The role of *Arabidopsis* transcription factors WRKY18 and WRKY40 in plant immunity

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Summary

Two related *Arabidopsis thaliana* transcription factors, WRKY18 and WRKY40, are induced upon infection with the obligate biotrophic powdery mildew, *Golovinomyces orontii* (*G. orontii*), during early stages of infection. WRKY18 and WRKY40 negatively regulate host resistance as *wrky18wrky40* double mutants are resistant towards this fungus. Differential expression of hormone biosynthesis and response genes between susceptible wildtype and resistant *wrky18wrky40* plants suggested a crucial role of hormone signaling during *G. orontii* infection. Investigating the potential contribution of hormonal changes to resistance during this plant-pathogen-interaction is one focus of this thesis. Although hormone measurements did not reveal major differences between susceptible wildtype and resistant *wrky18wrky40* plants, genetic studies demonstrated that SA biosynthesis is indispensable for resistance. Besides hormone-dependent defense responses, secondary metabolites, such as the indol-glucosinolate 4MI3G (4-Methoxyindol-3-ylmethylglucosinolat), have been shown to contribute to antifungal defense. Elevated levels of 4MI3G in infected *wrky18wrky40* plants indicate a potential role of this compound in resistance towards *G. orontii*.

Whereas WRKY18 and WRKY40 are negative regulators of resistance towards *G. orontii*, this was not the case for other powdery mildews. Hence, *wrky18wrky40* mutants do not exhibit a broad-spectrum but rather specific resistance towards *G. orontii* infection. Furthermore, comprehensive *wrky18wrky40* infection studies including different biotrophic, hemi-biotrophic and necrotrophic pathogens revealed a positive role of WRKY18 and WRKY40 in effector-triggered resistance towards avirulent *Pseudomonas syringae* DC3000 expressing the *AvrRPS4* effector gene. This response appears to be highly specific since it was not observed with bacteria expressing other tested *Avr* genes.

To further dissect roles of *WRKY18* and *WRKY40* in plant immunity and to uncover potential direct target genes of these transcription factors, global expression analyses of *wrky18* and *wrky40* single mutants upon *G. orontii* were performed. Overall, WRKY18 and WRKY40 function partly redundantly, but regulate highly diverse sets of genes. Direct binding of potential direct target genes will be analyzed by ChIP-PCR employing the newly generated WRKY18-HA complementation line. First results demonstrated WRKY18 feedback regulation on its own gene and the *WRKY40* gene during *G. orontii* infection.

In addition, a yeast 2-hybrid screen against a pathogen-induced cDNA-library revealed potential interaction partners of WRKY18 and WRKY40 that co-localize to the plant cell nucleus. In conclusion, this thesis contributes to further understanding the roles of WRKY18 and WRKY40 in plant immunity.

Zusammenfassung

Eine Infektion von *Arabidopsis thaliana* mit dem obligat biotrophen Mehltaupilz *Golovinomyces orontii* (*G. orontii*) induziert in der frühen Phase des Infektionsprozesses die Expression von zwei verwandten Transkriptionsfaktoren, WRKY18 und WRKY40. WRKY18 und WRKY40 haben einen negativ regulierenden Einfluss auf die Resistenz des Wirts, da *wrky18wrky40* Doppelmutanten resistent gegen diesen Pilz sind. Ein Schwerpunkt dieser Arbeit war die Untersuchung eines potentiellen Beitrags hormoneller Unterschiede während dieser Pflanzen-Pathogen Interaktion. Gene, welche in der Hormonbiosynthese und –antwort involviert sind, werden zwischen anfälligen Wildtyp und resistenten *wrky18wrky40* Pflanzen unterschiedlich exprimiert. Dies deutet auf eine entscheidende Rolle von Hormon-Transduktionswegen während der Mehltau-Infektion hin. Obwohl Hormonmessungen keine signifikanten Unterschiede zwischen anfälligen Wildtyp und resistenten Mutanten zeigten, konnte durch genetische Studien bewiesen werden, dass SA-Biosynthese für die beobachtete Resistenz unerlässlich ist. Neben solchen Hormon-abhängigen Abwehrreaktionen können auch sekundäre Pflanzenmetabolite, wie das Indol-Glucosinolat 4-Methoxyindol-3-ylmethylglucosinolat (4MI3G), zu einer Pilz-Abwehr beitragen. Erhöhte 4MI3G-Werte in infizierten *wrky18wrky40* Pflanzen deuten auf eine Rolle dieser Verbindung für die beobachtete Resistenz gegenüber *G. orontii* hin.

Während WRKY18 und WRKY40 negative Regulatoren der Resistenz gegenüber *G. orontii* sind, wurde dieser Effekt für andere gestestete Mehltaupilze nicht beobachtet. Somit weisen *wrky18wrky40* Mutanten keine Breitband-Resistenz gegenüber Mehltaupilzen, sondern vielmehr eine speziefische Resistenz gegen *G. orontii* auf. Darüber hinaus zeigten umfangreiche Infektions-Studien, die sowohl biotrophe und hemi-biotrophe, als auch nekrotrophe Pathogene umfassten, dass WRKY18 und WRKY40 eine positive Rolle in Effektor-vermittelter Resistenz gegen das avirulente Bakterium *Pseudomonas syringae* DC3000 AvrRPS4 einnehmen. Auch diese Immunantwort scheint hochspezifisch zu sein, da nach Infektion mit anderen getesteten avirulenten Bakterien keine verstärkte Anfälligkeit von *wrky18wrky40* Mutanten beobachtet werden konnte.

Ein weiterer Schwerpunkt dieser Arbeit war es potentielle Zielgene dieser Transkriptionsfaktoren zu identifizieren. Dazu wurden umfassende Expressionsstudien mit *wrky18* und *wrky40* Einzelmutanten nach *G. orontii* Infektion durchgeführt. Allgemein betrachtet agieren WRKY18 und WRKY40 teilweise funktional redundant, regulieren aber deutlich unterschiedliche Gruppen von Genen. In den Expressionsstudien identifizierte Kandidaten-Gene können mit Hilfe der neu generierten WRKY18-HA

Komplementationslinie auf direkte Bindung durch WRKY18 untersucht werden. Erste Ergebnisse konnten bereits WRKY18 ‚Feedback'-Regulation des eigenen Gens und des *WRKY40* Gens während *G. orontii* Infektion zeigen.

Des Weiteren wurden in einem Yeast 2-hybrid-Screen gegen eine Pathogen-induzierte cDNA-Bibliothek potentielle Interaktionspartner von WRKY18 und WRKY40 identifiziert, die im pflanzlichen Zellkern co-lokalisieren.

Insgesamt trägt diese Arbeit zu einem weiterführenden Verständnis der Rolle von WRKY18 und WRKY40 in der pflanzlichen Immunabwehr bei.

1. Introduction

1.1. The plant immune system

1.1.1 Non-host resistance

Plants are exposed to a multitude of pathogenic influences from the environment, including microbial pathogens, nematodes and insects. Combating these constant threats is essential for plants to ensure survival and reproduction. Plant pathogens are generally classified into biotrophic and necrotrophic pathogens, dependent on their feeding style (Glazebrook, 2005). While biotrophs entirely rely on living host cells and its metabolites, necrotrophs derive nutrients from dead plant tissue. Additionally, hemibiotrophic pathogens behave as both biotrophs and necrotrophs depending on the stage of their life cycle or the conditions they find themselves in. Some pathogens employ natural openings like stomata or wound sites to enter plant tissue and proliferate in the apoplast. Others employ specialized structures to penetrate and invade plant cells. Although potential pathogens have established various life styles and infection strategies, only a small number is actually able to infect plants successfully (Lipka et al., 2010; Nurnberger and Lipka, 2005). These pathogens are termed adapted as they have evolved mechanisms to overcome plant defense. In contrast, non-adapted pathogens fail to efficiently infect plants successfully.

Resistance of an entire plant species against all genetic variants of a non-adapted pathogen species is termed non-host resistance (NHR) and represents the most robust form of plant immunity (Lipka et al., 2008; Thordal-Christensen, 2003). NHR involves both constitutive barriers like the plant cell-wall but also inducible reactions, including the generation of reactive oxygen species (ROS), transcriptional reprogramming and the production of antimicrobial compounds (Nurnberger and Lipka, 2005). However, adapted pathogens that are able to infect a plant have evolved strategies to overcome the repertoire of protective mechanisms that should prevent its colonization and reproduction, representing the hallmark of basic compatibility (Lipka et al., 2008). Apart from preformed physical or chemical barriers, inducible defense reactions depend on the recognition of pathogenic threats by the plant.

1.1.2. MAMP-triggered immunity

Perception of pathogens that evade constitutive plant defenses is essentially dependent on plant membrane-resident pattern recognition receptors (PRRs). These receptors are characterized by the presence of an extracellular ligand-binding domain, a single membrane spanning domain and an intercellular kinase-signaling domain (Lipka et al., 2008; Segonzac

and Zipfel, 2011). More than 400 of these receptor-like kinases (RLKs) are present in the model plant *Arabidopsis thaliana* (*Arabidopsis*) but only few have been characterized in detail. RLKs can be subdivided into groups based on the composition of their extracellular ligand-binding domain. Besides LysM domains or epidermal growth factor (EGF)-like repeats, the largest group of RLKs contains leucine-rich repeat (LRR) motifs as extracellular recognition domain and a serine/threonine kinase-signaling domain, mediating activation of downstream signaling events following activation (De Lorenzo et al., 2011; Lipka et al., 2008).

