreated seedlings in the first 24 hours of JA treatment, after which they exhibit growth inhibition as observed at 48 hours. Our data indicates that *Lili* was more sensitive to JA then *Santiago*.

JA treatment is also known to induce anthocyanin accumulation in plants (Shan *et al*., 2009, Chini *et al*., 2016) and this was observed to a much greater extent in *Lili* compared to *Santiago*, 3-fold and 1.8-fold more accumulation respectively compared to the controls (fig 2.11 a,b).

Our data demonstrate that *Lili* is more responsive to JA resulting in greater root growth inhibition and with more pronounced anthocyanin accumulation compared to *Santiago*. The evidence indicates that the observed sensitivity to JA between the varieties might explain the greater resistance to Septoria in *Lili*.

2.6 JA signaling markers respond to treatment earlier in *Lili* than in *Santiago*.

To investigate the molecular basis of the differential JA responses in *Lili* and *Santiago,* we performed qRT-PCR analysis of JA marker genes (Gossens *et al*., 2015) in these varieties. Established molecular markers for JA biosynthesis, perception and signaling were analyzed. JA biosynthesis was monitored by analyzing expression of genes encoding Allene Oxide Synthase (*TaAOS*) and Lipoxygenase 2 (Ta*LOX2*). JA perception was monitored ascertaining gene expression of the JA receptor Coronatine-Insensitive 1(*TaCOI1*) and signal transduction through expressiong of genes encoding Jasmonate-Insensitive1(*TaJIN1-TaMYC2*) and Jasmonate-Zim-domain (*TaJAZ6*). Downstream response markers were monitored by analyzing expression of genes encoding Plant-Defensin 1.1 (*TaPDF1.1*) and Tyrosine-Aminotransferase (*TaTAT1*).

Tissue samples from leaves and root of JA treated or mock treated *Lili* and *Santiago*

were used to extract mRNA at 0, 24 and 48 hours time points where we observed the greatest differential JA response in root growth. For both genes encoding JA biosyntethic enzymes a drop in expression was observed in JA treated samples compared to untreated controls (fig 2.12 a, b, c, d). Remarkably the downregulation in Lili is detected at 24hrs while in Santiago at 48hrs after treatment (fig 2.12 a, b, c, d). Intriguingly gene expression of JA receptor *TaCOI1* were upregulated in both shoot and root dramatically in *Lili* at 24h after the treatment, 6-fold and 8-fold respectively (fig 2.12 e), while in Santiago *TaCOI1* gene is not significantly upregulated even 48h after JA treatment (fig 2.12f). The JA signal transduction marker gene *TaMYC2*, was not upregualted significantly in either varieties. The JA signaling repressor *TaJAZ6* is upregulated 14 fold and 10 fold in *Lili* shoot and root tissues respectively but only 7-fold and 4-fold in *Santiago* shoot and root tissues after 48 hours of JA treatment (fig 2.12 g, h, I, l). The downstream JA response genes *TaTAT1* is more active in *Lili* at 24 hours post JA treatment and *TaPDF1.1* gene activity is greater in *Lili* at 48 hours post JA treatment (fig 2.12 m, n, o, p).

Our data indicate that JA perception and response is dampened in *Santiago* compared to *Lili*. We demonstrate a novel inverse correlation between JA response and *TaWRKY10* gene expression in *Lili* and *Santiago* wheat varieties**.**

2.7 *TaWRKY10* gets downregulated after JA treatment.

In order to investigate the relationship between *TaWRKY10* and JA, gene expression analysis of *TaWRKY10* upon JA treatment has been performed, in both Lili and Santiago. The same time points sampling as indicated before was performed.

The results are displayed in fig 2.13 a for *Lili* and 2.13 b for *Santiago*.

A similar pattern of expression was detected for both the varieties examined; there were no changes for the untreated samples over the time course, but a remarkable downregulation of *TaWRKY10* upon JA treatment compared to controls. This downregulation of gene expression increases over time.

2.8 Silencing *TaWRKY10* leads to the upregulation of the JA receptor *TaCOI1* gene expression and increased JA response.

Given that *TaWRKY10* silencing leads to altered JA response in wheat and cultivars that show altered *TaWRKY10* levels have differential JA response, we wanted to explore the molecular mechanism underlying this *TaWRKY10* linked altered JA response. We performed qRT-PCR analysis of JA markers genes on 4-week old *TaWRKY10* silenced plants. Well established molecular markers for JA signaling has were chosen for gene expression analysis . We analyzed genes affecting biosynthesis (Ta*LOX1*), hormone perception (*TaCOI1*), signaling transduction (*TaMYC2*) and downstream response (*TaPDF1.1*) markers. The results shown in fig 2.14 demonstrate there is no significant difference in expression of *TaLOX1*, the gene encoding components of JA biosynthesis between the *TaWRKY* silenced lines and non-silenced control (fig 2.14a). Surprisingly we detected a 4 fold increase in *TaCOI1* transcript in the *TaWRKY10* silenced lines (fig 2.14 b). We also noticed that *TaCOI1* transcript is present at 10 fold higher level in *Lili* compared to *Santiago* in the empty vector controls. The upregulation of *TaCOI1* transcription is known to induce the expression of the downstream JA responsive transcriptional activator *MYC2* (Gossens *et al*., 2015) and we duly observed this in the *TaWRKY10* silenced lines (fig 2.14 c). Not surprisingly, *TaPDF1.1*, a downstream target gene of *MYC2* was also upregulated in the *TaWRKY10* knock down plants (fig 2.14 d). Our data indicate that *TaWRKY10* acts as a repressor of *TaCOI1* gene expression. We demonstrate that manipulating *TaWRKY10* gene expression in wheat cultivars leads to altered JA perception via changes in the JA receptor *COI1* gene expresison. Since JA is a major hormone that regulates defence against necroptrophic pathogens, our data provides a plausible mechanistic explanation for the differential Septoria susceptibility phenotypes observed in the wheat cultivars with altered *TaWRKY10* expression.

2.9 *TaCOI1* is involved in Septoria defense in both *Lili* and *Santiago*.

In order to validate the involvement of *TaCOI1* in the wheat immune response during Septoria infection, its expression profile was analyzed in a time-course fashion after pathogen inoculation. 15 days old seedlings were infected with Septoria and then *TaCOI1* expression was evaluated via qRT-PCR, as described by Lee *et al*., 2015 and Millyard *et al*., 2016, comparing infected and non-infected tissue, for both varieties *Lili* and *Santiago*.

Due to the results displayed in fig 2.15 a and 2.15 b it is indeed possible to conclude that *TaCOI1* plays a key role in immunity against Septoria.

In *Lili* (fig 2.15 a) there is no change in gene expression compared to controls until day 14 post infection, when the infected tissues show an upregulation of 3 fold-change compared to their control. *TaCOI1* maximum expression was detected at 16 days post-infection, in which an upregulation of 6 fold-change was observed. The level of expression then decreases at 18 days post infection, showing no difference compared to its own control at 20 days post infection. In *Santiago* (fig 2.15 b) the expression of *TaCOI1* follows the same pattern as seen in *Lili*.

TaCOI1 upregulation in Santiago starts at 12 days post infection, after 2 days the maximum peak is detected at 6 fold-change higher compared to its control, matching the control level of expression at 18 days after infection. A relatively small decrease in expression compared to the control is detected at 20 days post infection.

The data confirm the involvement of *TaCOI1*, validating its involvement during the necrotrophic phases of pathogen lifecyle (Chini *et al*., 2016). In both the varieties analyzed *TaCOI1* expression starts to increase which corresponds to the switch to the necrotrophic lifecyle in Septoria.

2.10 *Lili* shows a higher JA-Ile biosynthesis, but a lower SA level when compared to *Santiago.*

Phytohormones play key role in plants response to biotic stresses.

Jasmonic acid (JA) and salicylic acid (SA) have been associated with response against necrotrophic or biotrophic pathogens respectively, and it is a well established paradigm (Glazebrook *et al*., 2005, Seilaniantz *et al*., 2007). Therefore hormones produced by a certain cultivar could be informative on its own biotic stresses response capability. To asses one of the key factors that can describe immunity potential of the target varieties we quantified the amount of hormones present in control conditions, in 2 week-old seedlings. Rapid quantitative determination of hormone abundance by LC-MS/MS from crude plant extract was performed as described by Forcat *et al*., 2008.

Figure 2.16 displays the results of this quantification experiment, in which the absolute quantity of JA (fig 2.16 a), JA-ile (fig 2.16 b), abscissic acid (ABA) (fig 2.16 c) and SA (fig 2.16 d) have been evaluated.

Lili shows on average a higher but not statistically significant production of JA compared to *Santiago* (fig 2.16 a).

The measurements are not robust enough throughout the biological repeats to draw any major conclusion from this part of the experiment. This precision problem could have been due to the really low amount of JA producted by the plants at this particular growth stage.

JA-ile is the active compound of the JA, it is actually JA-ile that interact with *COI1* receptor triggering *JAZ*s degrading process (Wasternack *et al*., 2018).

Lili show a 4 times higher quantity of the above-mentioned phytohormone compared to *Santiago* (fig 2.16 b). This evidence led us to conclude that *Lili* is more resistant compared to *Santiago* not only because of a higher sensitivity to JA, but also because a higher JA-ile production.

ABA levels shows no noticeable differences between the varieties (fig 2.16 c).

Santiago shows a statistically significant higher SA production compared to Lilli (fig 2.16 d). It is possible to conclude that *Lili* and *Santiago* present inversely correlated JA-SA ratio and could their differential defence responses, as expected and previously reported for other plant species (Shinegaga *et al*., 2016).

2.11 Discussion.

STB is one of the most devastating wheat foliar diseases worldwide. Currently all elite wheat varieties show varying degrees of susceptibly to Septoria and STB is solely controlled by fungicide treatment. In recent years, outbreaks of fungicide resistant strains of Septoria have become more prevalent indicating that new control measures are vital for sustainable wheat production. A better understanding of wheat defence mechanism could lead to the discovery of novel components that could be exploited as STB resistance breeding targets.

The WRKY domain-containing transcription factor gene family is universally present in plants and is an integral part of plant defence signalling pathways (*Bakshi* et al., 2014). Several recent studies confirm the role of WRKYs as major regulators of immune responses in crop plants (Vo *et al*., 2018, Lee *at al*., 2018, Satapathy *et al*., 2018).

Here we identify *TaWRKY10* as a novel component of wheat immunity against Septoria. Among the *TaWRKY*s identified to date in wheat, *TaWRKY10* shows homology to only a single WRKY factor from *Hordeum vulgare*, indicating that *TaWRKY10* is *pooidae*-specific gene in *Triticum aestivum* and is only the second such gene to be characterised (Perochon *et al*., 2015). Interestingly the other *pooidae* specific-gene ,*TaFROG*, is also reported to be involved in immunity against a wheat pathogen suggesting that these genes may have evolved to play a common role in wheat.

Basal gene expression analysis of *TaWRKY10* across commercial elite wheat varieties indicates a pattern of variability that correlates with susceptibility to Septoria under field conditions. Two different varieties with extreme variability, *Santiago* (high*)* and *Lili* (low) were selected to understand the role of *TaWRKY10* in more detail. Initially, we demonstrate that these varieties show different degree of resistance to Septoria in laboratory conditions as well as in the field. *Lili* (low *TaWRKY10* transcript levels) is more resistant to Septoria than *Santiago* (high *TaRWKY10* transcript levels), mirroring the publicly available field scoring data (AHDB recommended list for cereals and oilseed 2017/18). This result confirms the capability of laboratory studies, such as the current report, to be a potent tool to understand molecular mechanisms that underpin phenotypes that occur in field conditions. These studies can pave the

way to the development of wheat varieties that show field effective Septoria resistance in the field.

To assess if *TaWRKY10* expression level decrease has direct correlation with wheat defence against Septoria, we performed gene silencing experiments to downregulate *TaWRKY10* expression. Silencing *TaWRKY10* in both *Lili* and *Santiago* varieties led to reduced sporulation but an earlier onset of the necrotrophic phase in Septoria similar to what we observed for another negative regulator of immunity *TaR1* in wheat (Lee *et al*., 2015). Thus, for both of the varieties the resistance to STB has increased as a consequence of silencing *TaWRKY10* underlining the importance of *TaWRKY10* for Septoria defence *TaWRKY10* mRNA levels are downregulated during Septoria infection approximately 2-3 days prior to the onset of the necrotrophic stage of Septoria. Since *TaWRKY10* is a negative regulator of JA signalling this suppression of gene expression indicates a response by wheat to upregulate JA-mediated defence.

Hormone signalling pathways play a pivotal role in plant response to environmental stimuli. JA is one of the main hormones involved in plant - pathogen interaction, and the presence of JA receptor *COI1* is necessary to propagate the hormonal signal and thus the plant response (*Gossens* et al., 2015, *Gimenez-Ibanez* et al., 2015 *Chini* et al., 2016, *Shigenaga* et al., 2016). Despite the wealth of knowledge of hormone signalling pathways in the model dicot *Arabidopsis thaliana*, little is known about hormone signalling in monocots, especially in wheat. The current study sheds new light into regulation of JA signalling in wheat.

It has been previously reported that different WRKYs play opposite roles in the JA mediated defence against the same necrotrophic pathogen. For example over-expressing a Wild Grape WRKY in Arabidopsis could lead to a downregulation of JA response during *Botrytys cinerea* infection leading to increased susceptibility (Wang *et al*., 2017). Whereas over-expressing of *Populus trichocarpa WRKY40* in poplar trees leads to increased JA response and increased resistance to *Botrytis cinerea* (Karim *et al*., 2015). This evidence indicates species specificity and functional diversity within the WRKY gene family.

The inhibitory effect of JA on root growth is well established (Staswick *et al*., 1992). We demonstrate that silencing *TaWRKY10* leads to root growth inhibition, mimicking the effect of enhanced JA signalling on root growth inhibition. Taken together with *TaWRKY10* silenced plants exhibiting increased defence our data demonstrate a

negative role in JA signalling both in defence and also in growth control for *TaWRKY10*.

Moreover via qPCR, we have been able to demonstrate that the downregulation of *TaWRKY10* transcript level leads to the upregulation of the JA receptor *TaCOI1* gene transcription, establishing an inverse correlation between the expression patterns of these two genes. It has been demonstrated that *COI1* transcript abundance is directly correlated with JA sensitivity in rice (Yang *et al*., 2012). In the current study upregulation of *TaCOI1* transcription has a consequence of activating JA signalling pathway due to increased sensitivity, leading to both root growth inhibition and increased resistance to Septoria. Therefore, *TaWRKY10* exerts a negative effect on JA signalling in wheat by suppressing *TaCOI1* gene expression. Since *TaWRKY10* only exists in the genus *pooidea* this is a novel mechanism for regulating JA signalling in wheat.

I demonstrated that not only JA sensitivity is a source of variation among our examined varieties, but also we detected a significant difference on the active JA biosynthesis. *Lili* is more effective at converting JA to its active form JA-ile compared to *Santiago*. This effect could be due to the increased activity of JASMONATE RESISTANT 1 (*JAR1*) (Staswick *et al*., 2002). JAR1 is the enzyme that converts JA to JA-ile. The higher level of active JA-ile and the higher JA-ile sensitivity explain the higher degree of resistance detected in *Lili* (Chini et al., 2016).

Until now there are no studies that examine the sensitivity of different wheat varieties to phytohormones. We have been able to demonstrate that a more rapid JA perception regulated by *TaWRKY10* mediated suppression of the JA receptor gene expression is coupled with best disease resistance performance, both in the lab and in the field. Our data suggests that breeding programmes that target improved JA perception mechanisms could lead to novel wheat germplasms better suited to withstand Septoria infestation resulting in greater yield.

2.12 Figures chapter 2.

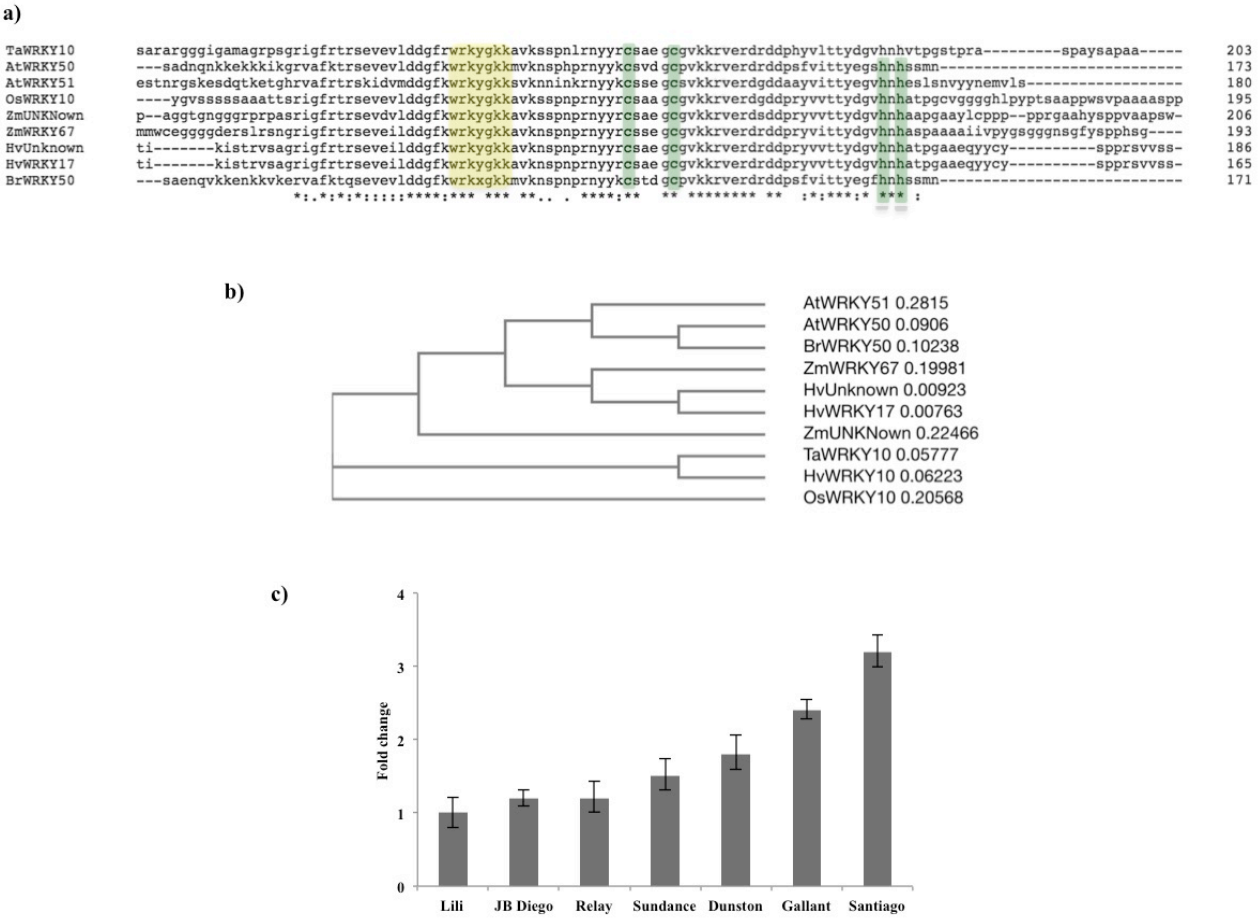
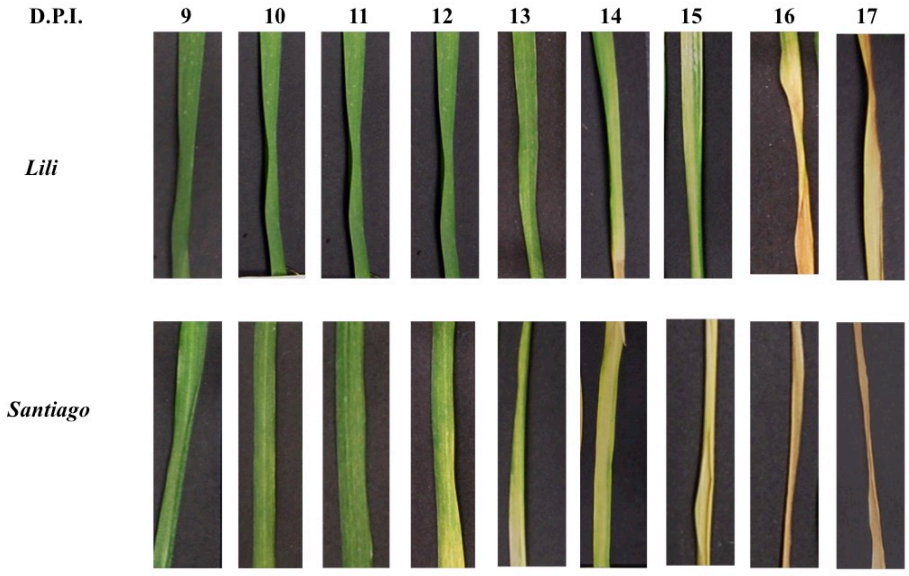


Figure 2.1



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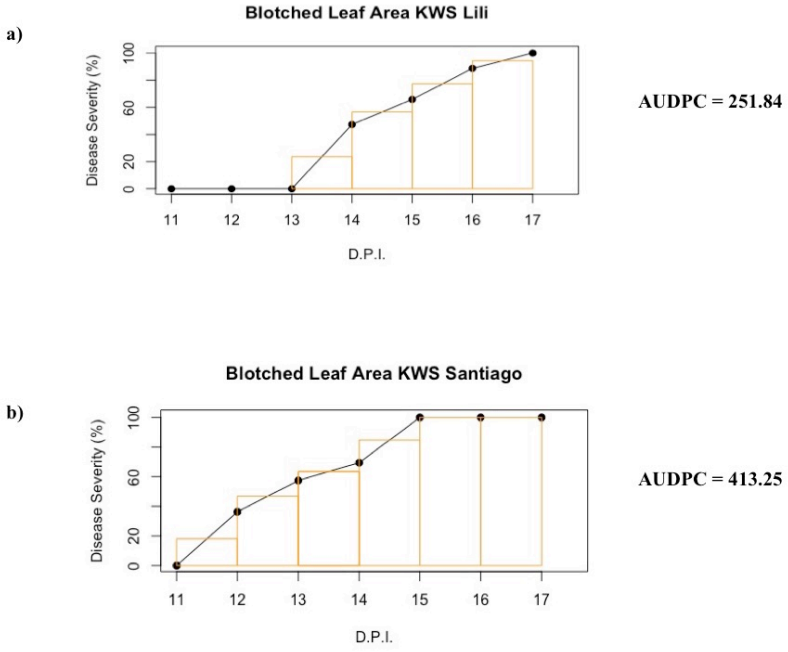

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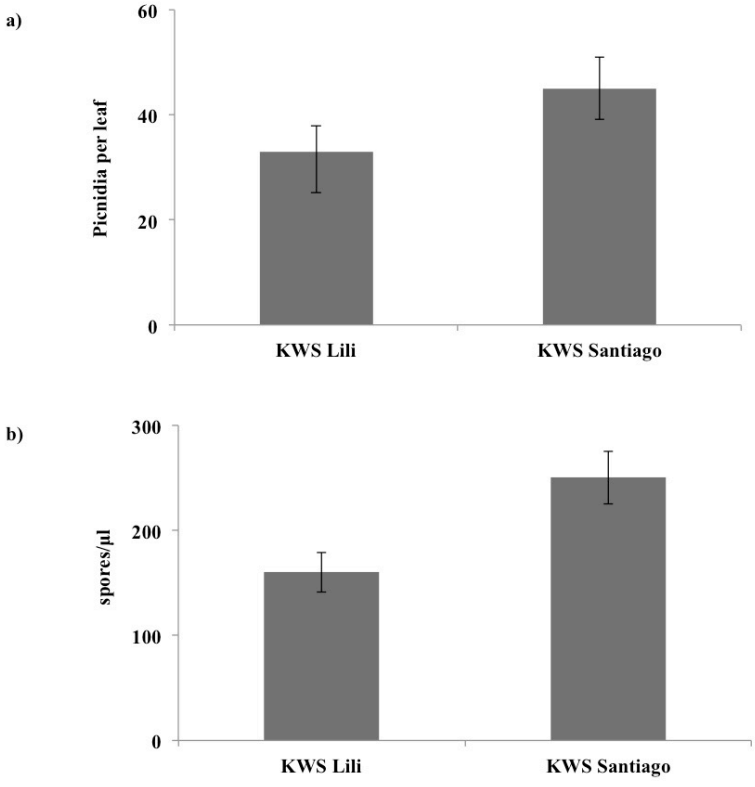
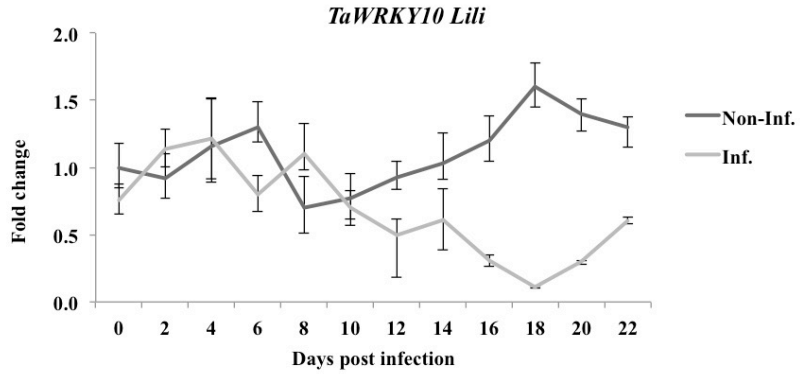


Figure 2.4

a)



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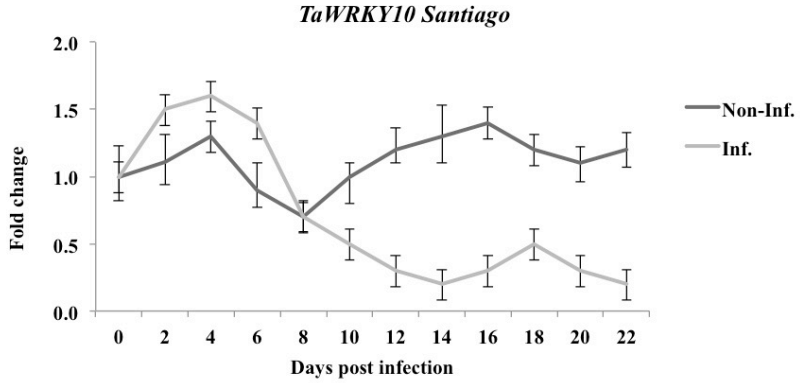

Figure 2.5

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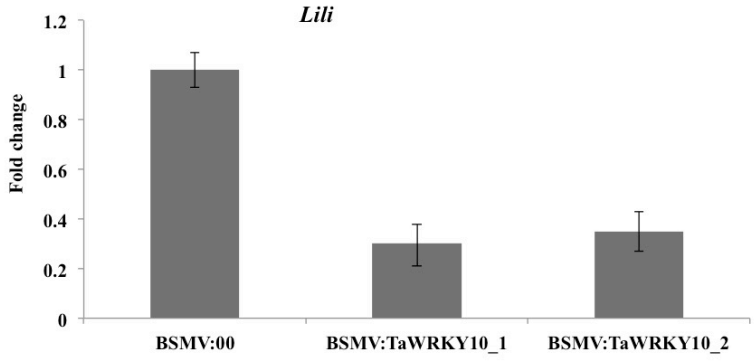

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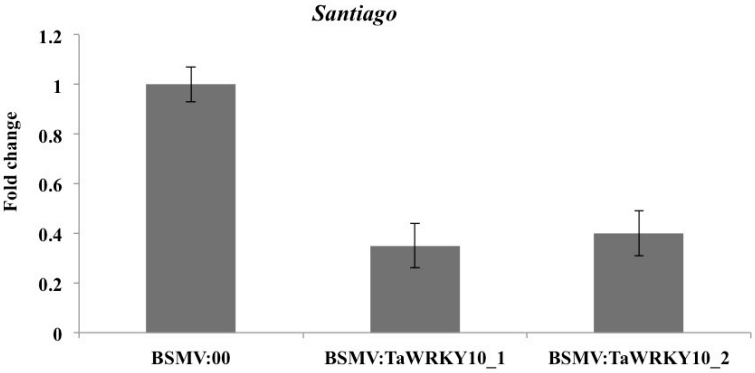

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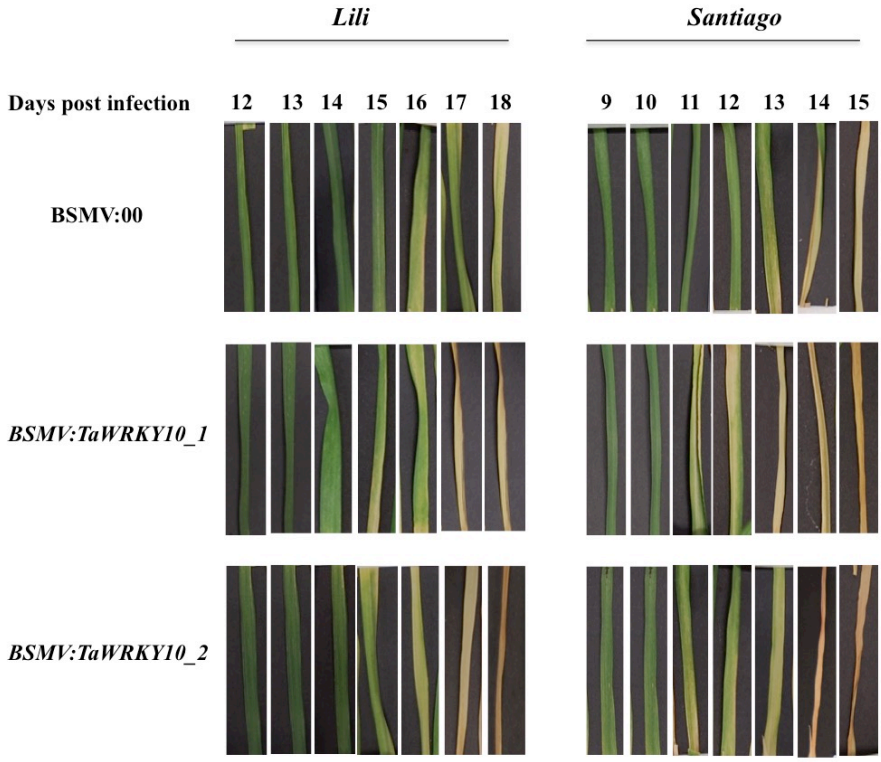

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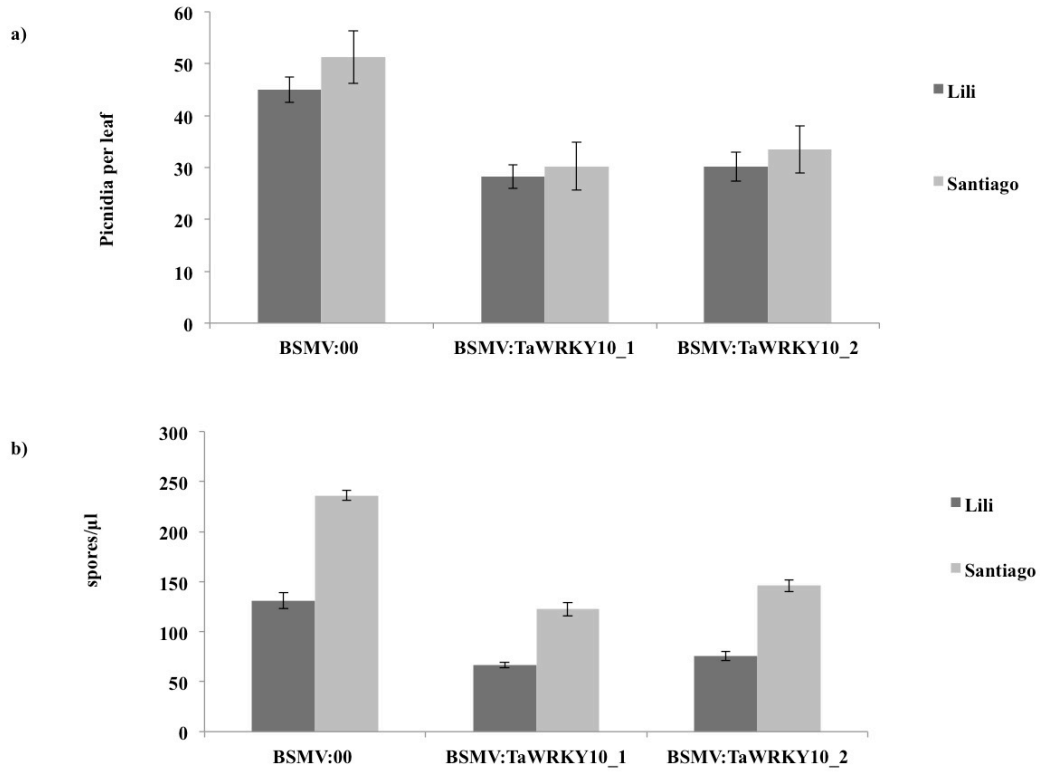

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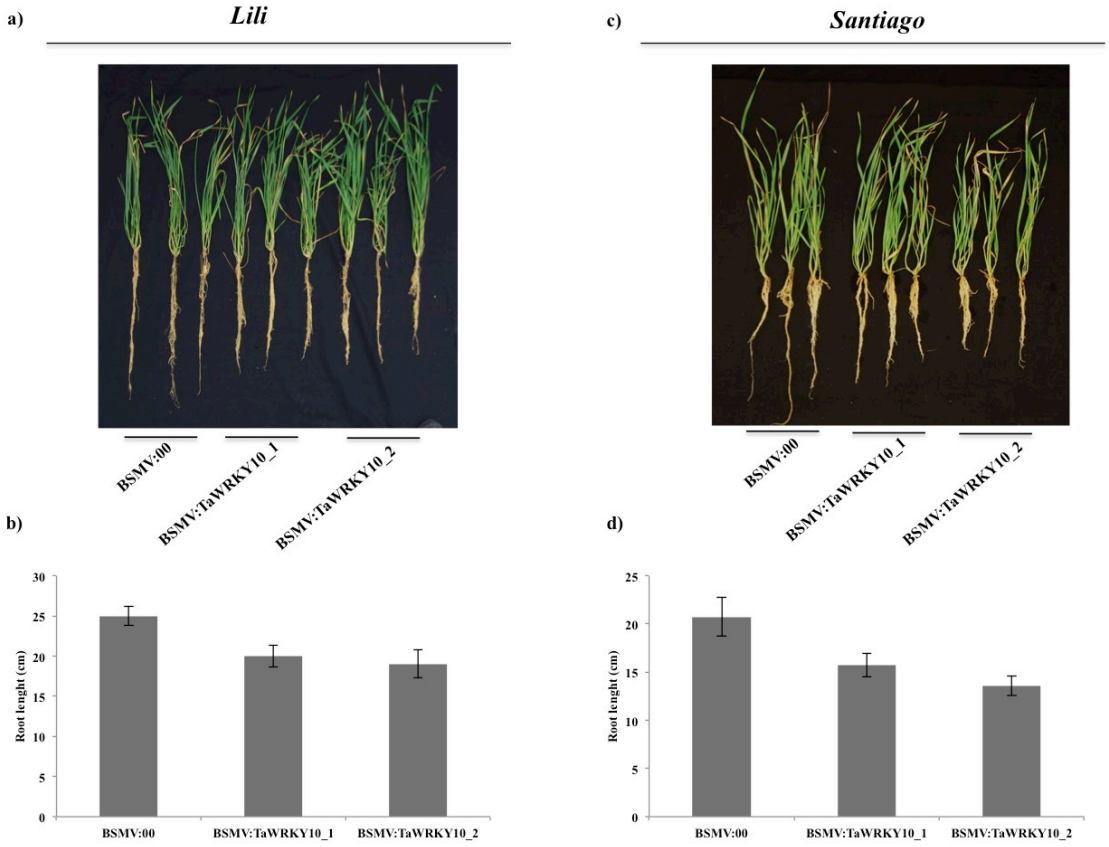

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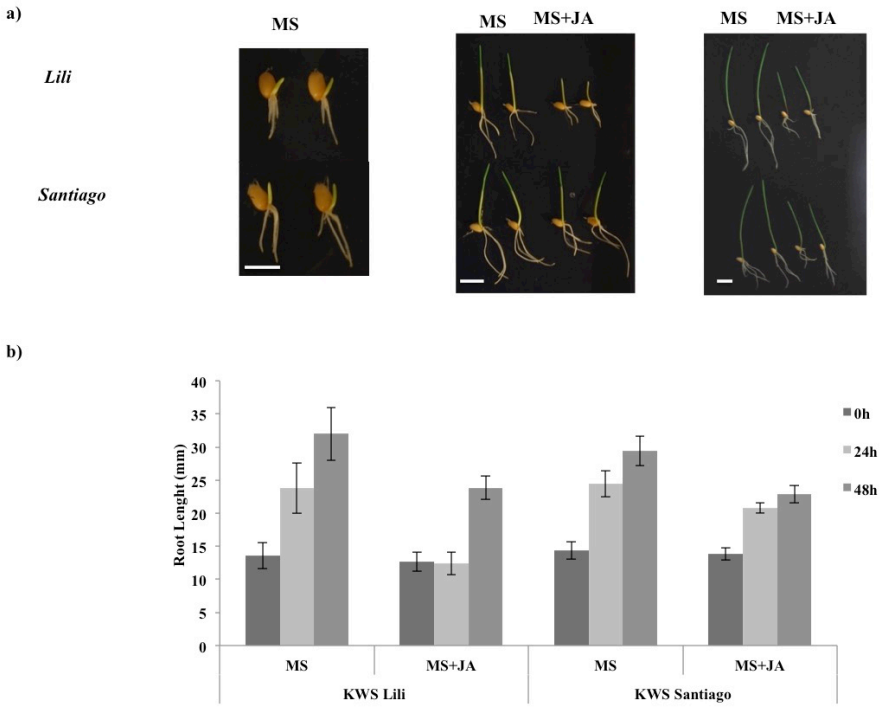