This first active layer of plant immunity is based on the discrimination of self and non-self-structures by detecting molecular components that are structurally highly conserved across a wide range of microbes and are normally not present in the host (Chisholm et al., 2006; Dodds and Rathjen, 2010). Perception of these slow-evolving microbe associated molecular patterns (MAMPS) by PRRs triggers downstream cell-autonomous responses leading to MAMP-triggered immunity (MTI). Best studied is the membrane-associated RLK FLS2 that is the *Arabidopsis* flagellin receptor, recognizing parts of the bacterial flagella (Gomez-Gomez and Boller, 2002). A 22 amino-acid peptide highly conserved in the amino terminus of flagellin is sufficient for receptor activation (Felix et al., 1999). Flagellin binding induces rapid ion fluxes across the plasma membrane, ROS and nitric oxide (NO) production and the induction of defense hormone pathways. It further involves the activation of mitogen-activated (MAP) kinase cascades leading to signal transduction to the plant-cell nucleus culminating in transcriptional reprogramming of defense related genes and ideally resulting in the induction of MTI (Asai et al., 2002; Chinchilla et al., 2007; Chisholm et al., 2006; Lipka et al., 2008; Meszaros et al., 2006). Another characterized PRR in *Arabidopsis* is the LysM-RLK CERK1 that binds the fungal cell wall component chitin and is required for its perception (Petutschnig et al., 2010). *Cerk1* mutants exhibit increased susceptibility towards fungal pathogens but also to bacteria, indicating that CERK1 also perceives another yet unidentified bacterial MAMP (Gimenez-Ibanez et al., 2009; Petutschnig et al., 2010). However, CERK1 and its induced phosphorylation upon ligand-binding are required for early defense responses and downstream signaling.

Although effective and durable in NHR, MAMP-induced defense responses in compatible plant-pathogen interactions are insufficient to stop infection. Nonetheless, it is referred to as basal resistance and forms an effective mechanism against the majority of potential plant pathogens (Nurnberger and Lipka, 2005).

1.1.3. Effector-triggered immunity

Some pathogens have evolved the ability to evade MTI by secreting specific effector molecules that suppress or interfere with the induction of MAMP-triggered immune responses. The bacterial type III secretion system (TTSS) enables the hemibiotrophic bacteria *Pseudomonas syringae* (*P.syringae*) to deliver effector proteins into the plant cell and achieve immune suppression using various strategies. By manipulating receptor signaling, blocking RNA pathways and vesicle trafficking and altering organelle function, *P.syringae* is able to successfully infect the plant (Block and Alfano, 2011). Moreover, fungal or oomycete parasites deliver effector molecules via a specialized infection structure, the haustorium, which invaginates the plasma membrane of epidermal cells (Panstruga and Dodds, 2009). Coevolution of plants and pathogens has subsequently led to the acquisition of plant proteins encoded by resistance genes (*R*-genes) that recognize pathogenic effectors. As this recognition usually occurs in a specific genetically defined pair-wise association between pathogen effector- and plant *R*-genes, this mechanism has been referred to as gene-for-gene resistance (Flor, 1971; Glazebrook, 2005; Keen, 1990). In general, NB-LRR proteins can recognize pathogen effectors either by direct physical interaction or indirectly by perceiving effector presence through an accessory protein. The latter is mediated by monitoring modifications of proteins, which may be effector virulence targets or structural mimics of those, termed decoys (guard/decoy model) (Dodds and Rathjen, 2010).

Most *R*-genes encode for a 'nucleotide-binding site plus leucine-rich repeat' (NB-LRR) class of proteins, which can be subdivided into two main classes based on their deduced N-terminal structures (Dangl and Jones, 2001). Some R-proteins contain a domain with homology to the *Drosophila* Toll and mammalian interleukin 1 receptors (TIR-NB-LRR), whereas others have a coiled-coil domain (CC-NB-LRR) at the amino-terminus. It is generally accepted, that resistance mediated by TIR-NB-LRR class proteins depend on the lipase-like protein ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1), whereas CC-type NB-LRR-mediated resistance is EDS1 independent but dependent on the plasma membrane anchored NON-RACE SPECIFIC DISEASE RESISTANCE (NDR1) (Aarts et al., 1998; Falk et al., 1999; Moreau et al., 2012). One well-studied example of *R*-gene mediated resistance is the interaction between *Arabidopsis* Columbia-0 (Col-0) plants with the avirulent bacterial strain *P.syringae* DC3000 expressing the avirulence gene *AvrRPS4*. Secretion of the effector protein AvrRPS4 via the TTSS leads to a perception of the respective effector by the plant TIR-NB-LRR protein RPS4, resulting in resistance towards this bacterial strain (Gassmann et al., 1999; Heidrich et al., 2011). Similar to MTI, perception of effectors by a cognate R-protein induces

a set of downstream events. Activation of signaling pathways, hormonal changes and transcriptional reprogramming result in a specific and robust effector-triggered immunity (ETI). Although activation of MTI and ETI triggers the induction of similar sets of genes and utilizes common signaling pathways, MTI is thought to provide a rather transient response and vulnerable immunity, whereas defense responses in ETI are more prolonged and robust against pathogenic perturbations (Tsuda and Katagiri, 2010). Together, MTI and ETI are interconnected parts of innate immunity in plants, although MTI may form the first active response to microbial perception (Chisholm et al., 2006). Thus, ETI forms a second layer of defense that constitutes a specific mechanism of the plant innate immune system to perceive and subsequently antagonize invading pathogens. However, one common feature of both MTI and ETI is the induction of hormone pathways that have been demonstrated to play pivotal roles for defense against pathogenic threats (Glazebrook, 2005; Tsuda et al., 2009). Infection of plants with diverse pathogens or pathogen-mimicking molecules results in changes in the level of different phytohormones involved in responses to abiotic and biotic stresses (Bari and Jones, 2009).

1.2. Plant hormones and secondary metabolites in plant defense

Jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) are three key plant hormones essential for the regulation of plant defense against pathogenic attacks (Thomma et al., 2001). JA is generally viewed as a signaling molecule in the defense against necrotrophic pathogens that usually kill the plant for nutrient acquisition, whereas SA is required for an effective defense against biotrophs and hemibiotrophs. In *Arabidopsis* biosynthesis of the small phenolic compound SA is based on the conversion of the primary metabolite chorismate via two distinct enzymatic pathways (Vlot et al., 2009). While only a small fraction of pathogen-induced SA is produced via a series of enzymatic steps initially catalyzed by PHENYLALANINE AMMONIA LYASE (PAL), the main proportion of SA is synthesized by a two-step catalytic process involving isochorismate. Chorismate is converted by the ISOCHORISMATE SYNTHASE 1 (ICS1) to isochorismate and further processed by ISOCHORISMATE PYRUVATE LYASE (IPL) to SA. *ICS1* mutants are severely compromised in pathogen-induced SA production and resistance and are therefore also referred to as SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2) (Garcion et al., 2008).
A major component of salicylic acid-mediated signaling during pathogenic attack is EDS1, acting upstream of SA in basal resistance to adapted biotrophic pathogens and *R* gene-mediated ETI (Gassmann et al., 1999; Wiermer et al., 2005). Nevertheless, expression of

EDS1 and its sequence-related interaction partner PHYTOALEXIN DEFICIENT 4 (PAD4) is induced upon SA accumulation (Jirage et al., 1999). Downstream signaling of the SA pathway is highly dependent on the function of NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1). NPR1 occurs in the cytosol where it plays a role in the crosstalk between SA and JA, as well as in the nucleus where it functions in SA-mediated induction of *PATHOGENESIS-RELATED 1* (*PR1*), a marker gene for SA-dependent defense responses (Mou et al., 2003; Vlot et al., 2009). SA accumulation upon biotic and abiotic stresses leads to the induction of *PR* gene expression, the establishment of systemic-acquired resistance (SAR), a plant immune response induced systemically in plant tissue upon local pathogen infection (Durrant and Dong, 2004), and contributes to the rapid local cell death termed the hypersensitive response (HR).

Another plant defense hormone is jasmonic acid (JA), belonging to the jasmonate class of plant hormones. Jasmonates are involved in the regulation of various developmental processes as well as responses to different biotic and abiotic stimuli (Fonseca et al., 2009). Derived from α-linolenic acid, JA is generated through a series of reactions in the chloroplast and peroxisome and is converted to a variety of derivatives. α-linolenic acid is processed by lipoxygenase 2 (LOX2), allene oxide synthase (AOS) and allene oxide cyclase (AOC) to 12-oxo-phytodienoic acid (OPDA), that is exported from the chloroplast by an unknown mechanism (Wasternack and Kombrink). Import to the peroxisome is mediated by an ABC transporter COMATOSE 1 (CTS1) presumably supported by an ion-trapping mechanism. Once in the peroxisome, OPDA is further processed via several enzymatic reactions, including the OPDA REDUCTASE 3 (OPR3), to JA. Finally, the amino acid conjugate synthase JASMONATE RESISTANT 1 (JAR1) catalyzes the conjunction of isoleucine (Ile) to JA that has recently been described as the molecularly active form of this hormone (Fonseca et al., 2009; Staswick et al., 2002). Perception of JA is based on the binding of JA-Ile to the Skp1-Cullin-F-box protein (SCF) CORONATINE INSENSITIVE 1 (COI1), designated the SCF^{COI1} complex. Like JAR1 mutants, *coi1* plants exhibit a strong JA insensitivity (Staswick et al., 2002; Xu et al., 2002).

Precise regulation of these hormone pathways is essential for an effective and well-defined response to biotic and abiotic stresses. A new class of proteins, the so-called jasmonate-ZIM-domain proteins (JAZ), has recently attracted attention as negative regulators of the JA pathway (Chini et al., 2009). In a non-induced situation, the basic-loop-helix-leucine zipper transcription factor MYC2 is bound by homo- or heterodimers of JAZ proteins suppressing the transcription of certain JA responsive genes. Upon biotic or abiotic stimuli, JAR1 converts

JA to JA-Ile that is perceived by the SCF^{COI1} complex. This leads to an ubiquitination of JAZ proteins by the SCF^{COI1} complex and subsequent degradation of the JAZ repressors by the 26S-proteasome. SCF^{COI1} in combination with JA-Ile and JAZ is currently viewed as the major JA receptor complex (Sheard et al.; Yan et al., 2009). The role of COII-mediated JAZ degradation is analogous to the auxin signaling pathway through the receptor F-box protein TIR1, which promotes auxin-dependent turnover of the AUX/IAA transcriptional repressors (Kepinski and Leyser, 2005).