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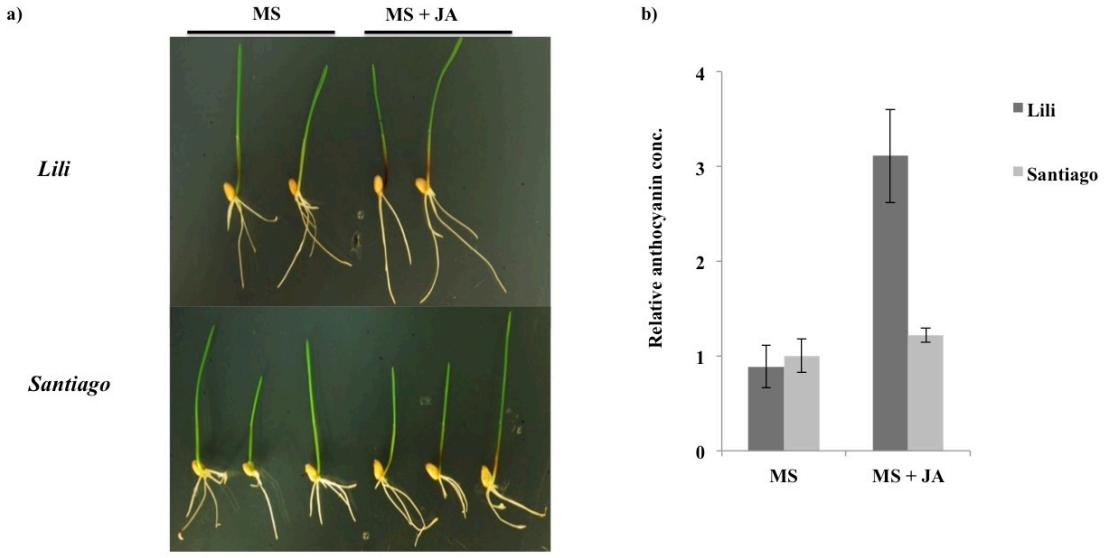

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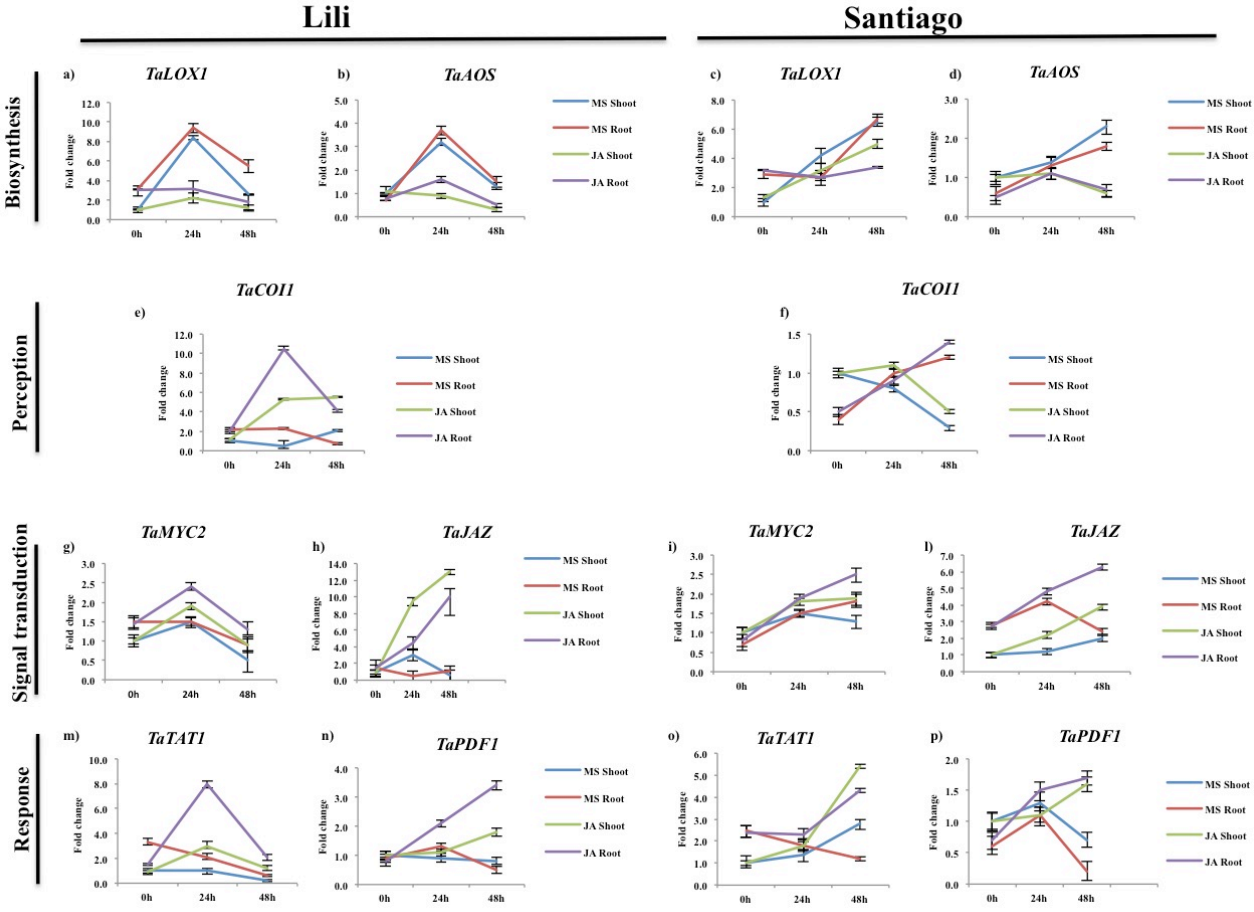

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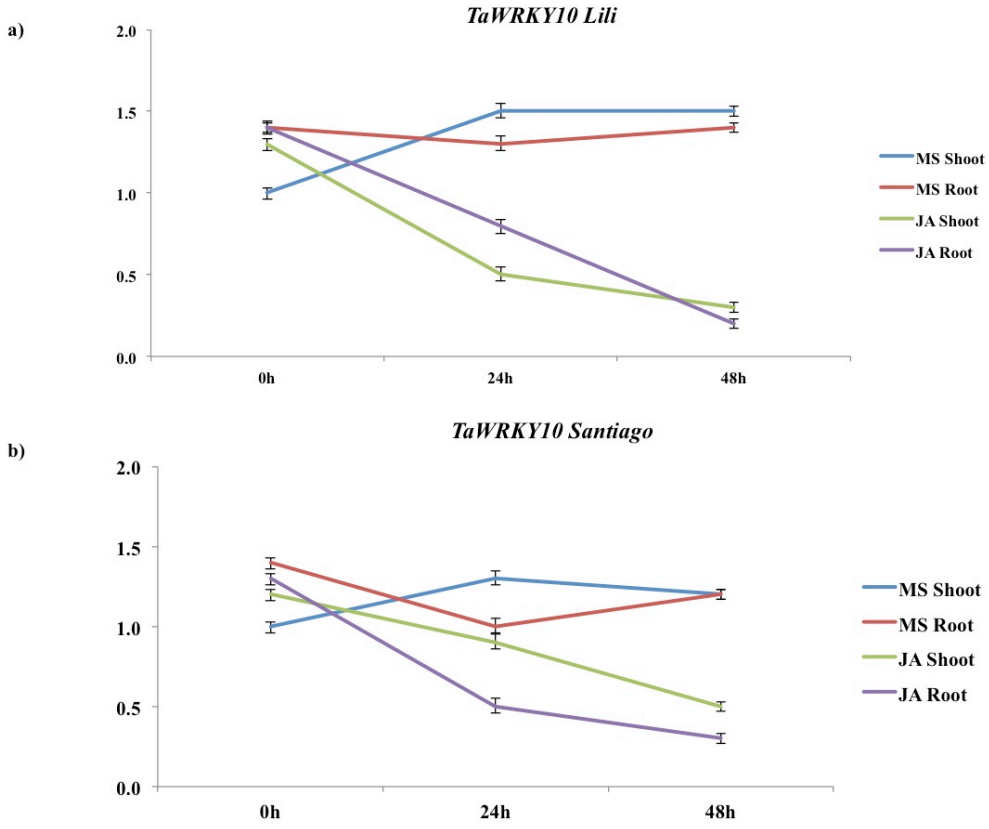

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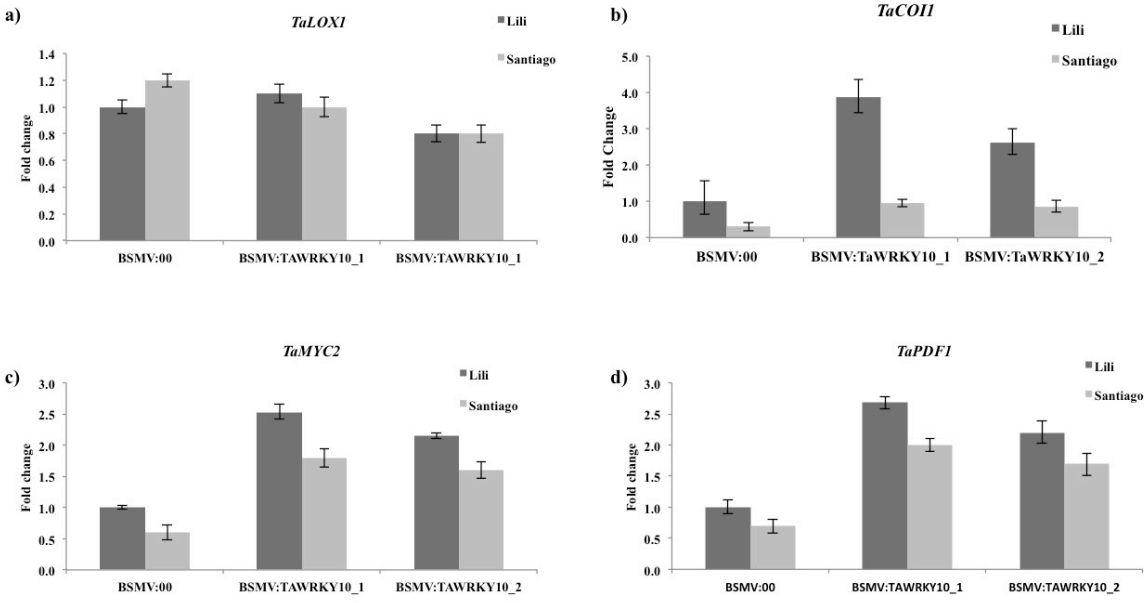

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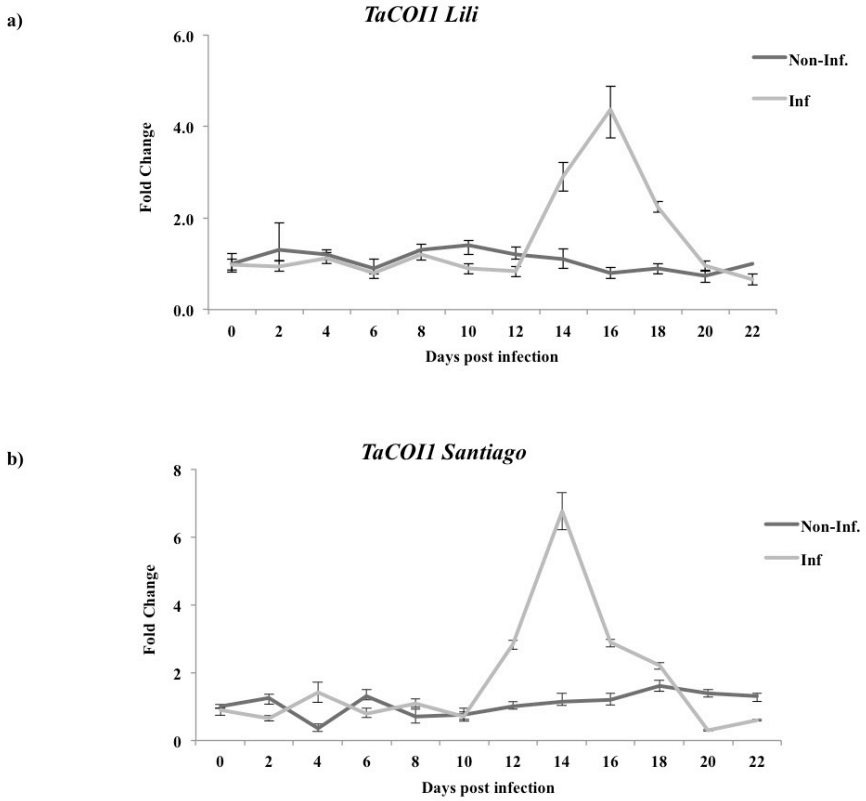

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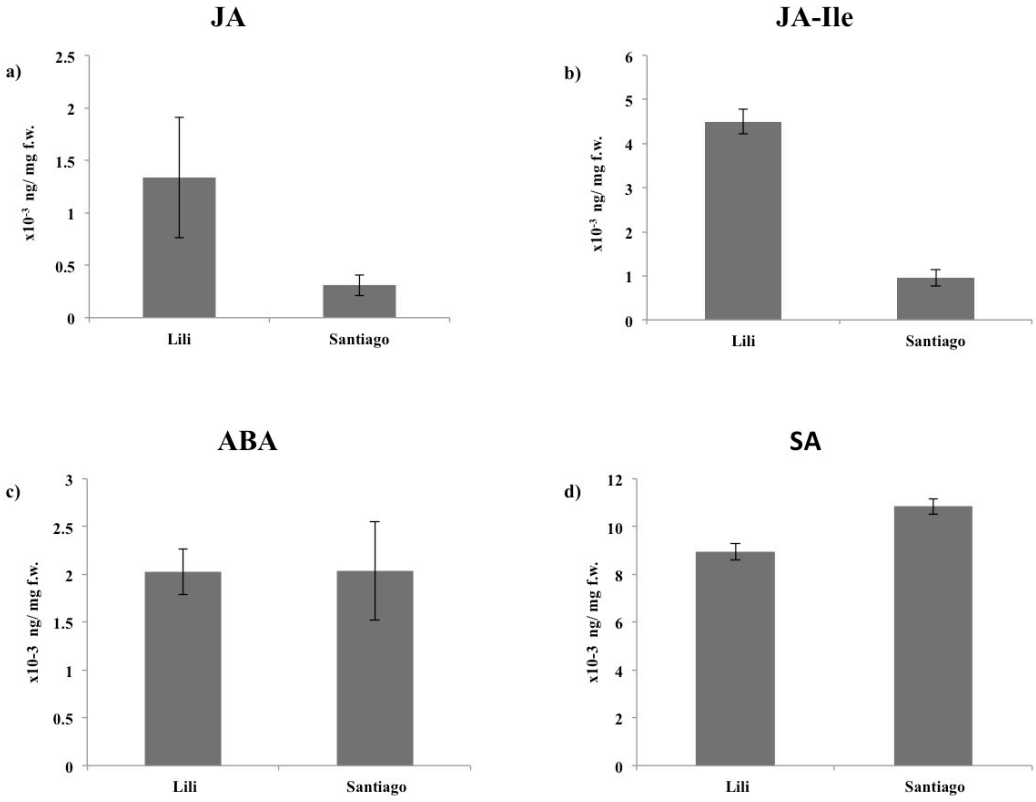


Figure 2.16

2.13 Figures legend chapter 2.

Fig 2.1 Characterisation of *TaWRKY10*.

(a) Multiple sequence alignment of *TaWRKY10* aminoacid sequences with its own nearest blast hits from other plants (Arabidopsis, Rice, Maize, Barley and Brassica). Alignment was obtained using ClustalOmega and then WRKY consensus sequence and zinc finger was recoloured. *TaWRKY10* belongs to the subfamily of type II WRKy TFs. (b) Phylogenetic tree generated exploting ClustalOmega. *TaWRKY10* display only one homolog in *Hordeum Vulgare*. (c) qRT-PCR performed on leaves of 2-week old wheat seedlings from different varieties (KWS *Lili*, KWS *Santiago*, , *JB Diego*, *Gallant*, *Relay*, *Dunston Sundance*). The data here demonstrate that *TaWRKY10* transcript level varies between cultivars. KWS *Santiago* display a 3 fold higher expression of *TaWRKY10* compared to KWS *Lili*. Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 2.2 *Zymoseptoria tritici* disease symptoms appear earlier in *Santiago* than *Lili*.

White light picture of a single leaf of KWS *Lili* and KWS *Santiago* respectively infected with Septoria. The pictures represent an infection timecourse from day 9 to day 17. It is clear that necrosis symptoms start to appear in KWS *Santiago* at day 12 DPI, while on KWS *Lili* the symptoms become clear at 14 DPI.

Fig 2.3 Area Under the Disease Progress Curve (AUDP) is higher in *Santiago* compared to *Lili*.

Area Under the Disease Progress Curve (AUDP) corresponding to KWS *Lili* (a) and KWS *Santiago* respectively. (b). The AUDPC was calculated by evaluating the percentage of blotched leaf area of both cultivars. We demonstrate that disease progression is quicker in *Santiago* compared to *Lili*. The mathematical calculation was performed exploiting the R package AUDPC (Madden *et al*., 2007).

Fig 2.4 *Lili* show an impaired picnidia formation and sporulation compared to *Santiago*

a) Average number of picnidia per leaf in KWS *Lili* and KWS *Santiago*. There is a significant difference in the picnidia production, demonstrating that KWS *Lili* is more resistant to Septoria than KWS *Santiago*. Error bars corresponding to standard error relative to 3 different biological replicates. Student's T-test p=0.0031 (statistically significant).

b) Spore wash was performed 32 days after initial Septoria inoculation. KWS *Lili* show and average of 50% less sporulation if compared to KWS *Santiago*.

Error bars corresponding to standard error relative to 3 different biological replicates. Student's T-test $p=2.20x10^{-4}$ (statistically significant).

2.5 *TaWRKY10* expression is downregulated upon Septoria infection for both *Lili* and *Santiago*.

Level of expression of *TaWRKY10* gene over a 22 days of Septoria infection time course, in both cultivars *Lili* (a) and *Santiago* (b). For both cultivars we identified a down regulation up to 10 fold in *TaWRKY10* transcript level. Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 2.6 *TaWKY10* can be effectively silenced.

qRT-PCR data shows that expression of *TaWRKY10* is reduced in virus-induced gene silencing (VIGS) treated plants silenced by *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2,* compared to BSMV:00 empty vector control in both *Lili* (a) and *Santiago* (b). The relative expressions are all normalized to BSMV:00 inoculated samples. Error bars, ±SE of the mean of raw data for 3 biological replicates.

Fig 2.7 Silencing *TaWRKY10* leads to earlier onset of symptoms for both studied varieties.

A single leaf of BSMV:00 mock silenced, *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2* silenced plants for both *Lili* (from day 12 to 18 post infection) and *Santiago* (from day 9 to day 15 after Septoria inoculation). The symptoms, namely necrotic lesions, in the silenced lines start to be visible 2 days earlier compared to the mock silenced controls for both the varieties analyzed. The experiment has been repeated for 3 biological replicates.

Fig 2.8 Silencing *TaWRKY10* leads to increased resistance against Septoria.

a) The number of picnidia produced on the leaves of *TaWRKY10* silenced plants shows about a 50% reduction compared to mock silenced plants for both the varieties examined. Error bars, ±standard error (SE) of the mean of raw data. Student's T-test show significance difference of the silenced lines compared to their own control (Lili *BSMV:TaWRKY10_1* $P=3.47x10^{-6}$, Lili *BSMV:TaWRKY10_2* $P=6.95x10^{-4}$; Santiago *BSMV:TaWRKY10_1* $P=2.52x10^{-9}$ Santiago *BSMV:TaWRKY10_2* $P=7.82x10^{-7}$). b) Spore washes performed 32 days after Zymoseptoria infection show 50% reduction in spores produced on *TaWRKY10* silenced plants. Error bars, ±standard error (SE) of the mean of raw data. Student's T-test show significance difference of the silenced lines compared to their own control (Lili *BSMV:TaWRKY10_1* $P=5.67x10^{-10}$, Lili *BSMV:TaWRKY10_2* $P=3.22x10^{-8}$; Santiago *BSMV:TaWRKY10_1* $P=8.33x10^{-13}$ Santiago *BSMV:TaWRKY10_2* $P=2.31x10^{-9}$).

Fig 2.9 *TaWRKY10* silencing leads to root growth inhibition.

a) white light picture of 7-week old plant cultivar *Lili*. Wheat has been germinated and then moved into sand pots. Virus-induced gene silencing has been performed at 10 days after germination. Silencing *TaWRKY10* leads to root growth inhibition in *Lili*. The effect is conserved also on cultivar KWS Lili (c). b,d) Root length quantified via ImageJ software on *TaWRKY10* silenced lines compared to control. The experiment has been repeated 3 times measuring root of 12 plants for each biological

repeat. Error bars, ±standard error (SE) of the mean of raw data. Student's T-test show significance difference of the silenced lines compared to their own control (*Lili*: *BSMV:TaWRKY10_1* P=1.1x10^{-4} , *BSMV:TaWRKY10_2* P=4.33x10^{-4} ; *Santiago*: *BSMV:TaWRKY10_1* P=5.25x10^{-4} , *BSMV:TaWRKY10_2* P=3.78x10^{-4}).

Fig 2.10 *Lili* is more sensitive to JA compared to *Santiago*.

(a) white light pictures of wheat seedling exposed to JA compared to their controls, for both *KWS* Santiago and *KWS* Lili cultivars. Seeds has been germinated on MS for 2 days, imaged (0h) and then moved either to MS or MS supplemented with 10 μM JA. Pictures were taken after 24 hours and 48 hours of treatment. (b) quantification of root elongation during JA treatment. Data was obtained by analyzing white light picture with ImageJ software. KWS Santiago is insensitive to JA treatment for the first 24h.

2.11 *Lili* displays enhanced anthocyanin production compared to *Santiago*.

a) white light picture of seedlings after 5 days of JA treatment. *Lili* show a more pronounced JA-dependent anthocyanin accumulation compared to *Santiago*. b) relative quantification of anthocyanin accumulation in seedlings via spectrophotometer. Error bars, ±standard error (SE) of the mean of raw data of 3 different biological replicates.

Fig 2.12 JA markers respond to treatment promptly in *Lili* but not in *Santiago*.

(a-d) There is no significant change in expression of genes that are implicated in JA biosynthesis between the two varieties after JA treatment.(e-f) expression of receptor *TaCOI1* is upregulated in both shoot and root dramatically in KWS *Lili* at 24h after the treatment, 6-fold and 8-fold respectively (e), in KWS Santiago *TaCOI1* gene is not significantly upregulated even 48h after JA treatment (f). (g-l) The JA signal transduction marker gene *TaMYC2* gets upregulated in KWS *Lili* by2.5-fold by 24h,

in KWS *Santiago* it reaches this upregulation level at 48h (g-i). The JA signaling repressor *TaJAZ6* is upregulated 14 fold and 10 fold in KWS *Lili* shoot and root tissues respectively. In KWS *Santiago* only 7-fold and 4-fold in shoot and root tissues after 48 hours of JA treatment(h-l). (m-p) JA response genes *TaTAT1* is more transcribed in KWS *Lili* at 24 hours post JA treatment but *TaPDF1.1* gene activity is greater in KWS *Lili* at 48 hours post JA treatment.

Fig 2.13 TaWRKY10 is downregulated upon JA treatment.

(a) Relative expression of *TaWRKY10* in JA treated seedlings, in *Lili* variety.

(b)Relative expression of *TaWRKY10* in JA treated seedlings, in *Santiago* variety. In both varieties *TaWRKY10* expression is downregulated promptly after JA application. We speculate this is needed to boost JA perception. The experiment has been repeated for 3 biological replicates.

Fig 2.14 *TaWRKY10* silencing causes perturbation on JA signaling pathway.

(a) qRT-PCR data shows the expression of *TaLOX1,* a gene involved in JA biosynthesis pathway. There is no change in the expression of this gene between the control and the silenced lines, demonstrating that TaWRKY10 silencing has no impact on the hormone synthesis. (b) The expression profile of the JA receptor *TaCOI1* is altered upon *TaWRKY10* silencing. *TaCOI1* transcription is upregulated by 4 times in KWS *Lili* and 3 times in KWS *Santiago*. This evidence demonstrates that *TaWRKY10* act as transcriptional repressor of *TaCOI1*. This fact leads to JA signaling boost. (c-d) As a consequence of *TaCOI1* upregulation we can detect as well a *TaMYC2* and *TaPDF1.1* gene expression upregulation in the *TaWRKY10* silenced lines.

2.15 *TaCOI1* expression is upregulated upon Septoria infection in both *Lili* and *Santiago*.

Level of gene expression of TaCOI1 changes during Septoria infection in *Lili* (a) and *Santiago* (b).

2.16 JA-ile and SA content are inversely correlated in Lili and Santiago.

Hormonescontent in uninfected control conditions could be used as a good proxy to analyse plant disease resistance capability. JA, JA-ile, ABA and SA were quantified in 2-week old *Lili* and *Santiago seedlings*. a) there is no statistically significant difference between *Lili* and *Santiago* JA content. b) *Lili* displays a 4 times higher production of active compound, JA-ile, compared to *Santiago*. c) no difference could be detected in ABA production among the two varieties analysed. d) Santiago show a higher content of SA than Lili, this evidence is in line with the general paradigm of inverse correlation between SA and JA phytohormones in plant. All data have been calculated with 3 different biological repeats, error bars represent Standard Error deviations. Each repeat consisted of 8 leaves from 8 different seedlings. Phytohormones content are represented as x10-3 ng/mg of tissue fresh weight, quantified by LC-MS/MS.

Chapter 3: Yeast-One-Hybrid screen

3.1 Yeast-one-hybrid.

Yeast one-hybrid screening (Y1H) is a powerful method to rapidly identify heterologous transcription factors that can interact with a specific regulatory DNA sequence of interest (the bait sequence). In this technique, the interaction between two proteins (bait and prey) is detected via *in-vivo* reconstitution of a transcriptional activator that turns on expression of a reporter gene.

Several biochemical methods have been developed to identify protein-DNA interactions, including gel shift, DNAse I footprinting, and chromatin-immunoprecipitation (ChIP) assays (Latchmann 1998; Orlando 2000). The above mentioned technique can address the question of which specific promoter is targeted by the protein of interest, contrarily Y1H can give information on which particular transcription factor interact with a specific DNA sequence, including *cis*-regulatory elements, origins of DNA replication, and telomeres (Li and Herskowitz, 1993; Lehmin *et al*., 1994; Kim *et al*., 2003).

Y1H relies on the general principles of the yeast two-hybrid assay (Y2H). In short, mammalian proteins are exogenously expressed in yeast and their interactions *in vivo* are measured by the downstream activation of reporter gene constructs. The main difference between Y1H and Y2H is in the interactions that are being measured. Y2H measures protein-protein interactions (Fields and Song 1989), while Y1H measures protein-DNA interactions.

In the Y2H system, two hybrid proteins are used. The bait protein (X) is fused to a DNA-binding domain (DB), and the prey protein (Y) is fused to a transcription activation domain (AD). When X and Y physically interact with each other, a functional TF is reconstituted and reporter gene expression is activated. In the Y1H system, a single hybrid protein, AD-Y, is used, and reporter gene expression is activated when Y interacts with the DNA bait. Although many predicted regulatory TFs contain an intrinsic AD, several TFs have a repressor domain or no activation/repressor domain at all. In addition, DNA-binding proteins that do not function in transcription (e.g., replication and DNA repair proteins) do not contain an AD. To enable the identification of a variety of DNA-binding proteins, a strong, heterologous AD is added to the prey protein.

This method is based on yeast mating. *Saccharomyces cerevisiae* (yeast) can stably exist as either a diploid or a haploid. Both haploid and diploid yeast cells reproduce by mitosis, with daughter cells budding off of mother cells. Haploid cells are capable of mating with other haploid cells of the opposite mating type (an a cell can only mate with an α cell, and vice versa) to produce a stable diploid cell (Haber 2012).

A cDNA library is cloned and transformed in haploid yeast of a specific mating type, while the prey is cloned into a vector (in our case pTUY1H), driving the expression of a specific auxotrophic marker (eg. HIS), and transformed in yeast of the opposite mating type. Both transformants for bait and preys are maintained by selective pressure of different auxotrophic markers in drop-out media (Reece-Hoyes and Walhout 2012).

The preys and the bait are forced to mate. While mating they exchange the genetic material they are carrying, so the bait and prey become in contact in the same cell. If plated on appropriate drop-out media selection of mated clones occures. The media is lacking the aminoacid that can be produced only by the protein whose gene expression is driven by the bait DNA sequence. In this case the surviving colonies are those in which the prey interact with the bait.

In this work a normalized and publicly available Arabidopsis TFs array library, called RR library, has been used (Castrillo *et al*., 2011), in order to investigate the existence of putative Arabidopsis homologs TFs that are capable to interact with *TaWRKY10* promoter. The use of an Arabidopsis normalized library can be useful to make the screen faster. There is no need to create a cDNA library, the procedure can be time consuming and complicated. Moreover When cDNA expression libraries are used as

preys, a limitation is that low abundant messengers, such as those derived from many TF encoding genes, tend to be underrepresented.
The TFs showing interaction can be match by homology to wheat TFs, in order to investigate how our target is transcriptionally regulated.
The Y1H system has shown great power in the field of biological research since it was established in 1993 by Wang and Reed. Application Y1H system has verified a number of known interactions between DNA and proteins and found a variety of new transcription factors. With the development of Y1H system, it will be more and more widely used in scientific research.

3.2 *TaWRKY10* promoter.

Previously we have been able, via genetic and molecular evidence, to establish a role of *TaWRKY10* and to define the targets of its transcriptional-regulation activity. Subsequently we wanted to investigate how *TaWRKY10* gene is itself regulated, in order to establish a hierarchical pathway. The first step was to isolate a sequence of 2 Kb DNA upstream of the start codon. I used the Plant Ensemble and the dedicated JBrowse software to identify this sequence in the wheat genome. The sequence was analyzed using the freely available online software PLACE (Plant cis-acting regulatory DNA elements) (Higo *et al*., 1999), to identify the conserved binding sites for different transcription factors (TFs) which could potentially interact with the WRKY10 promoter (fig 3.1).
As shown in fig 3.1, and as expected, there are no particular TF binding site which predominates on the WRKY10 promoter. The more represented binding sites are those corresponding to WRKY, MYB, DOF, ERF and GATA TFs.
The most efficient way to ascertain which TF could interact with specific *cis*-elements of a designated promoter region is to perform a (Yeast 1 hybrid) Y1H screen. A Y1H experiment using 2KB of the WRKY10 promoter was performed taking advantage of a previously existing, and publicly available, Arabidopsis TFs library (Castrillo *et al*., 2011). This approach is somehow limiting, as we did not use a *Triticum aestivum* TF library. However given the sequence conservation of the cis-elements it is likely to

identify Arabidopsis orthoglogs of wheat TFs that bind to WRKY10 promoter. Therefore any TFs identified in the screen will have to be exploited to identify the wheat orthologs.

3.3 p*TaWRKY10*-pTUY1H bait plasmid generation.

A 2 kb promoter region fragment upstream from the START codon of *TaWRKY10* was cloned in the Y1H bait vector pTUY1H (Castrillo *et al*., 2011). Primers used are listed in the primers table T1.

The subsequently generated bait plasmid *pTaWRKY10*-pTUY1H was first introduced into *E.coli* DH5-α for sequencing validation, and then in *S.cerevisiae* Y187α (mating type α). In the construct *pTaWRKY10*-pTUY1H TaWRKY10 promoter drives the expression of the auxotrophic selection marker –*HIS3* gene.

3.4 RR TFs array library.

The normalized and arrayed TFs library generated by Castrillo *et al*., 2011 (RR library) was obtained from NASC.

The use of an arrayed library gives advantages such as a fast and standardized protocol, using a 96-well format (replicates and liquid culture can be performed using the 96-replicator and 96-well standard plates for liquid culture propagation of yeast clones). More importantly such a library provides normalized representation of the different TFs, whereas in a cDNA derived library the TFs with a lower level of transcription are underrepresented, increasing the possibility to miss positive interactions of Tfs with low gene expression. The TF librabry clones were generated in pDEST22 plasmid, and then inserted into *Saccharomyces cerevisiae* YM4271 (mating type A).

3.5 Library screen.

The library has been screened as described by Castrillo *et al*., 2011. The protocol has been reported in the material and methods chapter.

The key step in this experimental procedure is the mating of the 2 different *S. cerevisiae* strains, containing respectively the bait plasmid (*pTaWRKY10*-pTUY1H) and the different clones of the RR library. The method exploited provide a high level of efficiency in mating. The outcome is represented in fig 3.2.

After the mating 10 positive interactions were isolated in the triple drop-out plates (-*TRP -LEU -HIS*) 11 and 15 (fig. 3.3). Series of negative controls was also included in order to rule out the possibility of contaminations during the earlier steps of the experimental setup. These controls consist of re-plating the isolated prays and the bait, before the mating, on double (-*TRP –LEU*) and triple drop-out (-*TRP -LEU –HIS*) medium. The outcomes are shown in figure 3.4, and as expected there is no notable yeast cell growth where is no interaction of promoter with TF.

3.6 Target validation via 3-Amino-1,2,4-triazole (3-AT) treatment.

Since positively identified yeast cells could be due to leaky expression of the *HIS3* gene in *S.cerevisiae*, a subsequent sub-culture of the positive clone cells was plated out on 3-Amino-1,2,4-triazole (3-AT) containing media. 3-AT is a competitive inhibitor of the *HIS3* gene product. The net effect of supplementing 3-AT to *S.cerevisiae* growing media is to suppress the effect of a low *HIS3* expression that can occur even in the absence of direct pray-bait interaction (Catrillo *et al*., 2011). Liquid cultures of the 10 positive colonies were grown on triple drop-out media. After 24 hours of growth 10µl of the culture was spotted on triple drop-out plates supplemented with increasing concentrations of 3-AT up to 60 mM (0, 5, 25, 60 mM). The outcome is to reduce the false positives and identify strong interactors on WRKY10 promoter which was narrowed down to six positive colonies, corresponsing to 6 positive interactions. The table 3.1 shows the TFs associated with a positive interaction with the bait.

3.7 Interactor clone 1: ZAT5 - C_2H_2.

Zinc finger proteins play a critical role in many cellular functions, including transcriptional regulation, RNA binding, regulation of apoptosis, and protein-protein interactions. They are classified according to the number and order of the Cys and His residues that bind the Zinc ion in the secondary structure of the finger (Klug *et al*., 1995, Mackay *et al*., 1998). According to *in silico* studies 0.7% of all Arabidopsis genes encode for a C_2H_2 zinc finger proteins (Englbrecht *et al*., 2001).

C_2H_2-type zinc finger proteins contain one of the best-characterized DNA-binding motifs found in eukaryotes. This motif consists mostly of about 30 amino acids and includes two conserved Cys and two conserved His residues bound to one zinc ion tetrahedrally, and is represented as $CX_{2\_4}CX3FX5$- $LX2HX_{3\_5}H$ (Pabo *et al*., 2001).

In Arabidopsis 176 different proteins contain the zinc finger domain, our target, *AtZat5*, belongs to the C1-2i subclass, together with other 19 representatives (Meissner *et al*., 1997, Pabo *et al*., 2011).

C_2H_2-type zinc finger proteins play a crucial role in many metabolic pathways as well as in stress responses and defense activation in plants. Recent studies emphasized the importance of C_2H_2-type zinc finger proteins with a putative repression activity to the defence and stress response of plants (Pabo *et al*., 2011, Ciftci-Yilmaz and Mittler 2008).

Other than the zinc finger domains, most members also share several putative nuclear localization sequences and an EAR motif [L/FDLNL/F(x)P] (that is thought to have an active repression activity and is found at the C terminus of the proteins (Ciftci-Yilmaz and Mittler 2008). The EAR motif was first identified in the AP2/ERF domain proteins (Ohta *et al*., 2001). AP2/ERF (or ERF proteins) domain proteins are plant-specific transcription factors that consist of a DNA-binding domain named the ERF domain (Allen *et al*., 1998, Hao *et al*., 1998). ERF proteins bind to the core sequence of a conserved ethylene-responsive element (GCC box) that is found in the promoters of many defence and stress response genes (Ohme-Takagi *et al*., 1995, Kazan *et al*., 2006). Studies suggested that EAR-motif containing repressors play a key role in plant defense and stress- response mechanisms by transcriptional repression of different defense or stress-response-related genes in the absence of stress (Kazan *et al*., 2006). For instance, the EAR repressor AtERF4 negatively

regulates the expression of PDF1.2 that encodes an antifungal peptide belonging to the family of plant defensins by modu- lating ethylene and jasmonic acid responses (McGrath *et al*., 2005, Yang *et al*., 2005).

Despite extensive studies on key member of the C1-2i subgroup such as Zat6, Zat7, Zat10/STZ, Zat12, AZF1, AZF2, and AZF3 (Ciftci-Yilmaz and Mittler 2008), very little is known about AtZat5. AtZat5 is closely related to another member of the family: AtZat18. *AtZat 18*, according to *in-silico* studies performed by Ciftci-Yilmaz and Mittler, 2008, could be implicated in immune response against *P. Infestans*. Recently AtZat18 has been linked to plant response to drought stress (Yin *et al*., 2017) But up to date there aren't any other exhaustive studies in literature about this gene.

3.8 Interactor clone 2: ABF1 .

Abscisic acid (ABA) is a vital mediator of responses in plants to various adverse environmental conditions like salinity, cold, drought, etc. Some of the ABA-mediated physiological responses are regulated by a group of basic leucine zipper (bZIP) transcription factors (Landschultz *et al*., 1988) that interact with a class of *cis*-acting DNA elements, collectively known as abscisic acid response elements (ABREs) (Busk *et al*., 1996). *ABRE binding factor -1* (ABF1) is a transcripton factor that binds to promoter ABRE sequences in response to ABA, activating downstream responses (Sarkar and Lahiri, 2013).

In response to water deficit, ABA, a well-known stress phytohormone, is rapidly induced, leading to the expression of stress-responsive genes and the activation of plants' cellular physiological adaptation to water stress (Fujii and Zhu, 2009; Cutler *et al*., 2010; Weiner *et al*., 2010).

On the other hand Salicylic acid is a phytohormone that plays a key role during plant developmental processes and responses to abiotic and biotic stress (Raskin, 1992; Bandurska and Stroinski, 2005; Khan *et al*., 2013). Surprisingly it has been reported that water deficit induces SA biosynthesis (Miura and Tada, 2014). Exogenous treatment with SA modulates plant drought resistance through multiple pathways such

as oxidative stress (Alam *et al*., 2013), stomatal conductance (Hao *et al*., 2010; Khokon *et al*., 2011; Habibi, 2012), antioxidant defense system (Hayat *et al*., 2008; Saruhan *et al*., 2012), and NO production (Hao *et al*., 2010; Khokon *et al*., 2011). Some Arabidopsis mutants that accumulate endogenous SA (*adr1, acd6*, *cpr5 myb96-1d,* and *siz1*) show both SA-mediated disease resistance and water deficit tolerance (Miura *et al*., 2013). Additionally, some SA- responsive genes are involved in plant response to water deficit such as those encoding MPK3, MPK4, MPK6, PR1, PR2 and PR5 (Ichimura *et al*., 2000, Ahlfors *et al*., 2004, Gudesblat *et al*., 2007, Liu *et al*., 2013).