Besides the transcriptional de-repression and resulting JA response upon specific stimuli, *JAZ* gene expression is also induced, leading to a contemporary termination of JA response gene expression via a negative regulatory feedback-loop mechanism. Key marker genes of the JA response pathway are *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *PLANT DEFENSIN 1.2* (*PDF1.2*) that are strongly up-regulated upon JA accumulation (Leon-Reyes et al., 2010; Spoel et al., 2009).

The ET and JA pathways are thought to operate mainly synergistically during plant defense as both hormones induce similar subsets of defense-related genes upon pathogen challenge (Bari and Jones, 2009). It has been shown that the transcription factor ethylene-response-factor 1 (ERF1) positively regulates JA and ET signaling and that MYC2 also regulates the interaction between JA- and ET-mediated defense signaling (Bari and Jones, 2009; Lorenzo et al., 2003). The fact that the JA/ET and SA defense pathways are often mutually antagonistic (Chisholm et al., 2006) underlines the complexity of plant defense regulation. However, the analysis of plant signaling networks, including the JA/ET and SA signaling pathways, revealed synergistic relationships between different hormone pathways (sectors) for MTI, whereas compensatory effects were observed during ETI upon biotrophic challenge (Tsuda et al., 2009; Yamasaki et al., 2005).

Besides plant hormones, plant secondary metabolites play important roles in plant defense (Sonderby et al.). Glucosinolates are sulfur-rich secondary metabolites with economically important roles in human nutrition and biological roles in plant defense. The *Arabidopsis* cytochrome P450 monooxygenase CYP81F2 is essential for the accumulation of tryptophan-derived 4-methoxyindol-3-ylmethylglucosiolate (4MI3G) upon pathogen challenge. 4MI3G is further activated by an atypical myrosinase PEN2 for antifungal defense (Bednarek et al., 2009). *CYP81F2* mutants impaired in pathogen-induced 4MI3G accumulation are more susceptible to non-adapted fungal pathogens. *PEN2* and the *PEN2 CYP81F2* double mutant showed indistinguishable resistance phenotypes compared to *CYP81F2* single mutants, indicating that CYP81F2 and PEN2 act in a common pathway (Bednarek et al., 2009).

CYP81F2 expression was also found to be induced after challenge with an adapted powdery mildew (Pandey et al., 2010), indicating that 4MI3G or its derivatives are also involved in defense during compatible plant-pathogen interactions.

Global expression analysis suggests that pathogens elicit an interconnected network of signaling cascades between MTI and ETI, showing the existence of a complex and highly regulated web of regulatory molecules comprising transcriptional activators and repressors (Eulgem and Somssich, 2007; Kalde et al., 2003; Pandey et al., 2010). Several transcription factor (TF) families involved in the regulation of gene expression upon pathogen challenge have attracted recent attention. The WRKY family of transcriptional regulators has been shown to regulate various developmental processes but prominently regulate gene expression during plant defense responses. Expression analyses revealed the induction of several *WRKY* genes after pathogen infection, treatment with pathogen effectors or by hormones triggering defense responses (Eulgem et al., 1999; Pandey et al., 2010; Xu et al., 2006).

1.3. WRKY transcription factors

The *WRKY* family comprises 74 expressed genes in *Arabidopsis* and represents one of the largest transcription factor families in higher plants (Eulgem and Somssich, 2007; Rushton et al., 2010). All WRKY proteins contain at least one highly conserved WRKYGQK amino acid motif and a zinc-finger motif forming the characteristic WRKY domain, responsible for binding of specific DNA elements. These binding sites share an invariant nucleotide composition, called the W-box (T/C-TGAC-T/C), that is specifically bound by WRKY proteins, although alternative binding sites have been identified (Ciolkowski et al., 2008; Pandey and Somssich, 2009). To ensure a certain degree of binding specificity, analyses have demonstrated that nucleotides directly adjacent to the W-box determine binding preferences of different WRKY proteins (Rushton et al., 2010).

WRKY transcription factors are divided into three groups based on the number of WRKY domains and the structure of their C-terminal zinc-finger motifs. Group I WRKY proteins contain two WRKY domains, whereas group II and III have a single WRKY domain. These groups are further subdivided with respect to the structure of their primary amino acid sequence (Rushton et al., 2010). WRKY proteins are integrated in a complex interconnected web of transcriptional activators and repressors. It has been shown that several members act as both repressors and activators in different plant processes (Miao et al., 2008). Moreover, WRKY proteins are involved in the transcriptional regulation of their own genes or of other WRKY members and show considerable rates of functional redundancy between individual

members (Pandey et al., 2010; Rushton et al., 2010). Regarding the large number of *WRKY* genes in *Arabidopsis* and the fact that some WRKY proteins form homo-and heterodimers (Eulgem, 2006; Xu et al., 2006) puts further complexity to the regulatory web of WRKY transcription factors.

To date, *WRKY* genes have not been found in yeast or animals but are present in the non-plant unicellular eukaryote *Giardia lamblia*, an early-diverging protozoan parasite that colonizes the intestinal tract of higher animals (Pan et al., 2009). Moreover, one WRKY gene was found in the eukaryote *Dictostelium discoideum*, a slime mold more closely related to the lineage of fungi and animals than to green plants, indicating an early origin of *WRKY* genes in primitive organisms representing the earliest branching among extant eukaryotes (Zhang and Wang, 2005). Alternatively it is conceivable that horizontal gene transfer may be the reason for their existence in these organisms. However, through duplication events and with an ongoing increase in complexity accompanied by the need for an excess of transcriptional regulation, *WRKY* genes have greatly expanded in higher plants (Pan et al., 2009; Rushton et al., 2010; Zhang and Wang, 2005).

In *Arabidopsis*, it has been demonstrated that *WRKY* genes play pivotal roles in seed and embryo development (Luo et al., 2005), auxin distribution during root development (Grunewald et al., 2012), plant senescence (Rushton, Somssich et al. 2010) and in regulating morphological processes during trichome formation (Johnson, Kolevski et al. 2002). Furthermore in rice (*Oryza sativa, Os*), WRKY factors have been shown to be involved in seed dormancy and germination (Rushton et al., 2010), whereas in *Medicago truncatula* these TF negatively regulated pith secondary wall formation, with mutants showing increased stem biomass (Wang et al., 2010). Besides the regulation of developmental processes, the main focus of WRKY research examines the role of *WRKY* genes in plant immunity as the last 15 years of WRKY research have demonstrated their outstanding role in transcriptional regulation of plant immune responses (Rushton et al., 2010).

1.3.1. WRKY transcription factors in plant defense

Over the past decade numerous global expression profiling studies in *Arabidopsis* have revealed that a large set of the *WRKY* gene family is responsive to pathogen challenge. In addition, promoter analyses revealed the over-representation of WRKY TF binding-sites in numerous *Arabidopsis* defense-related genes indicating an important role of zinc-finger-type WRKY transcription factors in the regulation of plant defense (Eulgem and Somssich, 2007). Within the past five years unequivocal evidence has been obtained supporting the crucial role of WRKY TFs in the regulation of plant defense mechanisms particularly in *Arabidopsis* but

also in other plant species (Eulgem et al., 2000; Eulgem and Somssich, 2007; Hwang et al., 2011; Ulker and Somssich, 2004; Zhang and Wang, 2005) and the analysis of specific WRKY proteins has just begun (Birkenbihl et al., 2012; Lippok et al., 2007; Ulker et al., 2007; Xu et al., 2006; Zheng et al., 2006).

The rice genome encodes more than 100 *WRKY* genes (Ramamoorthy et al., 2008; Shimono et al., 2012).Several of them have been associated with defense against the compatible rice blast fungus *Magnaporthe grisea* (*M. grisea*), causing serious yield losses and posing a constant threat to rice supplies (Qiu et al., 2007; Shimono et al., 2012; Zhang et al., 2008). *OsWRKY45* expression is induced upon SA treatment and infection with *M. grisea*. Also treatment with the SA-analog benzothiadizole (BTH), but not MeJA or gibberellin, strongly increased *OsWRKY45* transcript abundance (Shimono et al., 2007). Overexpression of *OsWRKY45* leads to an enhanced resistance against the rice blast fungus, whereas *Oswrky45* mutant plants did not reveal increased susceptibility. However, pre-treatment of rice plants with BTH results in induced resistance towards *M. grisea*, which is negated in *OsWRKY45* knockdown plants, suggesting a role of *OsWRKY45* in the SA signaling pathway (Shimono et al., 2012; Shimono et al., 2007). The lack of *OsWRKY45*-dependent resistance towards *M. grisea* in the absence of BTH-treatment is presumably caused by a time lag. Upon pathogen treatment, *OsWRKY45* expression is up-regulated 2-3 days after infection, whereas it is rapidly induced 24 h after BTH treatment. It likely requires *OsWRKY45* expression prior to, or in the early phase of fungal infection to exert an effective defense against *M. grisea* and its induction by plant activators like BTH (Shimono et al., 2012; Shimono et al., 2007).

Involvement of *WRKY* genes in specific defense-related plant hormone pathways was reported for various plant species (Atamian et al., 2012; Qiu et al., 2007; Skibbe et al., 2008; van Verk et al., 2011; Xie et al., 2007). In green chili pepper (*Capsicum annuum*, *Ca*), *CaWRKY1* expression was found to be strongly induced by SA within 1-2 h after treatment (Oh et al., 2008). Virus-induced gene silencing of *CaWRKY1* leads to reduced susceptibility towards the compatible bacteria *Xanthomonas axonopodis* pv. *vesicatoria*, indicating a negative role of *CaWRKY1* for resistance in this plant-pathogen interaction.

In *Arabidopsis*, positive and negative regulation of defense signaling pathways has also been demonstrated for WRKY33 (Birkenbihl et al., 2012; Zheng et al., 2006). This protein acts downstream of the MAMP-triggered signaling pathway (Andreasson et al., 2005) and is thought to interact with a protein, MKS1, linking the MAP kinase MPK4 with WRKY33 within the nucleus (Qiu et al., 2008). Moreover, interaction with MKS1 has also been demonstrated for the WRKY33-related WRKY25 protein. It has been shown that WRKY33

acts in the negative regulation of the SA pathway, functions as a positive regulator in the JA-dependent signaling pathway (Zheng et al., 2006) and is also involved in the crosstalk between the SA and JA pathways upon *Botrytis cinerea* infection (Birkenbihl et al., 2012). The analysis of *MPK4* mutants revealed a phenotype with dwarfed plant stature, whereas *WRKY33* mutants showed no obvious phenotype, suggesting a functional redundancy between the WRKY33 and WRKY25 proteins, which is a common phenomenon within the WRKY regulatory network. Moreover, it has been shown that *Arabidopsis* mutants lacking WRKY70 function are susceptible to different bacteria, fungi and the oomycete *Hyaloperonospora arabidopsidis* (*Hpa*), whereas it is also crucial for the regulation of the antagonistic interaction between SA- and JA-mediated defense responses (Bari and Jones, 2009; Knoth et al., 2007). Overexpression of *WRKY70* leads to a constitutive expression of SA-responsive *PR* genes and increased resistance against a biotrophic fungus, but repressed expression of JA-responsive marker genes, such as *PDF1.2* (Liu et al., 2006). Thus, WRKY70 acts as a positive regulator of resistance against pathogens and is also thought to function cooperatively with WRKY46 and WRKY53 (AbuQamar et al., 2006; Hu et al., 2012; Liu et al., 2006; Wang et al., 2006). Likewise, WRKY50 and WRKY51 have been shown to mediate SA- and low oleic acid (18:1)-dependent repression of JA signaling (Gao, Venugopal et al. 2011). Plants lacking the plastid-localized stearoyl-acyl carrier protein desaturase SSI2 are affected in both JA- and SA- signaling (Gao et al., 2011a). *Ssi2* mutants have significantly reduced 18:1-levels, accumulate high amounts of SA and *PR1* transcript and exhibit enhanced resistance towards bacterial and oomycete pathogens. Additionally, *ssi2* mutant plants are more susceptible to the necrotrophic fungal pathogen *B.cinerea* compared to wildtype plants (Nandi et al., 2005; Shah et al., 2001). Simultaneous knockdown of *WRKY50* and *WRKY51* in the *ssi2* mutant background, however, restored JA-responses and basal resistance to *B.cinerea*, suggesting WRKY50 and WRKY51 might serve as positive regulators of SA-mediated signaling but negative regulators of JA mediated signaling (Gao et al., 2011a). Both, *WRKY50* and *WRKY51* are rapidly induced upon SA treatment, which was not observed for *WRKY48* (Dong et al., 2003). Nevertheless, *WRKY48* gain-of function overexpressor plants exhibit an increased susceptibility phenotype when challenged with virulent hemibiotrophic bacterial strain *P. syringae* DC3000. Susceptibility was accompanied by pathogen-induced increase in SA levels and enhanced *PR* gene expression (Xing et al., 2008). On the contrary, *wrky48* mutant plants showed an increase in resistance towards this bacterial strain associated with a reduction of *PR1* transcript abundance, suggesting *WRKY48* acts as a negative regulator of resistance to *P. syringae*.

Also, enhanced resistance against *P. syringae* was observed for the single mutant *wrky7*, and the *wrky11wrky17* and *wrky18wrky40* double mutants (Eulgem and Somssich, 2007; Journot-Catalino et al., 2006; Kim et al., 2006; Xu et al., 2006), describing another negative regulation of resistance by these WRKY TFs. Similarly, *wrky18wrky40* plants also show increased resistance against the otherwise virulent powdery mildew *Golovinomyces orontii* (*G. orontii*) (Pandey et al., 2010; Shen et al., 2007).

1.4. The powdery mildew fungus *Golovinomyces orontii*

G. orontii belongs to the obligate biotrophic fungal plant pathogens of the phylum Ascomycete of the order Erysiphales. Powdery mildews have a broad spectrum of hosts within dicot and monocot plants and cause severe yield losses and agricultural damage. As biotrophic lifestyles depend on the availability of living host tissue, the fungus does not kill the plant. Instead, it penetrates the epidermal cell layer to gain access to required nutrients for completion of its asexual lifecycle (Figure 1). The classical symptom of compatible powdery mildew infections is a whitish, velvety powder formed mainly on infected leaf surfaces, but also on stems and flowers (Eichmann and Huckelhoven, 2008). After landing of an asexual

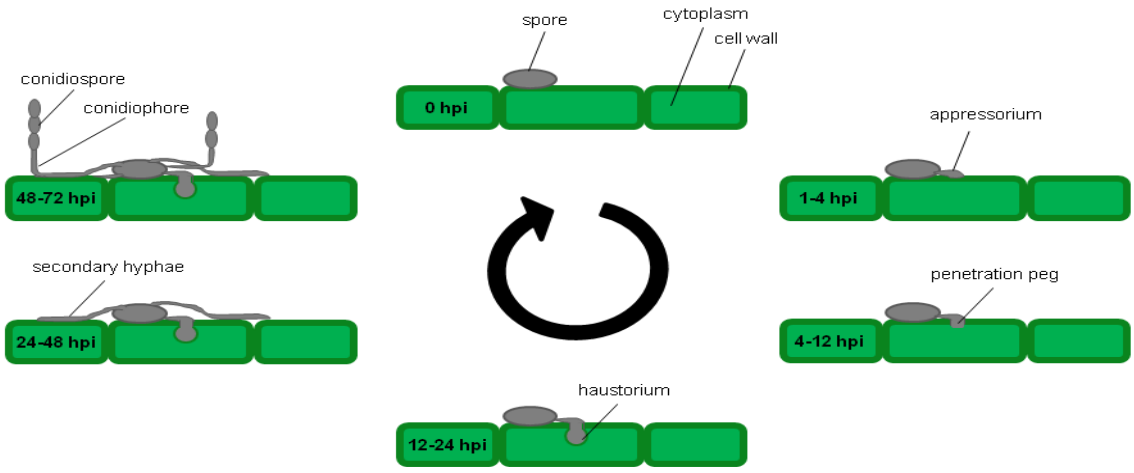


Figure 1: Schematic illustration of the asexual lifecyle of the powdery mildew fungus *G. orontii*. After landing of a spore on the plant leaf surface, an appressorium is formed which further develops into a a penetration peg. After 4-12 hpi, the fungus attempts to penetrate the cuticle and cell wall of a single epidermal cell. In a compatible interaction, the fungus invaginates the plasma membrane and forms a haustorium, the fungal feeding structure within 12-24 hpi. Epiphytic growth 24-48 hpi is achieved be elongation of seondary hyphe, from which it infects adjacent epidermal cells. The infection cycle is restarted with the formation of new conidiospores that emerge from conidophores 3 days after infection with spreading of new spores on other plant parts or surounding plants.

spore on the leaf surface, an appressorium develops within the first 1-4 hours post infection, leading to the penetration of the epidermal cell layer 4-12 hpi mediated by a structure called the penetration peg. Formation of the haustorium, the fungal feeding structure 12-24 hpi marks the next step in the powdery mildew life cycle (Chandran et al., 2009; Eichmann and Huckelhoven, 2008).

This appendage invaginates the plasma membrane, enables the fungus to acquire required nutrients from the penetrated host cell but also allows delivery of fungal effector molecules to manipulate host functions. Epiphytic growth of the fungus is attended by the formation and elongation of secondary hyphaes that evade further epidermal cells 24-48 hpi (Figure 1). Fungal development ends with the completion of its lifecycle within the next 2-5 days resulting in the formation of the reproduction structures known as conidiophores that contain conidiospores (Eichmann and Huckelhoven, 2008). Conidiospores can be subsequently spread by wind or animals and infect new plants or plant parts and the life cycle re-initiates.

Defense responses of *Arabidopsis* against the compatible *G. orontii* fungus are mainly restricted to single cells that try to avoid nutritional exploitation by execution of HR leading to rapid cell-death or by the formation of papillae, enforcements of the epidermal cell wall, to restrict fungal penetration and growth (Eichmann and Huckelhoven, 2008). Besides the relatively aggressive fungus *Golovinomyces orontii* (Plotnikova JM, 1998), also the powdery mildew species *Golovinomyces cichoracearum* (Adam and Somerville, 1996) and *Golovinomyces cruciferarum* (Koch E, 1990) are able to successfully infect and reproduce on *Arabidopsis* Col-0 wildtype plants (Eichmann and Huckelhoven, 2008), although infection is less severe. However, other *Arabidopsis* accessions possessing the atypical R-protein RESISTANCE TO POWDERY MILDEW 8 (RPW8) control resistance to a broad range of powdery mildew pathogens (Xiao et al., 2005).

In barley, mutations in *MILDEW RESISTANT LOCUS* (*MLO*) alleles result in a durable broad-spectrum resistance against all isolates of the compatible barley powdery mildew *Blumeria graminis f. sp. hordei* (*Bgh*) (Buschges et al., 1997). Likewise, *Arabidopsis* Col-0 *mlo2mlo6* mutants exhibit broad-spectrum resistance towards all three *Golovinomyces* species, demonstrating a conserved requirement of MLO for powdery mildew pathogenesis in monocots and dicots (Consonni et al., 2006).