Wang *et al*., 2018 proposed a model in which SA- and ABA-mediated responses are interconnected by PCaP2. PCaP2 is a plasma membrane-associated Ca^{2+}-binding protein, through its activity it can activate both ABA and SA response, activating not only water deficit response but also PRs and defense genes.

ABA signaling could be linked to plant response to biotic stress as well through JA pathway. The essential requirement for the transcriptional induction of JA-dependent genes is a functional ABA-signaling mechanisms (Anderson *et al*., 2004; Lorenzo & Solano, 2005; Niu, Figueroa, & Browse, 2011; Liu *et al*., 2014; Liu *et al*., 2016). ABA is absent in undamaged tissue but its synthesis is triggered by herbivore damage, which provides a direct link between the expression patterns of defense genes and the absence/presence of ABA (Erb *et al*., 2011, Consales *et al*., 2012, Vos *et al*., 2013). The JA- and ABA- signaling pathways can interact both synergistically and antagonistically (Anderson *et al*., 2004; Kilian *et al*., 2007; Huang, Wu, Abrams, & Cutler, 2008; Kazan and Manners, 2013; Savchenko *et al*., 2014; Rieman *et al*., 2015). In particular ABA-activated drought stress response is able to prime JA biotic stress responsive genes (Avramova, 2018). Those literature evidences, together with the capability of AtABF-1 to interact with *TaWRKY10* promoter, make a putative *TaABF1* as an interesting target to investigate the network underpinning the correlation between biotic and abiotic stresses.

3.9 Interactors clones 3 and 4: GATA19 and GATA23.

GATA factors are evolutionarily conserved transcription regulators that were named after their DNA-binding preference to the consensus sequence X-GATA-Y (X could be either thymidine (T) or an adenosine (A); Y could be either guanidine (G) or adenosine (A) (Reyes *et al*., 2004).

All GATA transcription factors from *Arabidopsis* have a type IV zinc finger with the consensus C-X_2-C-X_{17-20}-C-X_2-C (C, cysteine; X, any residue) followed by a highly basic amino acid stretch (Reyes *et al*., 2004).

GATA motifs are enriched in promoters of light-regulated genes and of genes controlled by the circadian clock (Arguello-Astorga and Herrera-Estrella, 1998). On top of that the GATA factor AreA from the fungus *Aspergillus nidulans* is a key regulator of nitrogen signaling, which suggested that studies of plant GATAs may also lead to advances in understanding nitrogen signaling in plants (Daniel-Vedele and Caboche, 1993; Scazzocchio, 2000). GATAs were initially defined following studies with the paralogous GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED) and GNL (GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR1) (Richter *et al*., 2010; Behringer and Schwechheimer, 2015).

In Arabidopsis 30 GATA transcription factors are present, they are further subdivided into 4 different sub-families, from A to D (Reyes *et al*., 2004).

GATA-19 belongs to the B-GATA family. This subgroup is defined by their highly conserved DNA binding domain and the presence of either an LLM- or a HAN-domain (Behringer and Schwechheimer, 2015). HAN-domain B-GATAs were first described in the Arabidopsis floral development regulators HAN (HANABA TARANU) and GATA19 (HAN-LIKE) (Zhao *et al*., 2004; Zhang *et al*., 2013). On the other hand GATA23 is a regulator of lateral root initiation from *Arabidopsis* that is closely related to GNC and GNL but has a degenerate LLM-domain that is seemingly specific for the *Brassicaceae* family (CLL instead of LLM) (De Rybel *et al*., 2010). GATA23 is induced by auxin, and it controls lateral root founder cell identity in the *Arabidopsis* root.

Since the expression of *GATA23* is impaired in gain-of-function mutants of the *AUX/IAA* gene *IAA28*, which is defective in lateral root formation, and since IAA28 interacts with several ARFs including ARF7 and ARF19, a model was

proposed, according to which auxin promotes lateral root initiation through degradation of the AUX/IAA IAA28 and subsequent ARF mediated *GATA23* expression (DeRybel *et al*., 2010). HAN containing B-GATA mutants display altered floral identity and impaired embryo development (Zhao *et al*., 2004, Nawy *et al*., 2010).
The biological role of the HAN domain is not clarified yet, but it is speculated that the domain could play a role in protein-protein interactions (Behringer and Schwechheimer, 2015).

3.10 Interactor clone number 5: CIB1.

The basic helix-loop-helix (bHLH) proteins form a large superfamily of transcriptional regulators that are found in organisms from yeast to humans and function in critical developmental processes as well as stress responses (Jones, 2004).
These proteins are defined by displying the bHLH domain which is composed of approximately 60 amino acids (Ferre-D'Amare *et al*., 1993). The basic region, an N-terminal stretch of approximately 15 to 20 residues typically rich in basic amino acids, is involved in DNA binding. Certain conserved amino acids in the basic region determine recognition to the so-called core E-box hexanucleotide consensus sequence 5′-CANNTG-3′, whereas other residues would provide specificity for a given type of E-box (e.g. the G-box [5′-CACGTG-3′]). In addition, flanking nucleotides outside the core have also been shown to play a role in binding specificity (Shimizu *et al*., 1997; Atchley *et al*., 1999; Martinez-Garcia *et al*., 2000; Massari and Murre, 2000). The domain function is to promote protein-protein interactions, enabling the formation of hetero- or homodimers (Massari and Murre, 2000).
Currently 119 bHLH have been mapped in Arabidopsis genome (Bailey *et al*., 2003).
bHLH class is sub-divided in 6 groups, according to their DNA-binding specificities and dimerization potential, conservation of residues in the other parts of the motif, and the presence or absence of additional domains (Murre *et al*., 1994, Ledent *et al*., 2002).

To date, 4 subclades of the bHLH family have been shown to play a role in JA signalling in Arabidopsis. Best characterized is the bHLH IIIe subclade, the members of which positively contribute to the general JA response, such as the centre of JA response, MYCs family (Pauwels and Goosens, 2011, Chini *et al*., 2016). In contrast, the recently characterized bHLH IIId subclade seem to exert a negative role in many JA-related responses. Members of this subclare are the proteins containing a the JA-ASSOCIATED MYC2-LIKE (JAM), the JAMs lack the canonical activator domain present on MYCs, therefore they act as JA response negative regulators (Fonseca *et al*., 2014, Qi *et al*., 2015)

Finally, bHLH IIIf subclade play a major role in JA-mediated anthocyanin accumulation and trichome initiation. Anthocyanin production is regulated by the R2R3 MYB proteins PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1), PAP2, MYB113 At1g66370 and MYB114 (Gonzalez *et al*., 2008, Qui *et al*., 2011). JA-induced trichome formation is driven by the activity of R2R3 MYB proteins MYB23 (At5g40330) and GLABRA1 (GL1) (At3g27920) (Matsui et al., 2008, Zhu et al., 2009)

3.11 Interactor clone 6: EDF3.

At3G25730, corresponding to *ETHYLENE RESPONSE DNA BINDING FACTOR 3* (*AtEDF3*), is among the positive interactors. It is an ethylene-responsive TF that is not well studied.

It belongs to the superfamily of APETALA2/Ethylene Responsive Factor (AP2/ERF). The AP2/ERF family is composed of 4 subgroups: ERFs, AP2, RAV and Soloist (Licause *et al*., 2013). EDF3 belongs to the RAV subgroup, defines by displaying an AP2 domain plus a B3 DNA binding domain (Swaminathan *et al*., 2008, Kagale *et al*., 2010, Licausi *et al*., 2013).

In its amino acid sequence a conserved RLFGV sequence motif , similar to that found in ERF/AP2 repressor proteins (Ikeda and Ohme-Takagi, 2009) was identified at its C-terminal. The presence of this sequence indicated that the *EDF3* gene could encode a transcriptional repressor that operates in presence of ethylene (Chen *et al*.,

2015). Previous studies demonstrated the synergistic connection between JA and ethylene, both during plant development and biotic stress response (Lorenzo *et al*., 2002; Zhou *et al*., 2005; Wunsche *et al*., 2011; Lahlali *et al*., 2014).

3.12 Homology analysis.

In order to try to couple the hits from this screen on Arabidopsis TFs with putative wheat targets, we carried out a basic homology analysis, blasting Arabidopsis hits proteins sequences against wheat proteome. Figures 3.6 to 3.11 represent the results of the protein sequence homology analysis between the positive Arabidopsis TFs and wheat TFs.

The protein sequences of Arabidopsis TFs were recovered from TAIR database and queried in PLANT ENSEMBLE BLAST-release 40 (Siever *et al*., 2011). The amino acid sequences from Arabidopsis was seracehd against the wheat proteome, and the sequences corresponding to the best hits were recovered from PLANT ENSEMBLE-JBrowse. The results are reported in table 3.2. The resulting sequences were fully aligned with the query sequences using Clustal-Omega (Siever *et al*., 2011).

In the figures the scores corresponding to the Blast analysis and the alignment are shown. The displayed scores correspond to: Blast score, E-value and %Identity shown correspond to the results obtained by blasting the Arabidopsis sequences against wheat proteome utilizing Plant-Ensemble Blast v40; the %Identity calc. represents the crude percentage calculated counting the amino acids displaying a perfect match in the 2 sequences. It has to be taken into account that this procedure was performed only to assign priorities among the putative interactors. To ascertain undoubtedly the homology between the proteins further investigation is needed.

The first protein that was analyzed is the C2H2 transcription factor ZAT5. It shows a score of 153, 2.9E-12 E-value and a 81.3% of identity. The %Identity calculated is 20.3% (fig 3.6).

Figure 3.7 display the results of the alignment of ABF1. The score is 245, 2.5E-24 the E-value and a 71% of identity. The calculated percentage correspond to 34.9%.

The transcription factor GATA23 is the one that shows the lowest values in terms of score, percentage of identity, E-value and calculated percentage of identity. The results are: 193 score, 1.5E-18 E-value and 55.7% of Identity and the calculated identity is 15.7% (fig 3.8).
GATA19 alignment scores are: score 220, E-value 2.7E-22 and %Identity 97.4, while calculated identity is of 22.5% (fig 3.9).
bHLH-CIB1 alignment display a score of 446, an E-value of 3.1E-51, a %identity of 80.9; the calculated percentage of identity is 33% (fig 3.10).
The alignment with the highest score is the one regarding EDF3 transcription factor. A score of 379, E-value 1.3E-43 and %Identity of 77.9. Also the calculated percentage of identity is the highest among the different putative interactors: 76%.
The results obtained via Plant-Ensembl BLAST v4 and ClustalOmega need to be validated via further experiments, such as direct interaction assay between the interactors and the target promoter or via silencing the interactor and estimating the target gene expression.

3.13 *TaEDF3* is upregulated upon Septoria infection.

TaEDF3 is the putative interactor displaying the highest score in both the Blast search and the alignment, therefore it has been chosen for further analysis. In order to start assessing *TaEDF3's role* in Septoria wheat defence, an expression analysis of *TaEDF3* transcript level during a Septoria infection time course was carried out. *Lili* and *Santiago* were both infected with virulent Septoria isolate, IPO323 (Arraiano and Brown, 2006). The transcript level of *TaEDF3* was evaluated in *Lili* and *Santiago* via qPCR (fig3.12 and 3.13). The expression pattern of *TaEDF3* appears to be rhythmic in both cultivars, in non infected control condition. This evidence can lead us to speculate that *TaEDF3* could be a gene involved in ethylene response during development (no stresses were applied in this conditions), these findings are consistent with previously published data (Lahlali *et al*., 2014).
The gene rhythmic transcription pattern leads us to think that it is regulated through a negative feedback loop system. During biotic stress, namely Septoria infection in this case, *TaEDF3* expression is upregulated during the early stages of infection (day 6 for

Lili and day 4 for *Santiago*) compared to non infected controls. The upregulation is consistent for both cultivars throughout the infection time-course.

Figure 3.14 indicates the level of expression by qPCR during Septoria infection for both *TaEDF3* and *TaWRKY10* in both cultivars, in order to make comparisons easier.

In *Lili* it is notable that there is an upregulation of *TaEDF3* gene expression of 2-fold change, starting from day 6 post-infection up to day 10. Notably *TaWRKY10* starts to downregulate its transcript level at day 10 in *Lili*, 2 days prior to the switch to necrtrophic growth in Septoria and 2 days after *TaEDF3* upregulation. At 12 days post infection there is the maximum variation of transcription between stressed and control tissue in *Lili*: 6 fold-change higher gene expression in favour of the stressed plant tissue. At day 14 the difference among infected and non-infected tissue is again 2 fold. At day 16 there is a difference of 2.5 fold. Interestingly at day 18 there is no different between infected and control, and this particular time-point is corresponding to the lowest reported level of *TaWRKY10* transcript. At day 20 a 6-fold change difference was detected.

In cultivar *Santiago* there is no perturbation of *TaEDF3* expression for the first 2 days, a 1.5 fold upregulation was detected at day 4 and 2 fold-change upregulation from day 6 (corresponding to the beginning of *TaWRKY10* gene expression drop).

At 8 days post infection *TaEDF3* expression is still 2 times higher in infected tissue compared to its own control, but no difference is detected at day 10. At day 12 there is only 1.5 fold-change difference, at day 14 a 2-fold change. Remarkably at day 16 the transcript level in the infected sample is lower compared to the control, but at day 18 an upregulation of 6 fold-change is detectable in the infected tissue compared to its own non infected control, the same difference is present at day 20. The first *TaEDF3* transcription upregulation peak is 2 days prior to the downregulation of *TaWRKY10* expression, for both cultivars.

As *TaEDF3* contain the repressor motif RLFGV (Ikeda and Ohme-Takagi, 2009), I suggest that this TF is a repressor of *TaWRKY10* expression. Even though during infection *TaEDF3* maintains a rhythmic expression profile, we can conclude that it has a role in immunity response because the upregulation of expression is statistically significant and consistent throughout the time course of this experiment.

3.14 Discussion.

Y1H screening is a widely used method for functional genomics studies (Sun et al., 2017), due to its capability to perform screening of cDNA libraries quickly. In the recent past the technique has improved, since a lot of different arrayed libraries are created and made publicly available by and for the research community (Mitsuda *et al*., 2010; Gaudinier *et al*., 2011; Ou et al., 2011; Castrillo *et al*., 2011). The array libraries enable one to adopt fast protocols (cDNA libraries preparation is avoided), and the avoidance of under-evaluation of less expressed TFs (Castrillo *et al*., 2011). Y1H screening has not been used only in Arabidopsis but as well in crops (Lopato *et al*., 2006; Xu *et al*., 2017) and ornamental plants (Han *et al*., 2017).

As *TaWRKY10* promoter region does not display any particular feature, nor enrichment of specific TFs binding sites, only a TFs library screen could give any detailed information on putative regulators of *TaWRKY10* expression. In this study the Arabidopsis arrayed RR library available from NASC and developed by Castrillo *et al*., 2011 was used.

A 2kb fragment upstream of *TaWRKY10* gene START codon was cloned into pTUY1H plasmid, giving rise to p*TaWRKY10*-pTUY1H construct, in which our target promoter drives the expression of *–HIS3* selection marker.

Mating was carried out successfully, after that the auxotrophic selection on triple drop-out media permitted the isolation of 10 putative positive interactors. The increasing stringency of the selection, with 3-AT supplemented media confirmed the interaction of 6 Arabidopsis TFs with *TAWRKY10* 2kb promoter region.

The 6 above mentioned transcription factors are: ZAT5, ABF1, GATA23, GATA19, CIB1 and EDF3. The common theme in this group seems to be that all of the targets are involved in Arabidopsis development, but they all seem to be connected to the JA response or biotic stresses.

ZAT5 and EDF3 seem to be connected to the ethylene response (Ciftci-Yilmaz and Mittler 2008, Ikeda and Ohme-Takagi, 2009). Previous studies demonstrated the synergistic connection between JA and ethylene, both during plant development and biotic stress response (Lorenzo *et al*., 2002; Zhou *et al*., 2005; Wunsche *et al*., 2011; Lahlali *et al*., 2014).

ABF1 transcriptional regulation activity is triggered by ABA presence (Sarkar and Lahiri, 2013).

It has been repeatedly reported that a functional and active ABA signal transduction machine is essential for a successful JA signal transduction (Anderson *et al*., 2004; Lorenzo & Solano, 2005; Niu, Figueroa, & Browse, 2011; Liu *et al*., 2014; Liu *et al*., 2016). Even though to date the mechanism of the reciprocity between the two pathways is not completely clarified.

GATA19 and GATA23 are respectively involved in flower development regulation and lateral root emergence (Zhao *et al*., 2004, DeRybel *et al*., 2010, Zhang *et al*., 2013). It has been proposed that JA has a key role in flower development (Mandaokar *et al*., 2008, Peng *et al*., 2013, Jibran *et al*., 2017). Even though has been reported that JA is a master regulator involved in root elongation control (Srivastava *et al*., 2018), this hormone is not yet been linked to lateral root formation.

Bioinformatics tools were used to identify putative homologous genes in wheat. Plant-Ensemble v40 BLAST (IWGSC consortium) has been used to isolate proteins that share similarity with the Arabidopsis proteins used as queries. The best BLAST search hit in wheat proteome was aligned with its putative Arabidopsis homologous protein sequence using ClustalOmega (Siever et al., 2011).

It needs to be remembered that all the BLAST algorithms perform only local alignments, therefore it is fundamental to understand that the score they calculate is locally based.

The effect is that sometimes sequences that share maybe only the conserved domains that define them could display a really high score, even if only a small part of the sequences is actually matching (Siever *et al*., 2011).

Due to the above-mentioned fact is important to take into account that the only way to define homology in our case is by performing further experiments to validate functional orthology. These experiments could be: direct interaction assays between the transcription factor and *TaWRKY10* promoter. This could be done as direct Y1H, prior to cloning the chosen transcription factor. Another possibility could be to perform an Electro Mobility Shift Assay (EMSA) (Hellman and Fried, 2009).

Moreover the best method to ascertain if a certain transcription factor activity can perturbate *TaWRKY10* transcript level (or one of the other putative targets) is to generate VIGS lines of the chosen target, and compare *TaWRKY10* transcription level with their own controls.

TaEDF3 was chosen for further study due to its higher BLAST search scores. Analyzing its expression upon Septoria infection we have been able to detect a specific expression pattern in both infected and non-infected conditions. Interestingly the expression is constantly upregulated in the Septoria infected samples. The fact that we have been able to detect differences in *TaEDF3* expression during Septoria infection is an evidence of *TaEDF3* involvement in necrotrophic pathogen response.
TaEDF3 contains the repressor motif RLFGV (Ikeda and Ohme-Takagi, 2009), therefore we can speculate that its role is to repress *TaWRKY10* expression, in order to boost JA signalling. It has been reported that AtEDF3 is activated by the presence of ethylene (Chen *et al*., 2015), we moreover speculate that, in wheat, TaEDF3 activation in infected condition could play a key role in fine tuning the interactions between JA and ethylene pathway, as those two pathways are intimately interconnected and act synergistically in both response to necrotrophic pathogens and response to developmental cues (Lorenzo *et al*., 2002; Zhou *et al*., 2005; Wunsche *et al*., 2011; Lahlali *et al*., 2014).