In many plant species resistance against powdery mildews is conferred by isolate-specific *R*-gene mediated immunity. One example is the barley polymorphic *R*-gene *MLA*, conferring isolate-specific resistance to the barley powdery mildew *Blumeria graminis f. sp. hordei* (*Bgh*). Perception of the *Bgh* effector A10 by the CC-NB-LRR-type immune receptor MLA10

is required for physical interaction of MLA10 with two TFs, *Hv*WRKY1 and *Hv*WRKY2, leading to a de-repression of basal defense and resistance towards *Bgh* (Shen et al., 2007). Thus, transcriptional knockdown of *HvWRKY1 HvWRKY2* results in an increased resistance against *Bgh*. Also, simultaneous mutations in the *Arabidopsis WRKY18* and *WRKY40* genes, sharing highest protein sequence-relatedness to *HvWRKY1* and *HvWRKY2*, result in resistance towards powdery mildew *G. orontii* (Shen et al., 2007). However, it is still unknown whether *wrky18wrky40* resistance towards *G. orontii* represents another example of broad-spectrum resistance against powdery mildews, as observed for e.g. *mlo* mutants. Moreover, WRKY18 and WRKY40 are thought to negatively regulate resistance towards *G. orontii* and *Pseudomonas syringae* DC3000 by basically repressing basal defense during MTI (Pandey et al., 2010; Xu et al., 2006). A direct role of WRKY18 WRKY40 in ETI has not yet been demonstrated, although various suitable *Pseudomonas* strains are available to address this question.

1.5. The bacterial pathogen *Pseudomonas syringae*

Pseudomonas syringae (*P.syringae*) is a hemibiotrophic Gram-negative plant pathogenic proteobacterium with a broad host range. Strains have been classified into pathovars (pv.) based largely on the host of origin (Block and Alfano, 2011). *P. syringae* lives on the surface and the apoplast of host plants, but lacks the ability to penetrate the epidermal cell layer. Hence, it enters the plant cell via natural openings like stomata and hydathodes or through lesions/wound sites on the plant leaf surface (Alfano and Collmer, 1996; Gimenez-Ibanez and Rathjen, 2010). Strain *P. syringae* pv. *tomato* DC3000 (*Pto*) is the causal agent of speck disease in tomato and also successfully infects *Arabidopsis* plants. In order to infect the plant, *Pto* must overcome the plant's innate immune system, activated by the perception of bacterial MAMPs, like flg22. Evasion of immune responses is accomplished partly by the biosynthesis of exopolysaccharides within the apoplast and the production of defense hormone analogs, e.g. coronatine that alter plant defense responses in a beneficial manner for the bacteria (Block and Alfano, 2011; Glazebrook, 2005).

However, a key factor of *Pto* pathogenicity is the syringe-like TTSS, enabling the bacteria to deliver effector proteins directly into host cells. These effector proteins cause pathogenicity by manipulating the host cell immune response leading to susceptibility or they evoke an HR in non-host plants, resulting in rapid cell-death of the infected cell and resistance. Effectors that cause susceptibility were called virulence (Vir) proteins, whereas effectors eliciting resistance were termed avirulence (Avr) proteins. In addition, newly identified type III

effector proteins (T3E) are named Hop (Hrp outer protein) to genetically indicate that they are secreted by the TTSS. Accordingly, it has been shown that *P. syringae* requires proteins encoded by HYPERSENSITIVE RESPONSE AND PATHOGENICITY / HRP-CONSERVED (*HRP*/*HRC*) genes for elicitation of HR in non-host or resistant plants and for pathogenesis in susceptible plants (Cunnac et al., 2009). *HRP*/*HRC* mutant bacteria lose pathogenicity and the ability to trigger HR (Block and Alfano, 2011). To date, some mechanisms of effector function are known. It has been shown that *Pto* effectors AvrPto and AvrPtoB target the MAMP receptor FLS2 to suppress MTI (Xiang et al., 2008). Moreover, the effector HopAI1 inactivates MAPKs through its phosphothreonine lyase activity and thereby suppresses MTI by altering MAPK-signaling (Zhang et al., 2007). However, it is widely believed that effectors manipulate both MTI and also ETI, if an appropriate R-protein is missing to confer resistance (Sohn et al., 2009). This was shown for the effector protein HopF2, which targets the plasma-membrane associated RPM1-INTERACTING PROTEIN 4 (RIN4) in *Arabidopsis*. Plants conditionally expressing HopF2 were compromised in effective ETI (Wilton et al., 2010), demonstrating that MTI and ETI are targets of bacterial manipulation through effector proteins.

Effector recognition by direct or indirect perception of effector presence through R proteins is the basis of effective ETI. One well-studied example of R-gene-mediated resistance is the interaction between *Arabidopsis* Col-0 plants expressing the TIR-NB-LRR immune receptor RPS4 and *Pto* DC3000 AvrRPS4 (*Pto* AvrRPS4), expressing the avirulence effector gene *AvrRPS4*, originating from *P. syringae* pv. *pisi* (Hinsch and Staskawicz, 1996). Perception of the AvrRPS4 effector by RPS4 leads to EDS1-dependent host-cell death and transcriptional reprogramming indispensable for resistance. Recent work demonstrates a direct physical interaction of the nucleo-cytoplasmic localized EDS1 protein with both RPS4 and AvrRPS4 inside plant nuclei after resistance activation, whereas no interaction was found for the effector and cognate R-protein (Heidrich et al., 2011). Thus, EDS1 is thought to act as a virulence target that is guarded by R-proteins like RPS4 and required for effective defense. Similar to RPS4-mediated resistance, defense against *Pto* AvrHopA1 is based on a TIR-type NB-LRR protein, RPS6, also showing EDS1-dependency (Bhattacharjee et al., 2011). *EDS1* mutant *Arabidopsis* plants exhibit increased susceptibility towards these two bacterial strains. On the other hand, resistance towards the avirulent strain *Pto* DC3000 AvrRPM1 is EDS1-independent and instead requires function of NDR1 (Day et al., 2006). AvrRPM1 targets RIN4 at the plasma membrane and mediates its phosphorylation. In a not yet clarified manner, CC-NB-LRR receptor protein RPM1 perceives this phosphorylation, leading to its activation

and effective defense, involving extensive transcriptional reprogramming (de Torres et al., 2003; Gao et al., 2011b; Mackey et al., 2002). WRKY TFs have been shown to regulate immune responses towards *Pto* (Hu et al., 2012; Zheng et al., 2007). Also, WRKY18 and WRKY40 are induced after infection with different *Pseudomonas* strains (Xu et al., 2006), indicating a role for WRKY18 and WRKY40 also in the interaction with this hemibiotrophic bacterium.

1.6. Thesis aims

In *Arabidopsis*, expression of both *WRKY18* and *WRKY40* is induced upon *G. orontii* infection compared to wildtype plants (Supplementary Figure 1), indicating a regulatory role of these transcription factors during this plant-pathogen interaction (Pandey and Somssich, 2009). Indeed, simultaneous transcriptional knockdown of *WRKY18* and *WRKY40* leads to resistance towards this biotrophic fungal pathogen, whereas both *wrky18* and *wrky40* single mutants show wildtype-like phenotypes, pointing to a functional redundancy between these two TFs. Resistance of *wrky18wrky40* mutants is accompanied by a decrease of fungal entry rates constituting an increase in pre-invasive resistance as well as cell-death induction in later infection stages (Pandey et al., 2010; Shen et al., 2007).

WRKY18 and WRKY40 together with WRKY60 belong to a subgroup of WRKY class II TFs, containing an additional leucine-zipper motif at their amino-termini. This enables the formation of homo- and heterodimers and puts another layer of complexity on the way these TFs can regulate gene expression (Xu et al., 2006).

Although mainly located within the nucleus, WRKY18 and WRKY40 protein was recently reported to localize to the chloroplast envelope and interact with the Mg-chelatase subunit H upon ABA treatment (Shang et al., 2010). This finding, if verified, indicates that apart from self-association WRKY18 and WRKY40 might be involved in various other nuclear and extra-nuclear protein-protein interactions. Hence, identification of new potential interaction partners of WRKY18 and WRKY40 was one goal of this thesis.

An important role of WRKY18 WRKY40 in early plant defense could be demonstrated in microarray analysis of wildtype and *wrky18wrky40* plants that revealed an extensive transcriptional reprogramming already eight hours after infection with *G. orontii* (Pandey et al., 2010). Significant up-regulation of positive and negative regulators of defense responses was already observed in non-infected mutant plants, indicating direct or indirect negative regulation of various defense-related genes by WRKY18 and/or WRKY40. Direct binding of WRKY40 to promoters of target genes *in vivo* was demonstrated in previous studies (Pandey

et al., 2010), whereas WRKY18 direct target genes are yet to be identified. Hence an additional aim of this thesis was to further dissect the roles of WRKY18 and WRKY40 and to uncover potential direct target genes of these TFs, but particularly for WRKY18.

G. orontii resistant *wrky18wrky40* double mutants revealed up-regulation of several genes related to defense hormone pathways compared to susceptible wildtype plants (Pandey et al., 2010). Little is known about dynamic changes of plant hormone accumulation and the expression of hormone pathway genes in early stages of this plant-pathogen-interaction. Global expression analysis demonstrated a crucial role of the SA pathway for the compatible interaction of *G. orontii* and *Arabidopsis* at least at later phases of the infection process (Chandran et al., 2009; Eichmann and Huckelhoven, 2008). Therefore, different defense-related hormone pathways were investigated upon early infection with *G. orontii* and compared between susceptible wildtype and resistant *wrky18wrky40* plants. Among the genes up-regulated in the *wrky18wrky40* double mutant compared to wildtype plants were several members of the *JAZ* family revealing elevated transcript levels already prior to infection. Likewise, increased expression of the *PHYTOALEXIN-DEFICIENT 3* (*PAD3*) gene catalyzing the final step in camalexin biosynthesis during *G. orontii* infection leads to a strong accumulation of this major phytoalexin in the resistant double mutant (Pandey et al., 2010). To better understand the effect of JA signaling during *G. orontii* infection with regard to susceptibility of wildtype and resistance of double mutant plants, *JAZ* gene expression was investigated during early *G. orontii* infection in both genotypes.