3.15 Figures chapter 3.

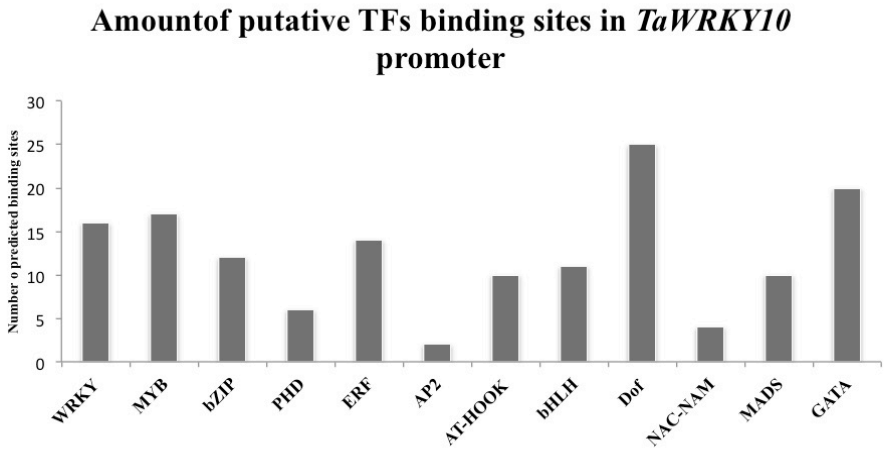


Figure 3.1

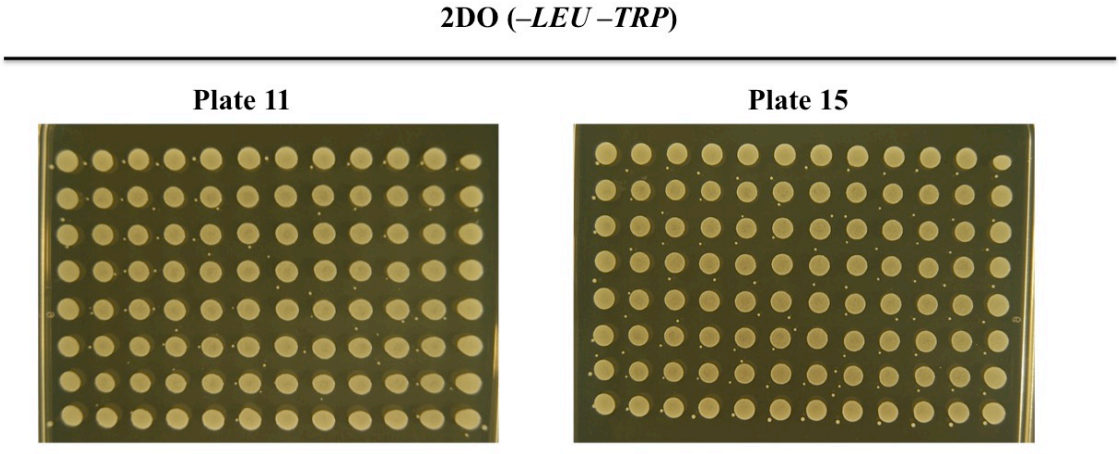


Figure 3.2

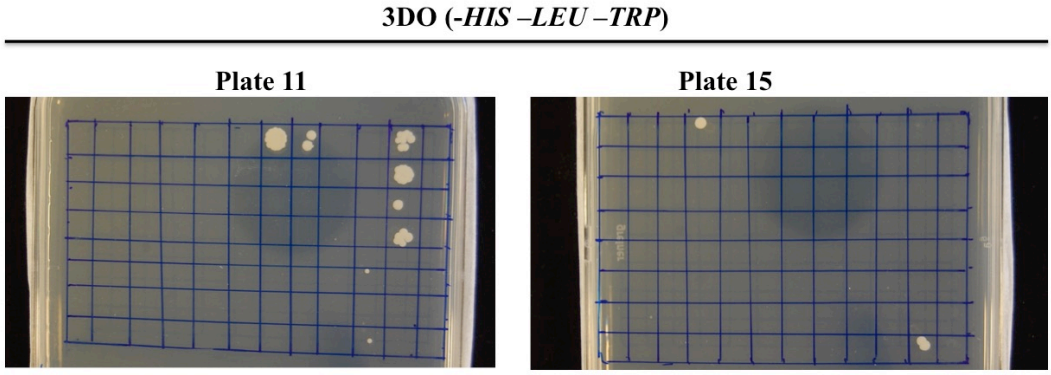


Figure 3.3

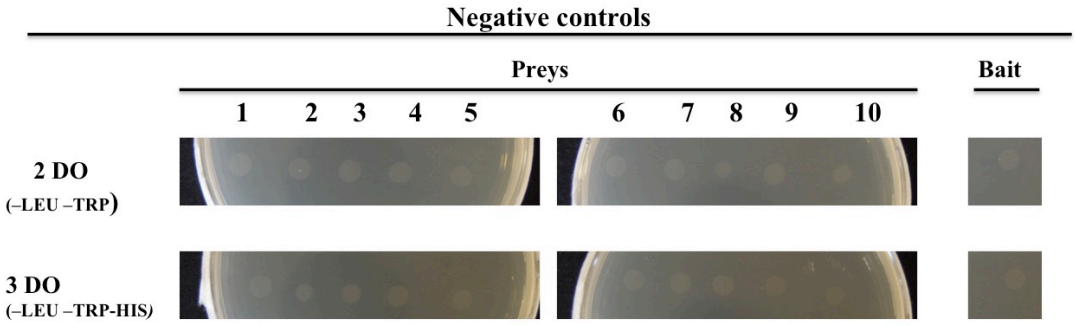


Figure 3.4

NUMBER CODE	GEND ID	Tf	GO/Function
1	At2G45050	GATA2	/
2	At1G08000	GATA10	/
3	At5G57520	ZFP2	NEGATIVE REGULATOR FLORAL ABSCISSION
4	At2G28200	C2H2	/
5	At1G49720	ABF1	ABA-ACTIVATED RESP
6	At5G43700	ATAUX211	AUX-RESPONSE
7	At5G26930	GATA23	LATERAL ROOT DEVELOPMENT
8	At4G36620	GATA19	FLOWER DEVELOPMENT
9	At1G68920	bHLH-CIB1	GROWTH REGULATION
10	At3G25730	EDF3	ETHYLENE ACTIVATED RESPONSE

Table 3.1

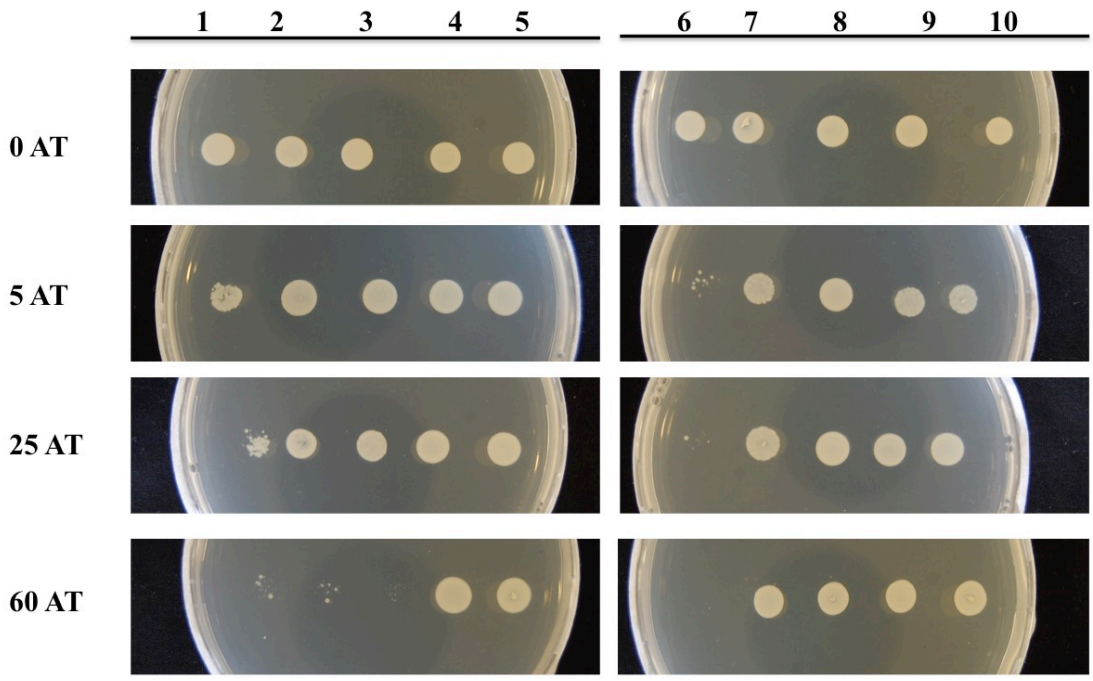


Figure 3.5

Arabidopsis thaliana IDs	*Triticum aestivum* IDs	Gene name
At2G28200	TraesCS6A01G404600	*ZAT5*
At1G49720	TraesCS5A01G237200	*ABF1*
At5G26930	TraesCS6D01G173000	*GATA23*
At4G36620	TraesCS3B01G269000	*GATA19*
At1G68920	TraesCS5A01G250600	*bHLH-CIB1*
At3G25730	TraesCS3B01G293000	*EDF3*

Table 3.2

At2G28200	C2H2-ZAT5

```
AtC2H2      --------MMMGQDE-----VGSDQTQIIKGKRTKRQRSSSTFVVTAATTVTSTSSSAGG       47
TaC2H2      MQASAMELVLFREEGAVDDRLQQRGGAVVKRKRTKRPRHQTPPVAAMA-----------       48
                    ::: ::      : .    ::* ***** * .:  *.: *

AtC2H2      SGGERAVSDEYNSAVSSPVTTDCTQEEEDMAICLIMLARGTVLPSPDLKN----------       97
TaC2H2      -------------CSSASSSESTTTEEEDMAHCLILLAQGAAPPGVVVDSRPPPATVPQE       95
                      . *:  : . * ****** ***:**:*:. *.  :..

AtC2H2      ----------------------------SRK---IHQKISSENSSFYVYECKTCNRTFSS       126
TaC2H2      ARQGTSSLPPAGPLPAPAVVSAKTERYTSRKYTEAATTADGVRAGFYVYECKTCNKCFPT       155
                                       ***      . .. .:.**********: * :

AtC2H2      FQALGGHRASHKKPRTSTEEKTRLP---------LTQPKSSASE----------------       161
TaC2H2      FQALGGHRASHKKPRLAGADDDSAANATSVAIAKLSKPPSTKTATPPLVQMTTASPPPPP       215
            *************** :  :.             *::* *: :

AtC2H2      ---------EGQNSHFKVSGSALASQASNIINKANKVHECSICGSEFTSGQALGGHMRRH       212
TaC2H2      PPQVDAAPDVTTVLSLNNGGSAV---CHAVNTNRLRVHECSICGAEFASGQALGGHMRRH       272
                      :: .***:   .  : .:  :********:**:************

AtC2H2      RTAVTTISPVAATAEVSRNSTEEEIEINIGRSMEQQRKYLPLDLNLPAPEDDLRE-----       267
TaC2H2      RPLHAPADRAIATTAVTAIAAST---TKKEKDSTPAGINLELDLNLPAPSDEECVSHPPP       329
            *   :  . . **: *:  ::.     :  :.       * ********.*:

AtC2H2      -------------------SKFQGIVFSATPALIDCHY      286
TaC2H2      PPPPSSAPPVVLGLGPFDSGKKRLMLTAASAALVDCHY      367
                         .* : :: :*: **:****
```

Score	E-value	%Identity	%Identity calc
153	2.9E-12	81.3	20.3

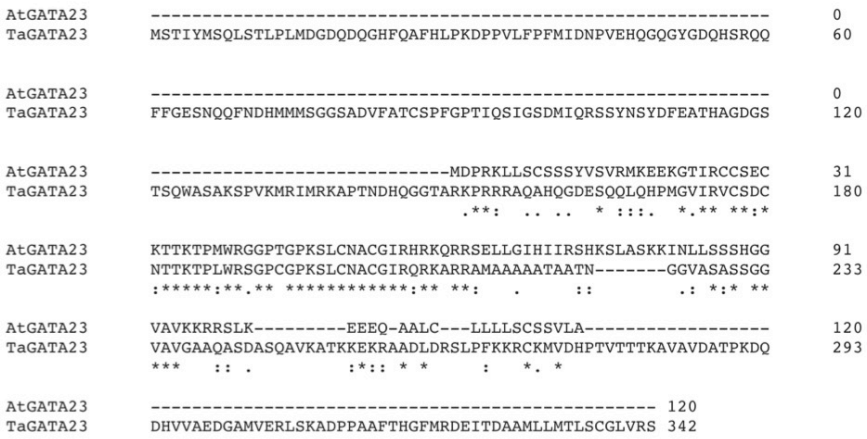
Figure 3.6

At1G49720	ABF1

Score	E-value	%Identity	%Identity calc
245	2.5E-24	71.0	34.9

Figure 3.7

At5G26930	GATA23



Score	E-value	%Identity	%Identity calc
193	1.5E-18	55.7	15.7

Figure 3.8

Figure 3.8

At4G36620	GATA19

```
AtGATA19      ------------------MGFSMFFSPEN-----------DVSHHSSPYASVDCTLSLGT      31
TaGATA19      MLHEAAPCTCGLLYGSCGGGCSMLFAAAPGDHHYYSKQCGDDGSYASYGGSVDCTLSLGT      60
                           * **:*:             * . ::*  .**********

AtGATA19      PSTRLCNEDDERRFSSHTSDTIGWDFLNG--------SKKGGGGGGHNLLARRCANCDTT      83
TaGATA19      PSTRRAEAG------------LPWEAVPSCNGRQDIGPARADQSNAGAASARRCANCDTT      108
              **** .: .            : *: : .          :.. ...    **********

AtGATA19      STPLWRNGPRGPKSLCNACGIRFKKEERRASTARNSTSGGGSTAAGVPTLDHQASANYYY      143
TaGATA19      STPLWRNGPRGPKSLCNACGIRYKKEERRAAAAAVAPTA----------LASDSGIEYAY      158
              **********************:*******::*  : :.          *  ::. :* *

AtGATA19      NNNNQYASSSPWHHQHNTQRVPYYSPANNEYSYVDDVRVVDHDVTTDPFLSWRLNVADRT      203
TaGATA19      GYAR--------QQQQQQQQWGCYGPAVAKAASY--GMFGDAATEDGPCLPWGLGVMPSS      208
              .  .        ::*:: *:   *.**  : :      . *  .  .* * * *.*   :

AtGATA19      GLV---HDFTM-----  211
TaGATA19      PAFGSVREMTSLFQYY  224
                .   :::*
```

Score	E-value	%Identity	%Identity calc
220	2.7E-22	97.4	22.5

Figure 3.9

At1G68920	bHLH-CIB1

```
AtCIB1        MDLSAKDEFSAEKRNPDNYDSVNNPSG----DWRVDSYPSENLISAGPASCSPSQMMDSF       56
TaCIB1        MDMNESSEKGMEG------NGSSGPGGGIPVEWQSQFSGGGG-FSA-HHHQQHPHMMDSF       52
              **:. ..* . *       :. ..*.*    :*: :   . . :**     .  :*****

AtCIB1        GQTLWYDPTSVQAVGYAGFNGGNASSSSFRGSIDRSLEMGWNLPNLLPPKGNGLFLPNAS       116
TaCIB1        ASGMWPAAS-------------------------------------QHGAGFLAP-VP       72
              .. :*   :                                       :* *:: * .

AtCIB1        SFLPPS--MAQFPADSGFIERAARFSLFSGGNFSDMVNQPLGNSEAIGLFLQGGGTMQGQ       174
TaCIB1        GFLPPPGLGGHFPVDSGFIERAARSSCFVGPGSGGGM-------MGAGAFGGAGDQHMGS       125
              .****    .:**.********** * * * . .. :        . * *  .*.   *.

AtCIB1        CQSNELNVGEPHNDVSVAVKESTVRSSEQAKPNVPGSGNVSEDTQSSGGNGQKGRETSSN       234
TaCIB1        A------FGEGY------LDHRRKEGGDKAEPELAGSGGVPSSEAAGGDCSSKGSD----       169
              .      .** :      :..   ...::*:*:: ***.* ..  :.*. ..** :

AtCIB1        TKKRKRNGQK-NSEAAQSHRSQQSEEEPDNNGDE-KRNDEQSPNSPGKKSNSGKQQGK-Q       291
TaCIB1        SKKRRRPSEVMGGDQVQSS--NVAADSANESAQSKDKGEESSPAT-GT-TTGGKSKGKGA       225
              :***:* .:  ..: .**   : : :. ::..:. .:.:*.** : *. :..**.:**

AtCIB1        SSDPPKDGYIHVRARRGQATNSHSLAERVRREKISERMKFLQDLVPGCNKVTGKAVMLDE       351
TaCIB1        KESSEKEDYIHVRARRGQATNSHSLAERLRREKISERMKLLQDLVPGCSKVTGKAVMLDE       285
              ...  *:.********************:**********:*********.***********

AtCIB1        IINYVQSLQRQVEFLSMKLATVNPQMDFNLEGLLAKDALQLRAGSSSTTPFPPNMSMAYP       411
TaCIB1        IINYVQSLQRQVEFLSMKLATVNPRLDLNIEGLLSKDLLRFPGVSSSSMGFSPEMMHPQL       345
              ************************::*:*:*****:** *:: . ***:  * *:*

AtCIB1        PLP-HGFMQQTLSSIG------RTITSPLSPMNGGFKRQETNGWEGDLQN-VIHINYGAG       463
TaCIB1        QLSQPGLMQGGAAAMANSDVFRRIMQAQLGAKDG-SHSQMAHALNGPFSDHVAQMAYPSM       404
               *   *:**   :::.      * : : *.  :*  : * ::. :* :.: * :: * :

AtCIB1        DVTPDPQAAATASLPAANMKVEP-------      486
TaCIB1        G---------SSHSHSQDLSIRPSQDAYQM      425
              .         ::   : ::.:.*
```

Score	E-value	%Identity	%Identity calc
446	3.1E-51	80.9	33

Figure 3.10

At3G25730	EDF3

```
AtEDF3      MDAMSSVDESS----TTTDSI--PARKSSSPASLLYRMGSGTSVVLDSEN-------GVE        47
TaEDF3      MDSTSCLADDTSSGGASTDKLKALAAAAAAAAAGPLERMGSGASAVLDAAEPGSEADSGGA       60
            **: *.: :.:    ::**.:   *  ::: *. * *****:*.***: :       *

AtEDF3      VEVEAESRKLPSSRFKGVVPQPNGRWGAQIYEKHQRVWLGTFNEEDEAARAYDVAAHRFR       107
TaEDF3      GAGRAAAGKLPSSRFKGVVPQPNGRWGAQIYERHQRVWLGTFAGEADAARAYDVAAQRFR       120
               .* : ************************:*********  * :*********:***

AtEDF3      GRDAVTNFKDT-----TFEEEVEFLNAHSKSEIVDMLRKHTYKEELDQRKRNRDGNGKET       162
TaEDF3      GRDAVTNFRPLADADPDAAAELRFLAARSKAEVVDMLRKHTYFDELAQSKRAFAASAALS       180
            ********:           *:.** *:**:*:********* :** * **   ...  :

AtEDF3      TAF----ALASMVVMTGFKTAELLFEKTVTPSDVGKLNRLVIPKHQAEKHFPLPLGNNNV       218
TaEDF3      APTTSRGADGHASTPSPAAAREHLFDKTVTPSDVGKLNRLVIPKQHAEKHFPLQLPAAGG       240
            :      * .   . :   : * **:*******************::******* *   .

AtEDF3      SVKGMLLNFEDVNGKVWRFRYSYWNSSQSYVLTKGWSRFVKEKRLCAGDLISFKRSND--       276
TaEDF3      ESKGLLLNFEDAAGKVWRFRYSYWNSSQSYVLTKGWSRFVKEKGLGAGDVVGFYRSAAGS       300
            . **:******. ***************************** * ***::.* **

AtEDF3      --QDQKFFIGWKSKSGLD-----------LETGRVMRLFGVDISLNAVVVVKETTEVLMS       323
TaEDF3      TGEDTKLFIDCKLRPDTNSPASADPVDQSAPVQKAVRLFGVDLLTAPASP-------EQG       353
              :* *:**. * : . :            . :.:******:    .          .

AtEDF3      SLRCKKQRVL--------------------      333
TaEDF3      MPGCKRARDLVKSPPPKVAFKKQCIELALA      383
               **: * *
```