Besides negative regulation of resistance towards *G. orontii*, only sparse knowledge about the role of WRKY18 and WRKY40 in other plant-pathogen interactions is available. Therefore, *wrky18wrky40* mutants were tested against various biotrophic and necrotrophic pathogens, as well as addressing the question of broad-spectrum resistance of *wrky18wrky40* plants towards powdery mildews.

Altogether, this work contributes to the further understanding of *wrky18wrky40* resistance towards *G. orontii* and attempts to broaden the view on WRKY18 and WRKY40 function in plant immunity.

2. Results

2.1. Transcriptional activation of *JAZ* genes during *G. orontii* infection

JAZ genes were recently shown to negatively regulate the JA signaling pathway by repressing the expression of JA-responsive genes. Comparative microarray analysis between wildtype (Col-0) and *wrky18wrky40* plants revealed an up-regulation of five members of these transcriptional regulators already in non-challenged tissue (Pandey et al., 2010). To verify the up-regulation of these genes before challenging the plants with *G. orontii* and their transcriptional regulation during early stages of the infection process, qPCR analysis of *JAZ* genes was performed in an infection kinetic. All known *JAZ* family members were tested for their expression at 0, 4, 8, 12, 24 and 48 hours post infection relative to the expression in the non-challenged wildtype sample set to 1. All *JAZ* genes showed a constitutive higher expression in *wrky18wrky40* plants at 0 hpi, with >5-fold elevated levels observed for *JAZ1*, *JAZ5*, *JAZ7* and *JAZ8* (Figure 2 A). Whereas *JAZ3*, *JAZ4*, *JAZ6*, *JAZ11* and *JAZ12* showed only weak inductions and only subtle differences between both genotypes during the infection process, other *JAZ* members are strongly up-regulated in the double mutant. Interestingly, only one gene, *JAZ1*, was strongly induced solely at 4 hpi, whereas *JAZ2*, *JAZ7*, *JAZ8* and *JAZ10* showed increased transcript abundance at 12 hpi. Notably, *JAZ7* showed highest constitutive levels at 0 hpi as well as being the most strongly induced (25-fold induction) 12 hpi compared to wildtype 0 hpi. Additionally, *JAZ5* and *JAZ9* showed a biphasic induction pattern with maximal levels reached at both 4 and 12 hpi (~8-fold higher transcript levels than wildtype 0 hpi plants).

Recently, Yan and colleagues (Yan et al., 2009) described *JAZ10* overexpressing seedlings as being insensitive towards methyl-jasmonate (MeJA) treatment, and also showing a similar root-phenotype as *coi1* or *jar1*. Elevated transcript levels of almost all *JAZ* gene members in the *wrky18wrky40* double mutant prior to infection raised the question of whether this mutant exhibits constitutive JA-insensitivity. To address this question, wildtype and *wrky18wrky40* plants were used in a MeJA root-growth assay (Figure 2 C). Col-0, *WRKY18* and *WRKY40* single mutants, and *wrky18wrky40* double mutants were grown on MS-agar plates containing different concentrations of MeJA. Because the expected JA-insensitive phenotype might be weaker than observed by Yan and colleagues, seedlings were grown on plates containing 0, 1, 2.5, 5 and 12.5 µM MeJA. Root-length was determined after 10 days under long-day conditions and compared to JA-insensitive *jar1* mutant, which served as a control. Although *wrky18wrky40* plants exhibited elevated constitutive levels and strong induction of various

JAZ genes, root growth on MeJA-containing medium did not reveal a JA-insensitive phenotype. Even *WRKY18* and *WRKY40* single mutant plants in which stronger induction of *JAZ* gene expression was partially observed compared to *wrky18wrky40* plants (data not shown) did not show any obvious root phenotype comparable to *jar1*. All tested genotypes showed a constant decrease in root-length with increasing MeJA-concentrations (Figure 2 C). As expected, the effect on *jar1* plants was significantly weaker compared to all other genotypes, confirming the JA-insensitivity of this mutant and the functionality of the assay. From all tested *JAZ* genes, expression analysis of *JAZ7* revealed the strongest induction in transcript abundance in *G. orontii* resistant *wrky18wrky40* plants and the second highest induction levels in wildtype plants (Figure 2 A). If *JAZ7* expression plays a negative or positive role in defense against *G. orontii*, then *jaz7* plants may show differences in resistance or susceptibility towards this fungus. Moreover, *JAZ10* when ectopically overexpressed has been shown to exhibit JA-insensitivity. As the JA pathway is generally associated with defense against necrotrophic pathogens, overexpression of *JAZ10* may induce the antagonistic SA pathway and contribute to resistance against infection with biotrophic fungi. Hence, two available transgenic *JAZ* lines, *JAZ10* overexpressing (*JAZ10ox*) plants and plants carrying a mutation in the *JAZ7* (*jaz7*) gene, were tested with respect to altered resistance or susceptibility towards *G. orontii*, respectively.

Phenotypic characterization and fungal penetration counts were performed on these lines with susceptible Col-0 and resistant *wrky18wrky40* plants included as controls (Figure 2 B). Neither *JAZ10*ox nor *jaz7* plants showed any statistically significant differences in penetration numbers compared to wildtype plants, showing an overall penetration rate of ~80%. Regarding fungal proliferation, both *JAZ* genotypes also did not show any obvious differences compared to susceptible wildtype plants (Figure 2 B).

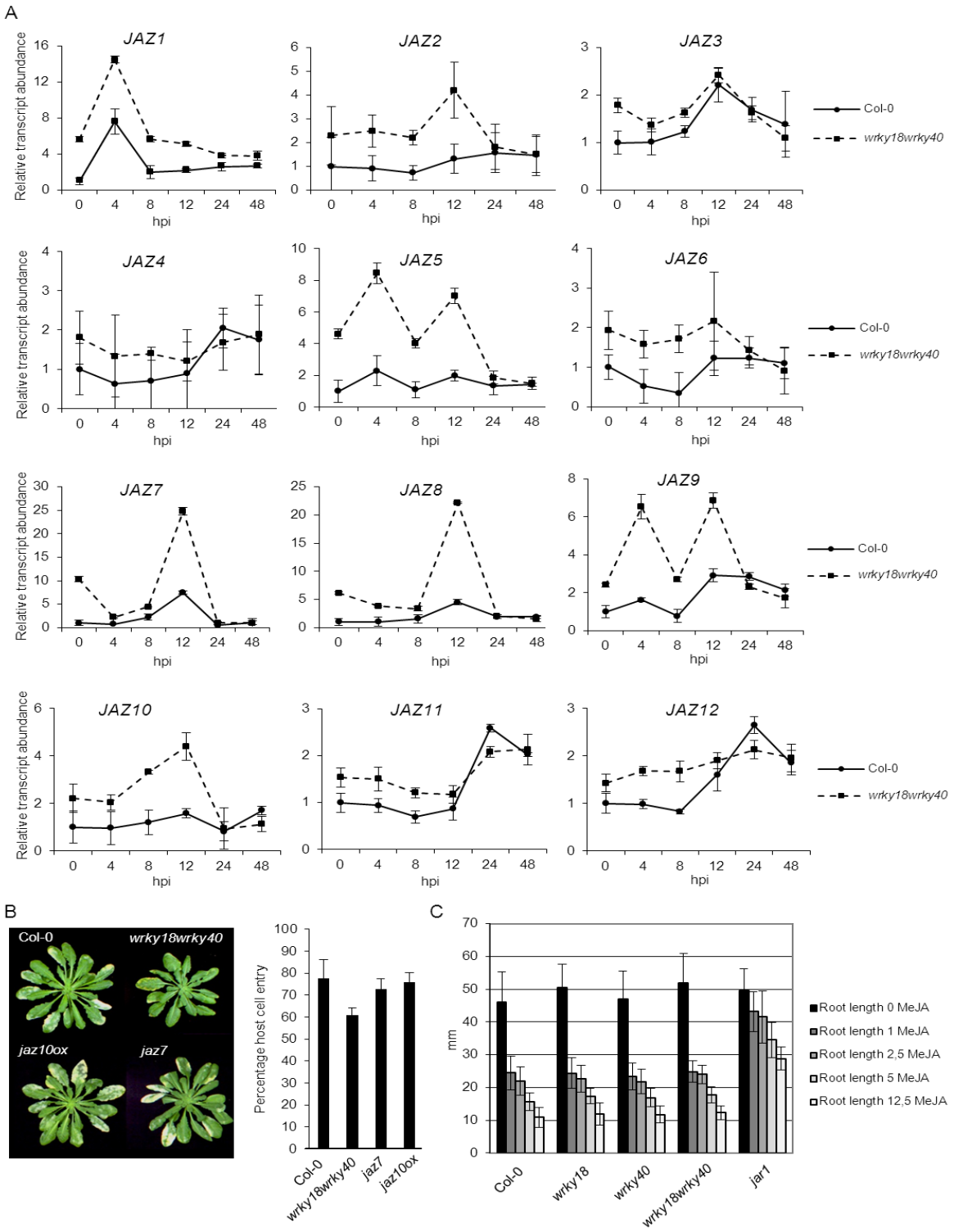


Figure 2: Differential expression of the *JAZ* gene family during early stages of *G. orontii* infection and characterization of *JAZ*-mutants upon *G. orontii* infection. (A) qPCR analysis of transcript levels of *JAZ* genes were measured after infection with *G. orontii* at indicated time points (hpi). After normalization to gene *At4g26410* (Expressed protein), fold changes in transcript abundance in wildtype (Col-0, solid line) and *wrky18wrky40* (dashed line) plants were calculated relative to untreated Col-0 samples set to 1. Error bars represent standard deviation (SD, n=3). **(B)** Characterization of *JAZ10* overexpressing and *jaz7* mutant plants upon *G. orontii* infection compared to susceptible wildtype (Col-0) and resistant *wrky18wrky40* plants. Pictures were taken 7 dpi and rate of host cell entry of fungal structures was determined 48 hpi. Error bars represent SD (n=4). **(C)** Root growth assay of indicated genotypes upon treatment with different MeJA-concentrations. Seedlings were grown under long-day conditions on MS-phytogel plates supplemented with 0-12.5 µM MeJA and root length was determined 10 days after treatment. Error bars represent SD (n=20).