Score	E-value	%Identity	%Identity calc
379	1.3E-43	77.9	76

Figure 3.11

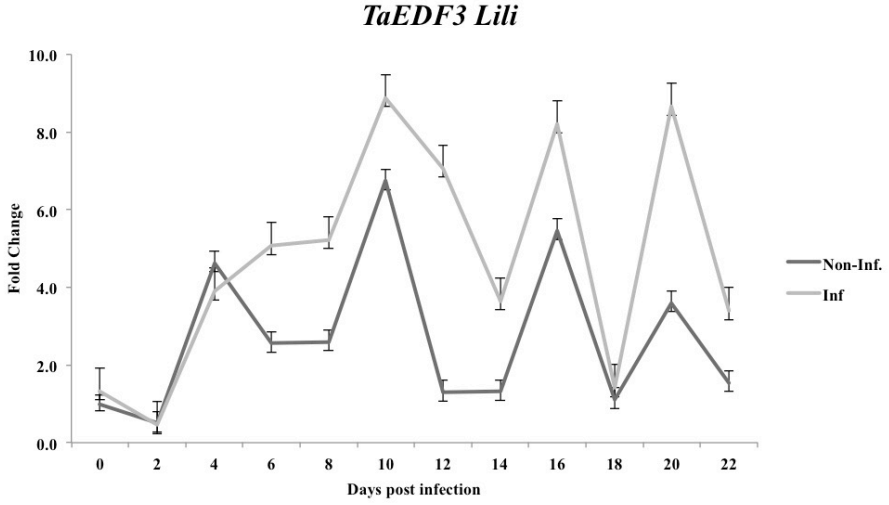


Figure 3.12

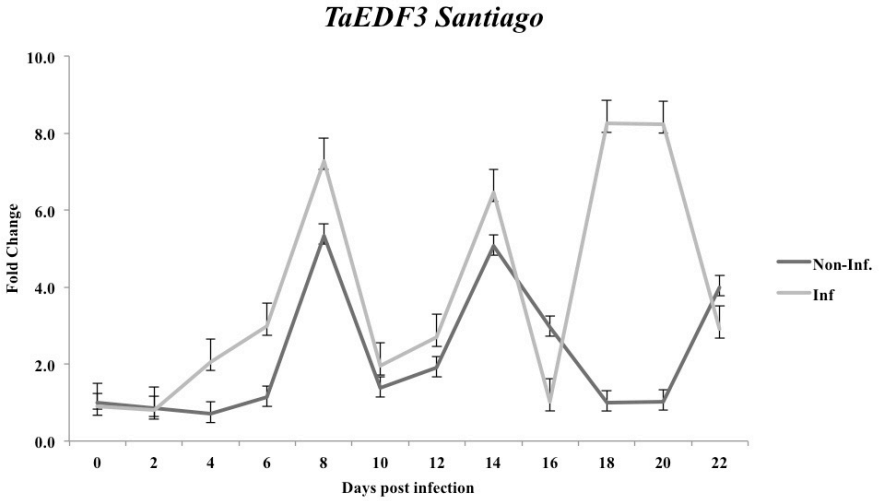


Figure 3.13

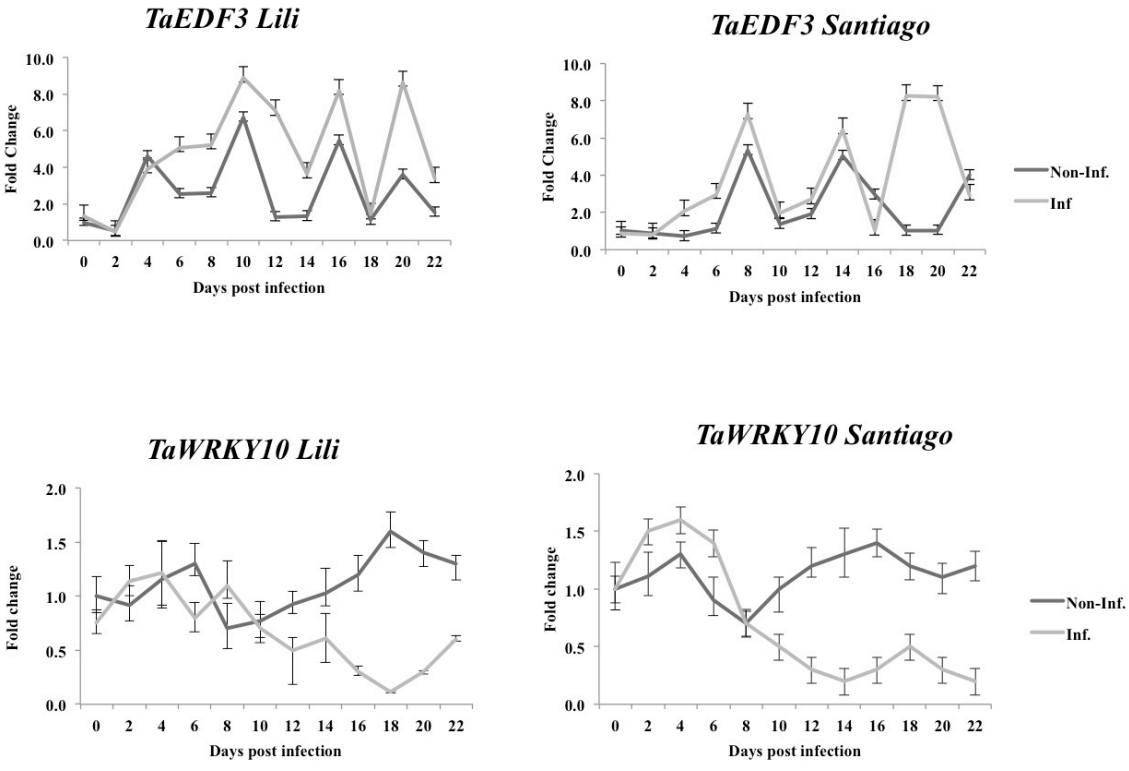


Figure 3.14

3.16 Figures Legend chapter 3.

Fig 3.1 cis element analysis of the *TaWRKY10* promoter region.

A 2kb fragment of the region upstream of the START codon of *TaWRKY10* was submitted to the free online available software PLACE (Plant cis-acting regulatory DNA elements by Higo *et al*., 1999). The TFs binding site was quantified and plotted. The most represented binding sites are those corresponding to WRKY, MYB, DOF, ERF and GATA TFs binding sites.

Fig 3.2 Mating control on double drop-out media.

As described by Castrillo *et al*., 2011 the mating between preys and the bait has been carried out in liquid YPAD rich media, and then plated on synthetically defined AGAR medium. As the preys carry a *–TRP* and the bait *–LEU* auxotrophic markers, the cultures was plated on DOB-LEU-TRP, letting growth only on mated cultures. The figure shows that both in plate 11 and 15 all the clones have grown, proving the success of the mating procedure.

Fig 3.3 10 prey clones are able to activate *–HIS3* reporter gene.

The positively mated clones was replica-plated on triple auxotrophic selection medium. *TaWRKY10* promoter drives the expression of *-HIS3* gene, therefore the selection for interactions was carried out on triple drop-out media (DOB-LEU-TRP-HIS). 10 positive interactions were isolated.

Fig 3.4 Negative controls.

The positive preys from the RR library as well as the bait only has been plated on double drop-out (DOB-LEU-TRP) as well as triple drop-out (DOB-LEU-TRP-HIS). Those controls were carried out in order to rule out the possibilities of leaky expressions on any of the auxotrophic markers gene as well the possibility of contaminations.

Table 3.1 Summary of positive interactors.

Table provides the list of the 10 positive interactors, with gene ID, gene name and GO terms associated with it if available. Enlighted in green the 6 positives to 3-AT treatment.

Fig 3.5 Six interactors are positive to 3-Amino-1,2,4-triazole (3-AT) screen.

In this procedure is essential to re-plate the positive interactions on triple drop-out plates supplementit ed with increasing amount of 3-Amino-1,2,4-triazole. A liquid subculture of the 10 positive interactors has been made in triple drop-out, and then drop-inoculated on plates supplemented with increasing concentrations of 3-AT as shown in figure. After two days 6 colonies were still able to grow on the higher concentration of 3-AT, allowing us to conclude that those are the strongest interactors of our promoter target region.

Table 3.2 Arabidopsis-wheat correspondences.

Once the prey-bait has been validated, Arabidopsis sequences has been recovered from TAIR database and then blasted against wheat proteome. The table is reporting the best BLAST hits obtained via PLANT-ENSEMBL v40 release (IWGSC).

Figs 3.6-3.10 protein alignements wheat-arabidopsis sequences.

The protein sequence alignement between the Arabidopsis positive interactor and the correspondant wheat best Plant Ensembl Blastv40 is shown. The alignement has been performed utilizing Clustal Omega (Sievers *et al*., 2011).

The score, E-value and %Identity shown correspond to the results obtained by blasting the Arabidopsis sequences against wheat proteome utilizing Plant-Ensemble Blast v40; the %Identity calc. represents the crude percentage calculated counting the amminoacids displaying a perfect match in the 2 sequences.

3.6. The alignement between Arabidopsis C2H2-ZAT5 and its wheat correspondant display a score of 153, a 2.9E-12 E-value and a 81.3% of identity. The %Identity calc is 20.3%. **3.7.** ABF1 alignement show as results 245, 2.5E-24 and 71.0% for score, E-value and %Identity respectively. The calculated percentage correspond to 34.9%. **3.8.** GATA23 analysis display the lowest score among the group analyzed: 193 score, 1.5E-18 E-value and 55.7% of Identity and the calculated identity is 15.7%. **3.9**. The output of the alignement of AtGATA19 against TaGATA19 harvests the subsequent results: score 220, E-value 2.7E-22 and %Identity 97.4, while calculated identity is of 22.5%. **3.10.** bHLH-CIB1 alignement display a score of 446, an E-value of 3.1E-51, a %identity of 80.9; the calculated percentage of identity is 33%. **3.11.** The blast search against wheat proteome and the subsequent alignment between Arabidopsis and wheat EDF3 protein is the one that show the highest score. A score of 379, E-value 1.3E-43 and %Identity of 77.9. Also the calculated percentage of identity is the highest among the different putative interactors: 76%.

Fig 3.12 *TaEDF3* transcription is constantly upregulated during Septoria infection in cultivar *Lili*.

TaEDF3 expression analysis has been carried out comparing non-infected and infected *Lili* leaves. A wavy pattern can be identified in non-infected controls tissues, suggesting a role in development and a negative feedback loop based regulation.

If infected the plant tissue show an upregulation of *TaEDF3* mRNA level, the rhythm in conserved, but is visible an increase of transcript of at least 2-fold, starting from day 8. Notably at 18 days post infection the infected and the control tissues display

the same amount of *TaEDF3* transcript, but it increase again suddenly at 20 days post infection.
Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 3.13 *TaEDF3* mRNA level is higher during Septoria infection in cultivar *Santiago*.
In order to establish if *TaEDF3* plays a key role during Septoria defence in wheat cultivar *Santiago*, a qPCR transcript analysis has been carried out throughout a 22 days infection time-course. The expression pattern remains similar to that of a non-infected tissue, but with a slight but constant upregulation in all of it, a part on day 16 post infection. Interestingly between day 18 and 20 in both infected and non infected samples the transcript level remain the same within the same sample, but in the infected tissue we detected a TaEDF3 expression 8 fold change higher compared to the control.
Error bars, ±standard error (SE) of the mean of raw data for 3 biological replicates.

Fig 3.14 Comparison of transcript levels of *TaEDF3* and *TaWRKY10* for both cultivars *Lili* and *Santiago.*
In order to facilitate reading and data interpretation an extra figure has been created reporting together the expression patterns of *TaEDF3* and *TaWRKY10* for both cultivars *Lili* and *Santiago. TaWRKY10* data has been collected from the previous chapter.

Discussion.

World population is rapidly growing, consequently the food demand worldwide is dramatically increasing. The Food and Agriculture organization of the United Nations (FAO) estimates that 70% increase in food production is needed by 2050. Agriculture plays a key role in food production, therefore plant science plays a fundamental part in this major challenge.

Triticum aestivum (bread wheat) is one of the major food sources in many parts of the world, providing approximately 20% of calories consumed by humans (Dvorak and Akhunov 2005). This aspect makes clear that it is fundamental to perform in depth investigations utilizing wheat as a model plant, in order to generate knowledge that could be rapidly translated to elite cultivar improvement.

Zymoseptoria Tritici (also known as *Mychosporella Graminicola*, *Septoria tritici* or commonly known as Septoria) is the causal pathogen of Septoria Tritici Blotch (STB), one of the most threatening wheat foliar diseases across temperate regions, causing up to 40% yield loss if untreated with fungicide (Orton *et al.,* 2011). Due to its considerable genome plasticity Septoria is indeed a rapidly evolving pathogen (Siah *et al*., 2013). This trait enables the pathogen to quickly overcome the genetic resistance of elite wheat varieties (Kettles and Kanyuka 2016). Understanding the molecular mechanisms that underpin Septoria-wheat interaction will be crucial for generating new control strategies against STB.

Plant hormones are essential regulators of growth and immunity (Shinegaga *et al*., 2016). In particular, Jasmonic acid (JA) plays a central role during defence against

necrotrophic pathogens (Okada *et al*., 2015). JA-dependent defence has been widely studied in the model system *Arabidopsis thaliana* challenged with the necrotrophic pathogen *Botrytis cinerea* (Zhang *et al*., 2017, Wang *et al*., 2017, Smirnova *et al*., 2017) and in *Oryza sativa* against *Magnaportha oryzae* (reviewed by Nasir *et al*., 2017). It is widely accepted that growth regulation is intimately and inversely linked to plant immunity (Bergelson and Purrington, 1996).

JA in recent years is emerging as a critical agent acting to modulate the growth–defence trade-offs (Bodenhause *et al*., 2007; Pauwels *et al*., 2008; Zhang *et al*., 2008). Therefore JA and its signalling pathway components are good targets to identify mechanisms to improve resistance against pathogens without paying the plant fitness costs associated with defence activation. Unlike Arabidopsis or rice, the knowledge on JA pathway and JA-triggered events are not well studied in wheat.

WRKYs transcription factor gene family is one of the largest and plant specific transcriptional regulators (Bakshi and Oelmuller, 2014). WRKYs are part of an intricate plant signaling web and regulate multiple pathways from stress responses to growth (Bakshi and Oelmuller, 2014). It has been established through multiple studies that WRKY Tfs are central regulators of innate plant immunity in plants (Rushton *et al.,* 2010).

Here we have been able to identify two members of the WRKY family in wheat and establish their role in immunity against Septoria. For one of them, namely *TaWRKY10*, we have been able to identify the hormonal pathway that is affected by its activity, along with 6 putative regulators of WRKY10 transcription.

Initially we identified and mapped the WRKY gene family in the wheat genome.

It is important to state that during this procedure the information available on the wheat genome and proteome was limited. Genomic databases and search algorithms that are freely available use incomplete genome information. Therefore in order to obtain a complete set of WRKYs in wheat we had to utilize and compare different databases such as PLANTTFDB, NCBI GenBank, Plant-Ensemble (Jin JP *et al*., 2013, Benson *et al*., 2005, Kinsella *et al*., 2011). Consequently we have been able to isolate a total of 135 amino acid sequences possessing a WRKY domain via BLAST search algorithm.

Only 71 WRKY genes had complete DNA sequences. In the near future a similar bioinformatic analysis will result in a much better and more complete genomic

information on the WRKY gene family. A new and complete assembly of wheat genome and proteome has just been released by IWGSC in August 2018, and all the information have been gathered together in Plant-Ensemble v40 website(Kersey *et al*., 2018). This is going be the best genomic tool in the future to gain insight into wheat genomic and proteomic information.

Literature and bioinformatics analysis enabled us to generate a shortlist of WRKYs likely to be involved in immune response. Experimentally 2 WRKY targets were identified as major role players during Septoria infection of wheat, namely *TaWRKY13* and *TaWRKY10*.

TaWRKY13 shows a clearly perturbated transcription upon Septoria infection.

However when *TaWRKY13* VIGS-silenced lines were generated, the phenotype detected, even though statistically significant, was only marginally different compared to the controls. In particular the knock-down of TaWRKY13 lead to only a small increase in susceptibility, a phenotype that is different from reported effects on Septoria resistance of other genes knock-down (Lee *et al*., 2015, Millyard *et al*., 2016). Thus we speculate the existence of an another gene/s orthologous to *TaWRKY13*, function which is compensating for the decrease in TaWRKY13 activity. This is definitely one of the scenarios where the new IGWSC gene assembly could be really helpful in order to boost better our understanding of WRKY gene family function.

On the other hand *TaWRKY10* shows a more prominent role during plant immunity.

TaWRKY10 is a *poieadae*- specific WRKY, it is present only in wheat and barley.

Barley is particularly susceptible to a fungal pathogen closely related to Septoria: *Rhynchosporium commune*. Rhyncosporium is the causal agent of barley leaf blotch.

It is important to note that both Septoria and Rhyncosporium are hemibiotrophic fungus, with the same life cycle, and both are specific to wheat and barley respectively (Zhan *et al*., 2008, Walters *et al*., 2012). Therefore we speculate that *TaWRKY10* and *HvWRKY10* both evolved in order to influence Septoria and Rhyncosporium induced immune response in wheat.

TaWRKY10 is downregulated prior to Septoria's switch to necrotrophic growth.

Silenced lines that knock down *TaWRKY10* transcript level display an increased resistance to Septoria, reducing its sporulation capability therefore impairing the pathogen fitness, even though the necrosis in infected leaves gets anticipated compared to the controls.