2.2. JA levels and response to early *G. orontii* infection

The JA signaling pathway is generally involved in defense against necrotrophic pathogens. To investigate the induction of JA biosynthesis and response genes, qPCR-analyses of genes encoding JA pathway components were analyzed during the first 48 hours after *G. orontii* infection. Expression analysis of the JA biosynthesis gene *LOX2* revealed an induction of transcript abundance in both wildtype and *wrky18wrky40* plants upon challenge with *G. orontii* (Figure 3 A). In both genotypes, *LOX2* was induced up to 8-fold compared to uninfected control plants. In addition, *OPR3*, encoding the enzyme that catalyzes the final step of JA biosynthesis, was also up-regulated in both genotypes. Expression of both genes, however, peaked at 4 hpi, with similar kinetics, although slightly higher expression in *wrky18wrky40* plants was observed. To test whether the expression of JA biosynthesis genes correlate with the accumulation of the respective product, JA levels were determined 0, 4, 8, 12, 24, 48 and 72 hpi, representing early stages of the powdery mildew infection process. Equal JA levels were observed for both genotypes in uninfected controls (0 hpi), followed by a first peak in JA accumulation at 4 hpi that did not significantly differ between susceptible wildtype and resistant *wrky18wrky40* plants (Figure 3 B). While JA levels decreased subsequently during the course of infection in *wrky18wrky40* until declining to control levels, wildtype plants accumulated twofold higher JA levels than the mutant at 24 hpi, marking the peak of JA accumulation. Moreover, accumulation of the bioactive form JA-Ile resembled the accumulation pattern of its precursor JA (Supplementary Figure 2). At 24 hpi, wildtype plants accumulated ~6-fold more JA-Ile than *wrky18wrky40* plants. In disagreement with this observation, the early JA-responsive gene *VSP2* and late responsive gene *PDF1.2* were significantly higher expressed in the resistant double mutant (Figure 3 C). Whereas *VSP2* showed a strong increase in relative transcript abundance at 8 hpi, *PDF1.2* expression peaked at 24 hpi in *wrky18wrky40* plants and showed formidable fold-changes in transcript abundance compared to control wildtype plants (up to 100-fold). However, *PDF1.2* expression was also induced in wildtype plants up to 12-fold at 24 hpi (Figure 3 C). The current model of hormone signaling places the induction of downstream hormone-responsive genes temporally after hormone accumulation. Indeed, expression of the early responsive gene *VSP2* in wildtype plants followed the accumulation of JA, whereas the late responsive gene *PDF1.2* showed a delay in transcriptional induction (Figure 3 C).

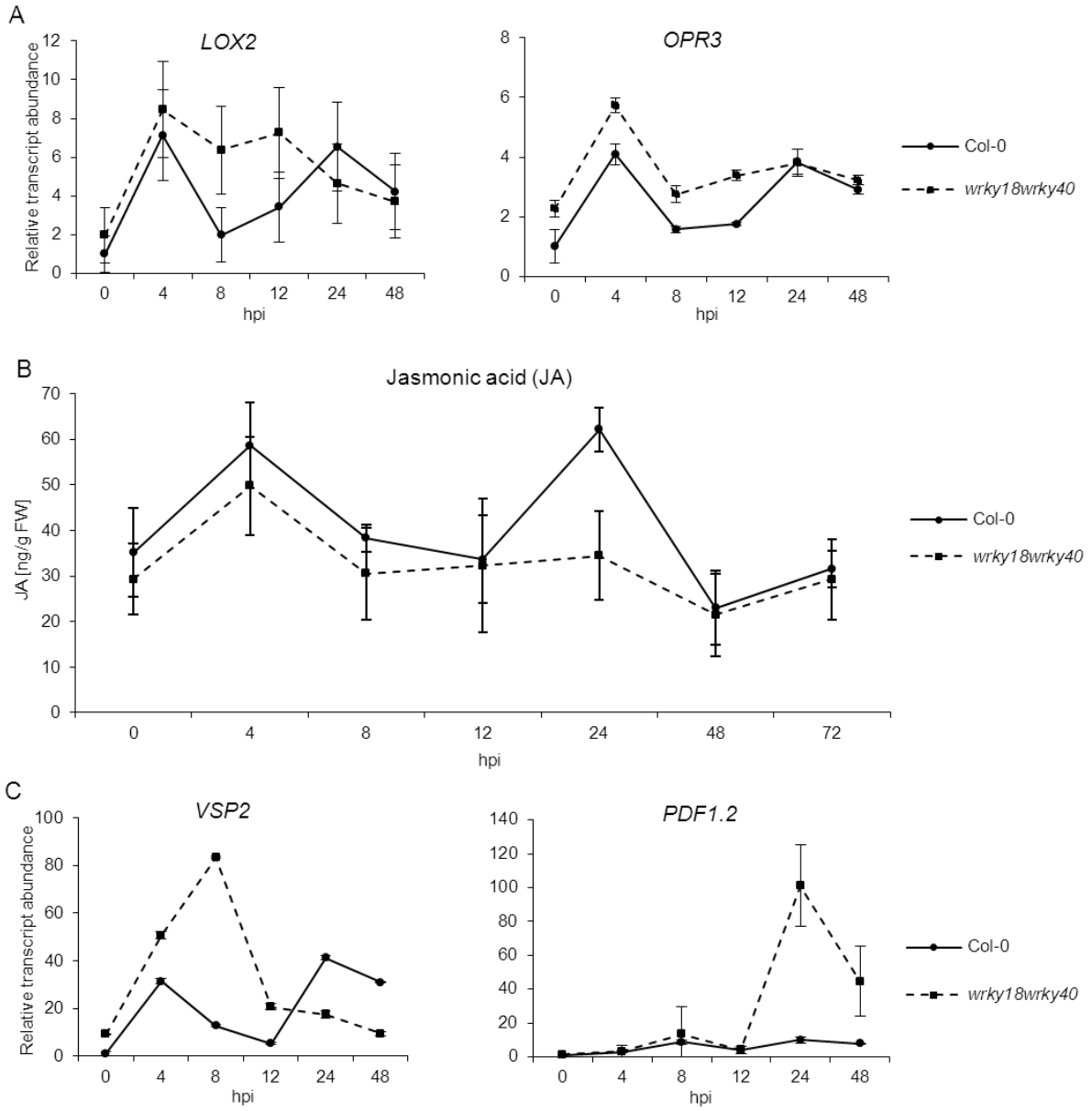


Figure 3: Activation of JA-related genes and JA levels during early *G. orontii* infection. (A) qPCR analysis of the expression of JA biosynthesis genes (*LOX2/OPR3*) at indicated time points during early *G. orontii* infection in susceptible Col-0 (solid line) and resistant *wrky18wrky4*0 (dashed line) plants. Error bars represent standard deviations (SD, n=3). Fold changes are relative to non-challenged Col-0 samples set to 1. **(B)** Accumulation of JA in susceptible Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants during the first 72 hours after infection with *G. orontii*. Error bars represent SD (n=4). **(C)** qPCR analysis of the expression of JA-responsive genes *VSP2* and *PDF1.2* at indicated time points during early *G. orontii* infection in susceptible Col-0 (solid line) and resistant *wrky18wrky4*0 (dashed line) plants. Error bars represent SD (n=3).

In contrast, expression in *wrky18wrky40* appeared to be more delayed with respect to JA accumulation. Whereas *LOX2* expression did not significantly differ between wildtype and *wrky18wrky40* plants with regard to fold-changes, a maximum of 10-fold difference in *PDF1.2* transcript abundance and a >2-fold difference for *VSP2* was observed between wildtype and *wrky18wrky40* plants.

In summary, only weak JA accumulation was observed for both susceptible and resistant genotypes during early *G.orontii* infection. However subtle differences were found at 24 hpi with wildtype plants accumulating twice as much JA compared to *wrky18wrky40* plants. Nevertheless, resistant double mutants show exaggerated responses to JA by strong induction of downstream JA marker genes.

Although JA and SA are thought to act antagonistically, an impact of the SA signaling pathway during the early interaction of wildtype and *wrky18wrky40* with *G. orontii* has not been described to date. Thus, SA measurements were performed to help elucidate its possible contribution to the establishment of the resistant phenotype of the *wrky18wrky40* mutant.

2.3. SA levels and response to early *G. orontii* infection

The SA signaling pathway is generally associated with the defense against biotrophs. Induction of SA-responsive genes was observed for numerous biotrophic pathogens and the induction of crucial SA biosynthesis genes, such as *SID2*, marks the starting point of SA accumulation. Indeed, *SID2* was transcriptionally upregulated in both wildtype and *wrky18wrky40* plants after *G. orontii* infection (Figure 4 A). *SID2* slowly starts to accumulate in both genotypes 24 hpi and is strongly induced 48 hpi. To confirm that elevated *SID2* expression correlates with the biosynthesis and subsequent accumulation of SA, hormone measurements were performed. To investigate the temporal dynamics of SA production during early events of powdery mildew infection, samples were taken at 0, 4, 8, 12, 24, 48 and 72 hpi for both wildtype and *wrky18wrky40* plants after *G. orontii* infection. Starting from similar basal levels, total SA accumulated to similar extent in susceptible wildtype and resistant *wrky18wrky40* plants, revealing a > 2.5-fold increase in total SA accumulation at 48 hpi (Figure 4 B). Accordingly, expression analysis of a robust marker gene of the SA signaling pathway, *PR1*, did not show any conspicuous differences. In both genotypes, *PR1* expression was induced 48 hpi. The SA signaling pathway is closely associated with EDS1 signaling. Acting upstream of SA, EDS1 and its interacting partner PAD4 are major components of SA-mediated defense responses and essential for the perpetuation of resistance towards many plant pathogens. Differences in transcript abundance between resistant *wrky18wrky40* and susceptible wildtype plants for *PAD4* and *EDS1* were observed during *G. orontii* infection. While transcript levels in *wrky18wrky40* increased 48 hpi, expression of *EDS1* and *PAD4* in wildtype plants were considerably lower (Figure 4 A).