Wheat grain filling is a critical step to obtaining high yields (Monpara, 2011).
The majority of the photosynthetic potential needed in this phase is mainly produced by the flag leaf (Sanchez-Bragado *et al*., 2014, Borril et al., 2015, Carmo-Silva et al., 2017). In fact Septoria becomes a real threat to the yield when it reaches the flag leaf (Ponomarenko *et al*., 2011). Therefore limiting Septoria sporulation, even at the cost of diminishing the plant photosynthetic potential during the seedling stage via reducing *TaWRKY10* expression, could be an interesting crop protection strategy to investigate further. Moreover *TaWRKY10* silenced lines were phenotypically similar to Arabidopsis plants artificially supplemented with JA or genetically hypersensitive to JA (Chini *et al*., 207, Srivastava *et al*., 2018).
Further investigations on the transcriptional profile of specific components of the JA signalling pathway on the silenced lines, will allow us to demonstrate that *TaWRKY10* exerts its activity via regulating *TaCOI1* receptor transcription. In particular TaWRKY10 is a transcriptional repressor of *TaCOI1* expression, limited *TaCOI1* expression leads to JA insensitivity (Gossens *et al*., 2016). *TaWRKY10* silencing leads to a higher transcription of TaCOI1, which in turns leads to a higher activation of all JA pathway, therefore an enhanced resistance against necrotrophic pathogens (Yang et al., 2012, Chini *et al*., 2017). To date it is not demonstrated yet if TaWRKY10 transcription factor acts directly on *TaCOI1* promoter or if the interaction is indirect.
In order to better understand *TaWRKY10* and JA biological roles in wheat we used to two different wheat varieties in parallel for our experiments. The two varieties were chosen based on their variability in Septoria resistance and in *TaWRKY10* transcriptional profile.
The variety *Lili* (low *TaWRKY10* transcript levels) is more resistant to Septoria than the variety *Santiago* (high *TaRWKY10* transcript levels), mirroring the publicly available field scoring data (AHDB recommended list for cereals and oilseed 2017/18). Utilizing two different varieties with different traits, we have been able to demonstrate that their different degree of susceptibility to Septoria is correlated with their *TaWRKY10* expression, which leads to a higher or lower JA pathway activation.
To further uncover the pathway that regulates JA signalling upstream of TaWRKY10, Y1H screen was performed. Y1H enables us to determine which transcription factors specifically interact with a given promoter region of a gene (Kim *et al*., 2003).
Taking advantage of a publicly available arrayed Arabidopsis transcription factors library (Castrillo et al., 2011), the screen was performed utilizing *TaWRKY10*

promoter region. Six putative interactors were isolated, all of them correlated with developmental cues connected to phytohormone signalling . Each one of them seem to possess a putative homolog in wheat, but their activity needs to be further investigated in order to understand their specific roles in mediated any potential effects on Septoria defence.

Among the identified interactors we have major player in developmental response as well as biotrophic stress response, all of them driven by different but correlated hormonal activity.

At this stage we decided to further investigate only one of them, the one that showed the higher score after our homology analysis: *TaEDF3*.

We examined the transcriptional profile of *TaEDF3* during Septoria infection. *TaEDF3* mRNA level is always higher in infected sample compared to non-infected controls. This is only a first step to validate its role, but this very promising result asserts the potency of Y1H screening as well the possibility to perform Y1H utilizing well established resources meant to be used in Arabidopsis and then generating parallelism with wheat.

Taken all the results together we can affirm that the main objectives of this thesis work have been accomplished.

We have been able to identify and map WRKYs in wheat genome, and investigate in depht the function of one of them, utilizing both new and well established molecular and phenotyping techniques.

Moreover we established an inverse correlation between *TaWRKY10* expression in control condition and Septoria resistance. This demonstrated intimate relationship can be exploited to assess quickly the performance of novel varieties prior the field experiments.

In any case larger scale validating experiments need to be performed to further enhance the specificity and sensitivity of the proposed tool.

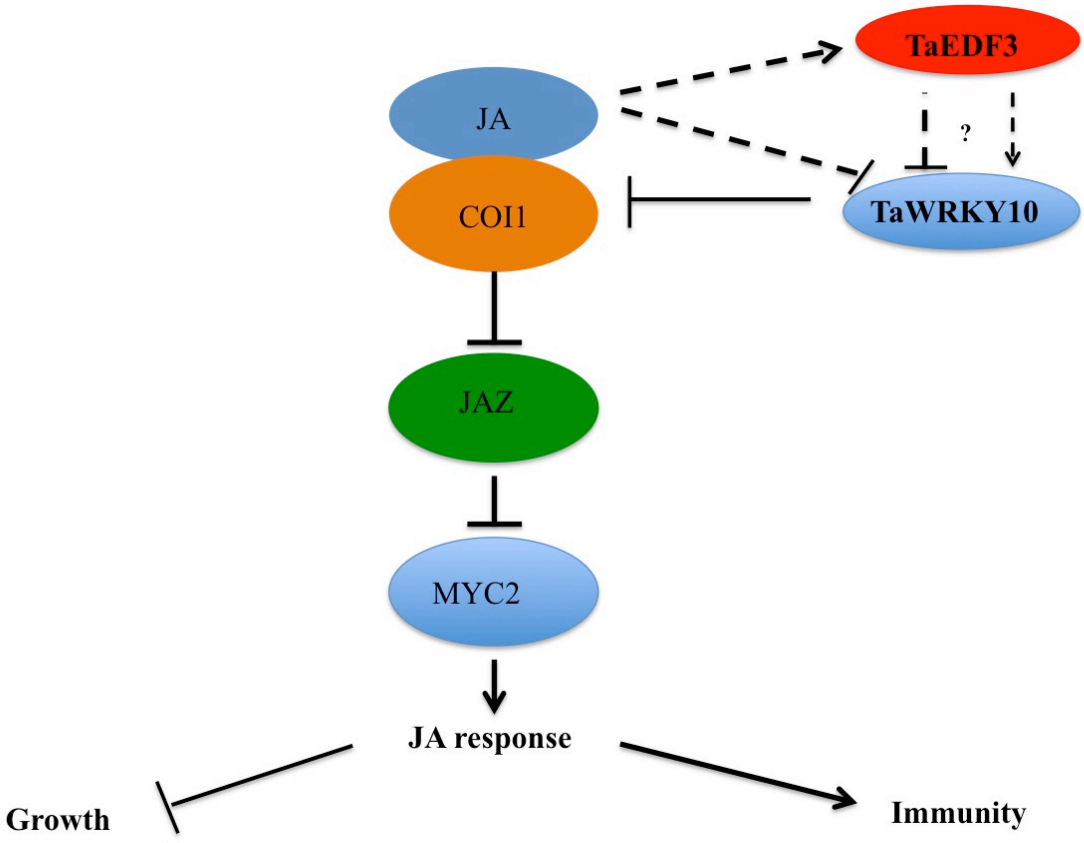
Putative upstream targets regulating *TaWRKY10* expression have been identified, but further studies are required.

To date in depht studies that investigate the sensitivity of different varieties to phytohormones are lacking.

With this work we have been able to establish a precise correlation between hormone sensitivity and immunity in wheat.

We are confident that innovative breeding programs that target both JA production and perception can improve the elite varieties output both in terms of pathogen resistance and yield.

Subsequently we present a model resuming this work.



Experimental procedures

Plant materials and growth conditions

Nicotiana benthamiana plants were grown in environmentally controlled cabinets at 24°C at long day conditions (16 h : 8 h, light:dark cycles). *Triticum aestivum* cvs KWS *Lili* and KWS *Santiago* plants were grown in a temperature controlled room at 24°C at long day conditions (16 h : 8 h, light : dark cycles).
The samples for transcription analyses were collected at 8 hour into the 16 hours light cycle.

Quantitative Real-time PCR (qRT-PCR)

All quantitative real-time polymerase chain reactions (qRT-PCRs) were performed in a 10 μl volume reaction containing Agilent SYBR Green PCR Master Mix (Qiagen) with (1 : 10 v/v) first-strand cDNA as template. The reactions were carried out in a ABI one step plus machine (Applied biosysthem). The endogenous control mRNA used for normalization were those encoding *TaEF1* and *Ta26S*, the geometric mean of the Ct value of the two were used to normalize the samples. Three independent

biological repeats and three independent technical replicates were performed for each of the time points. Primers used are listed in Supplementary table1.

Pathotests

Zymoseptoria tritici isolate IPO323 was used for all pathology assays as described previously by Keon *et al*., 2007. Spores were grown on YPD (yeast extract peptone dextrose plates) for 7 d at 18°C. For plant infection, spores were suspended in water containing 0.1% (v/v) Tween20 at a density of 7.5 x 10^6 spores ml^{-1}. Wheat plants were inoculated with Septoria spores 15 days after VIGS silencing. Replicates of five leaves each were used to determine the de novo spore production within pycnidia after 28 days of infection. Spores counts was carried out using a light microscope and a haemocytometer after spores washing as previously described by Lee *et al.,* 2014. Area Under the Desease Progression Curve has been obtained on the average leaf blotched area displayed by the leaves, and calculated using a AUDPC calculation R package.

Sequence analysis

To retrieve genes and protein sequences used in this study the public available databases GenBank, SwissProt, PLANTTFDB, NcbiBlast, Plant-Ensembl were used (Jin JP *et al*., 2013, Benson *et al*., 2005, Kinsella *et al*., 2011, Kersey *et al*., 2018). Domain sequences were aligned using ClustalOmega (Sievers *et al*., 2011).

Virus induced gene silencing (VIGS)

Reverse genetics studies in *Triticum aestivum* were carried using the virus-induced gene silencing (VIGS) platform (Baulcombe, 1999). based on the Barley Stripe Mosaic Virus (BSMV) as previously described (*Yuan et al*., 2011). Two independent silencing fragments were designed for each gene, in order to minimize the off-target effect. Primers used to generate the BSMV construct are listed in Supplementary table1. At the moment of silencing wheat seedlings were 10 days old.

Hormone treatments

Wheat seeds were germinated on Murashige and Skoog medium (Duchefa) and grown for 3 days. After germination the seedling were moved individually into glass test tubes containing either MS or MS supplied with Jasmonic acid (Sigma) at the concentration of 10 μ M. Samples for transcriptional analysis and pictures to evaluate root elongation were taken daily. Three independent biological replicates have been performed. Root growth inhibition was calculated using the software ImageJ. Regarding the root growth evaluation, 25 plants each treatment for each genotype have been analyzed.

Root growth measurement

To evaluate the effect of *TaWRKY10* silencing on root growth, wheat seeds were germinated on plates as previously described, and then moved to sand pots. The nutrients were supplied using ½ Hoagland. 7 weeks after germination the sand was removed by washing with tap water and pictures were taken and analyzed using ImageJ software.

Anthocyanin relative quantification.

Anthocyanin extraction and quantification has been done collecting 3 gr of tissue each sample, then extraction and quantification has been done as previously described by Neff *et al*., 1998. Three biological replicates have been performed, with three technical replicates each. Anthocyanin extraction has been performed by incubating the samples overnight in 150 µL of methanol acidified with 1% HCl. After addition of 100 µL of distilled water, anthocyanins and clorophilles were separated by adding 250 µL of chloroform.
Total anthocyanins were determined by measuring the$A_{530}$and A_{657} of the aqueous phase using a spectrophotometer (Gene Quant 1300, GE). By subtracting the A_{657} from the A_{530}, the relative amount of anthocyanin per sample was calculated. The experiment has been performed for 3 biological repeats, with 2 technical repeats for each biological replica.

***TaWRKY10* promoter cloning.**

TaWRKY10 regulatory region sequence was obtain by free online software JBrowse implemented by TGAC (reclover 2.1 assembly). A pair of primers was designed to amplify a region 2 kb upstream of the gene START codon (primers are listed in Primer table T1). The primers were designed with restriction sites of *XmaI* and *XbaI* respectively for forward and reverse primer, to clone the desired region upstream the *HIS3* reporter gene. Genomic DNA extracted from *Lili* was used as template.
Amplification has been carried out with Q5 proof-reading enzyme (NEB) as the manufacturer's instructions. The resulting product, once tested via gel electrophoresis for correct size, was gel-purified via Zymo clean PCR recovery kit (Zymo). After a double digestion with the restriction enzymes (NEB), performed following manufacturer's guidelines, the amlicon was ligated (via T4 DNA ligase, NEB) into pTUY1H vector, previously digested with the same restriction enzymes. The resultant construct, pTUY1H-p*TaWRKY10*, has been transformed in *E. coli* DH5α strain, and

plated onto LB supplemented with ampicillin for selection. Positive colonies were sequenced.

***Saccharomyces cerevisiae* transformation.**

A single sequencing-positive clone was used to transform *S. cerevisiae* Y187α, using the small scale LiAC method. The method is schematically described below, including the preparation of *S. cerevisiae* competent cells, as they need to be freshly prepared and transformed:

1. Inoculate 1 ml of YPD or SD with several *S. cerevisiae* colonies, 2–3 mm in diameter.
2. Vortex vigorously for 5 min to disperse any clumps.
3. Transfer this into a flask containing 50 ml of YPD or the appropriate SD medium.
4. Incubate at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD_{600}>1.5).
5. Transfer 30 ml of overnight culture to a flask containing 300 ml ofYPD. Check the OD_{600} of the diluted culture and, if necessary, add more of the overnight culture to bring the OD_{600} up to 0.2–0.3.
6. Incubate at 30°C for 3 hr with shaking (230 rpm). At this point, the OD_{600} should be 0.4–0.6.
7. Place cells in 50-ml tubes and centrifuge at 1,000 x g for 5 min at room temperature (20–21°C).
8. Discard the supernatantsa nd thoroughly resuspend the cell pellet in sterile TE. Pool the cells into one tube (final volume 25–50 ml).
9. Centrifuge at 1,000 x g for 5 min at room temperature.
10. Decant the supernatant.
11. Resuspend the cell pellet in 1.5 ml of freshly prepared, sterile 1XTE/1X LiAc.
12. Add 0.1 μg of plasmid DNA and 0.1 mg of carrier DNA to a fresh 1.5-ml tube and mix. Add 0.1 ml of yeast competent cells to each tube and mix well by vortexing.
13. Add 0.6 ml of sterile PEG/LiAc solution to each tube and vortex at high speed for 10 sec to mix.

14. Incubate at 30°C for 30 min with shaking at 200 rpm.

15. Add 70 µl of DMSO. Mix well by gentle inversion.

16. Heat shock for 15 min in a 42°C water bath.

17. Chill cells on ice for 1–2 min.

18. Centrifuge cells for 5 sec at 14,000 rpm at room temperature. Remove the supernatant.

19. Resuspend cells in 0.5 ml of sterile 1XTE buffer.

20. Plate 100 µl on each DOB agar plate that will select for the desired transformants (DOB-

in our case).

21. Incubate plates, up-side-down, at 28°C until colonies appear (, 2–4 days).

***S. cerevisiae* selecting plates preparation.**

The selective auxotrofic marker for pTUY1H plasmid is *LEU* for integration of the plasmid, the prey library carry a *TRP* marker and the bait marker associated with *TaWRKY10* promoter is *HIS3*.

The minimum defined yeast media used was DOB, obtained from MP Biomedicals, the reagents to prepare the media lacking the selected amminoacid/s were obtained as well from MP Biomedicals, and all the selective plates has been prepared as manufacturer's instruction.

The plates with their selection and objective are described below schematically.

-TRP : RR library propagation.

-LEU : *S. cerevisiae* transformants carrying to pTUY1H-p*TaWRKY10.*

-LEU -TRP (2DO) used to asses the positive mating between prays and bait.

-LEU -TRP -HIS (3DO) to screen positive interaction between the prey and the promoter analyzed.

RR library.

The normalized array used in this work TFs library is supplied by NASC, deposited by Castrillo *et al*., 2011.
The library is in the format of 96-wells plates, each well containing a different prey clone, maintained in *Saccharomyces cerevisiae* strain YM4271, auxotrofic marker associated is TRP. To propagate it a specially designed replicator stamp with 96 pins has been used, to replicate the glycerol plates provided by NASC. The replicator has been used throughout all the screen procedure, to make sure to always identify the clones associated with a certain coordinate in every single plate.
The library has been replicated out from the glycerol stocks on agar supplemented DOB-*TRP*, growth at 28°C for 3 days and then utilized.

Library screen.

Library and bait clones were grown for three days on DOB-TRP and DOB-LEU (MP Biomedicals) plates from their corresponding frozen stocks. A 1 L flask containing 200 ml of YPAD medium was inoculated with bait cells and incubated overnight at 28°C with shaking (200 rpm). In parallel, 100 ml of YPAD was aliquoted into 96-flat bottom well plates (Starlab) by using a multichannel pipette and a replicator was used to inoculate them with their corresponding library colonies. After overnight incubation with vigorous shaking (500 rpm) at 28°C (HiGro shaker; Genemachines), 100 ml of the bait culture was added to each well of the 96-well plates with a multichannel pipette and mating was allowed 48 h by incubating at 28°C without shaking. Settled cells were resuspended by hitting the bottom of the wells with the pins of the replicator and used to inoculate another set of 96-flat bottom well plates containing 200 ml of diploid selection media (DOB-LEU-TRP). The replicator was able to transfer about 5 µl of liquid in each pin and each well was inoculated twice, always sterilizing by flaming with 70% ethanol between plates (3 minutes after the flaming was allowed to cool the replicator). After one day of growth at 28°C and

vigorous shaking, the cells were plated on two different plates: 2DO (mating control) and 3DO (promoter Tf interaction selection) each of them.

The colonies showing a positive interaction (visible growth on both 2Do and 3DO) were spotted later on increasing concentration of 3-amino-1,2,4-triazole (3AT, Sigma), in order to rule out false positives due to leaky expression of the *HIS3* gene.

Primers table

Gene	Forward	Reverse	Purpose
TaWRKY13	AAG GAA GTT TAA GGTCACTTACTTCAACCATC	AAC CAC CAC CAC CGT GAAACTGGTGACCGTAAG	BSMV:TaWRKY13_1
TaWRKY13	AAG GAA GTT TAA CTTACGGTCACCAGTTTC	AAC CAC CAC CAC CGTGAAAGTACTCGATGCATCTC	BSMV:TaWRKY13_2
TaWRKY13	TTC CTA CCC CAG CGA CCT G	TTG TTT TGT CGC AGG GCA CTT C	qPCR
TaWRKY10	AAGGAAGTTTAA CCTACTGAACTGAGCTACTGATC	AACCACCACCACCGT TCGTGTACA TGCATCCGTGA	BSMV:TaWRKY10_1
TaWRKY10	AAG GAA GTT TAAAGCTCGTCT GTGCAGTGCAC	AACCACCACCACCGT TCACCGGCTTGGAAGTTGTA	BSMV:TaWRKY10_2
TaWRKY10	TTATGGCAGCTTCGCTGGGAC	TACATGTTCATCGCCTCGCC	qPCR
Ta26S	GGGTCAACCTATGGTGTA	ACGAGAACTTGTATAGAGAGGGATTT	qPCR
TaEF1	ACCTGAAGAAG GTCGGCTACAA	ATCTGGTCAAGCGCCTCAAG	qPCR
TaLOX1	TGTTGATAGACTGGTGCTGTG	TGAGGATTAACGCTTAGGATCG	qPCR
TaAOS	TCCCGAGAGCGCTGTTTAAA	GACGATTGACGGCTGCTATGA	qPCR
TaCOI1	CATTGTGCGAGTGAACTGTGACA	CGCGGAAACCAGACAAGCT	qPCR
TaMYC2	CCGGGGAAAACACCTAAAAT	TGCTCCAGGCTCTCTTTCTC	qPCR
TaJAZ6	CCGTAGCACGGTCTTACCAT	ATATGAGGCGAGCAACTTGG	qPCR
TaTAT1	GTGGCAGAGCACTTGTCG	TTTATGATGACCATCGCAGTT G	qPCR
TaPDF1.1	ATG GCA TCC CCT CGT CGC AT	GAAAGTTCTCGGTGCGGCA	qPCR
TaWRKY10	CCCCCCGGGGGGGTA CTA ATT ACT ACT CGC AAC	TGCTCTAGAGCA AAG TGC ACT GCA CAG ACG A	Promoter cloning
TaEDF3	AGC TAG GCG GCG GTT TTT G	GGA TAT GCT TCA GTT TTT GAC AAG	qPCR

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