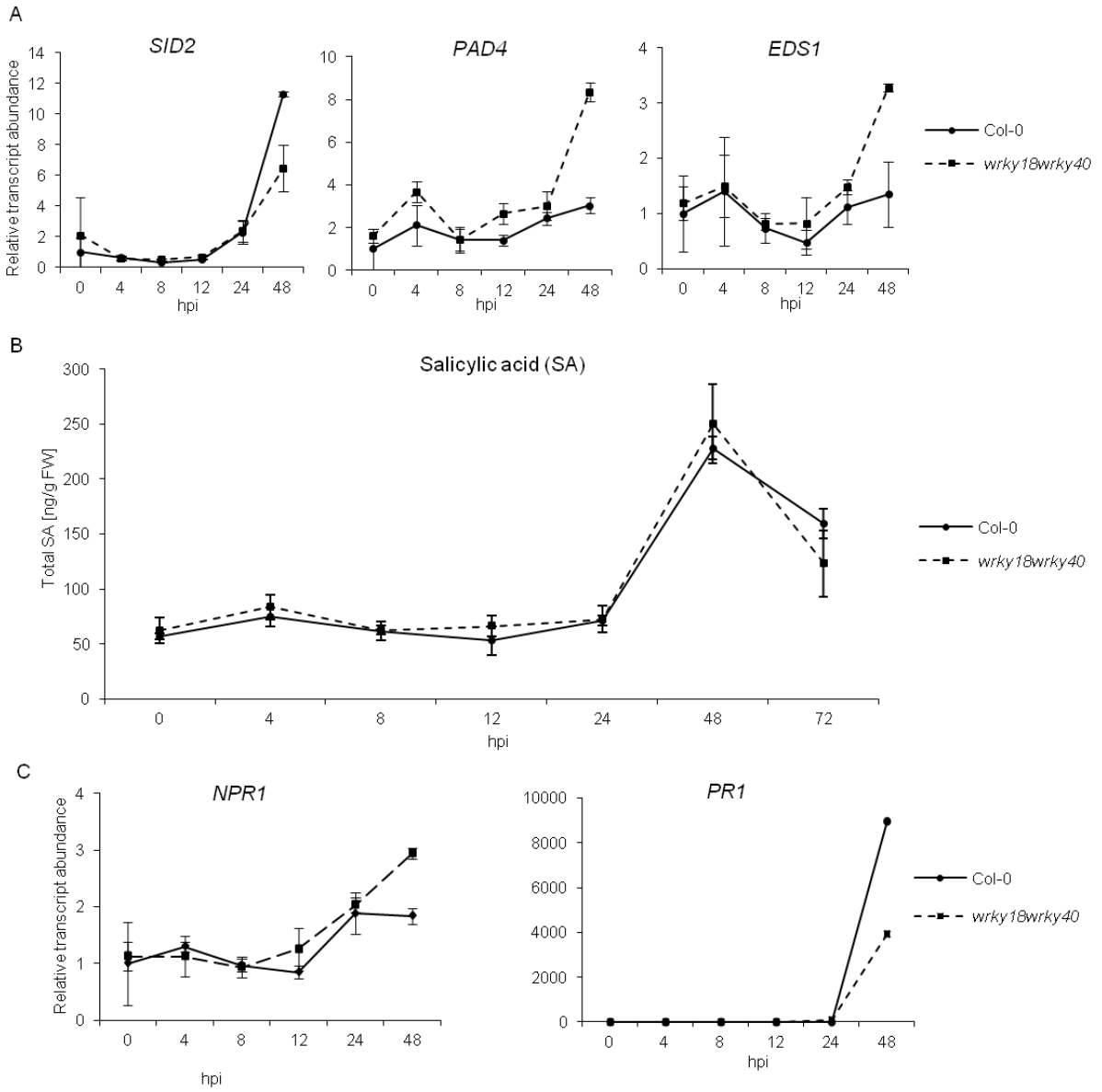


Figure 4: Activation of the SA/EDS1 signaling pathway and SA levels during early *G. orontii* infection. **(A)** qPCR analysis of the expression of the SA biosynthesis gene (*SID2*) and the SA-dependent EDS1-signaling pathway genes *EDS1* and *PAD4* at indicated time points during early *G orontii* infection in susceptible Col-0 (solid lines) and resistant *wrky18wrky40* plants (dashed lines). Error bars represent SD (n=3). Fold changes are relative to non-challenged Col-0 samples set to 1. **(B)** Accumulation of SA in susceptible Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants during the first 72 hours after infection with *G. orontii*. Error bars represent SD (n=4). **(C)** qPCR expression analysis of downstream SA marker genes *NPR1* and *PR1* at indicated time points during *G. orontii* infection in susceptible Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants. Error bars represent SD (n=3). Fold changes are relative to non-challenged Col-0 samples set to 1.

Another key component required downstream of SA for *PR1* gene activation that is also involved in the antagonistic cross-talk between JA and SA is NPR1. Expression of *NPR1* also showed a weak induction at 48 hpi in *wrky18wrky40* plants.

Overall, temporal analysis of SA accumulation, SA biosynthesis and response revealed synchronous dynamics between both SA biosynthesis and SA accumulation in *wrky18wrky40* and wildtype plants. This also held true for the expression of downstream marker gene *PR1* in

response to SA. However, it is still unclear which role SA plays for resistance towards *G. orontii* in *wrky18wrky40* mutant plants. To elucidate whether SA accumulation is required for resistance of *wrky18wrky40* plants, the double mutant was crossed with the SA-biosynthesis mutant *sid2*.

Homozygous F3 triple mutants were grown under controlled short-day conditions. No obvious morphological leaf phenotype was observed for the triple mutant compared to wildtype Col-0 plants (Figure 5 A). To test whether resistance in *wrky18wrky40* is dependent on SA biosynthesis gene *SID2* and thereby on the observed SA accumulation, 4 weeks old plants were infected with *G. orontii* and macroscopically characterized 9 dpi (Figure 5 A).

Figure 5: Leaf infection phenotype of *wrky18wrky40sid2* triple mutants upon *G. orontii* infection. (A) Macroscopic phenotype of indicated genotypes of 4 weeks old plants before and 9 days after infection with *G. orontii*. **(B)** Percentage of host cell entry rate of indicated genotypes upon infection with *G. orontii*. Host cell entry rate was determined 48 hpi.

Susceptibility of wildtype Col-0 plants and *sid2* single mutants was associated with successful reproduction of *G. orontii* on the leaf surface, whereas resistance of *wrky18wrky40* plants was accompanied with occasional fungal sporulation on leaf margins but yellow necrotic leaf areas restricting fungal proliferation. However, *wrky18wrky40sid2* triple mutants exhibited a wildtype-like phenotype with successful reproduction of *G. orontii* on the leaf surface. For *wrky18wrky40*, resistance towards *G. orontii* was associated with a reduction in effective host cell entry to ~ 60% (Figure 5 B) compared to wildtype and *sid2* mutant plants showing ~80%. Similarly, percentage of host cell entry rate in the *wrky18wrky40sid2* triple mutant was restored to wildtype-like levels, leading to a fungal entry rate of ~80%.

Apart from plant hormones, secondary metabolites have recently been shown to play a key role in plant defense. One metabolite catalyzed by the cytochrome P450 enzyme CYP81F2 from tryptophan is the antifungal glucosinolate 4MI3G. Microarray analysis indicated an up-regulation of crucial 4MI3G biosynthesis genes in *wrky18wrky40* plants (Pandey et al., 2010). Hence, the accumulation of this secondary metabolite was determined during the course of *G. orontii* infection.

2.4. Accumulation of 4MI3G during early *G. orontii* infection

Activation of essential biosynthesis genes of biological active compounds is an indication for the synthesis of associated products. *CYP81F2* expression was found to be induced in both susceptible wildtype and resistant *wrky18wrky40* plants (Figure 6 A). Whereas wildtype plants showed a biphasic expression kinetics with almost 10-fold increase at 4 and 12 hpi during *G. orontii* infection, respectively, *wrky18wrky40* plants accumulated 45-fold higher levels of *CYP81F2* transcript at 12 hpi when compared to wildtype non-challenged samples, and >4.5-fold more transcript than wildtype infected plants at this similar time point. To investigate the accumulation of 4MI3G and the correlation between expression of the biosynthesis gene and product accumulation, 4MI3G levels were determined. Plants were infected with *G. orontii* and 4MI3G levels were measured at 0, 4, 8, 12, 24, 48 and 72 hpi. Indeed, *wrky18wrky40* plants accumulated higher levels of 4MI3G during the first 72 hours after infection than wildtype plants. Additionally, resistant mutant plants showed an earlier accumulation of 4MI3G compared to wildtype plants and the increase was stronger. After 72 hours, 4MI3G accumulation starts to decline in wildtype plants whereas *wrky18wrky40* plants reveal a continuous rise up to this time point with >3-fold higher 4MI3G levels compared to wildtype non-challenged samples and ~25% more than in the corresponding wildtype sample (Figure 6 B).

Currently no direct role for 4MI3G in defense against fungal pathogens has been reported but activation of 4MI3G by PEN2 is postulated to result in the accumulation of an antifungal compound (Bednarek et al., 2009). Thus, expression of *PEN2* was monitored during *G. orontii* infection. However, expression of *PEN2* was not significantly affected upon powdery mildew infection and no obvious differences were observed between wildtype and *wrky18wrky40* plants (Figure 6 A).

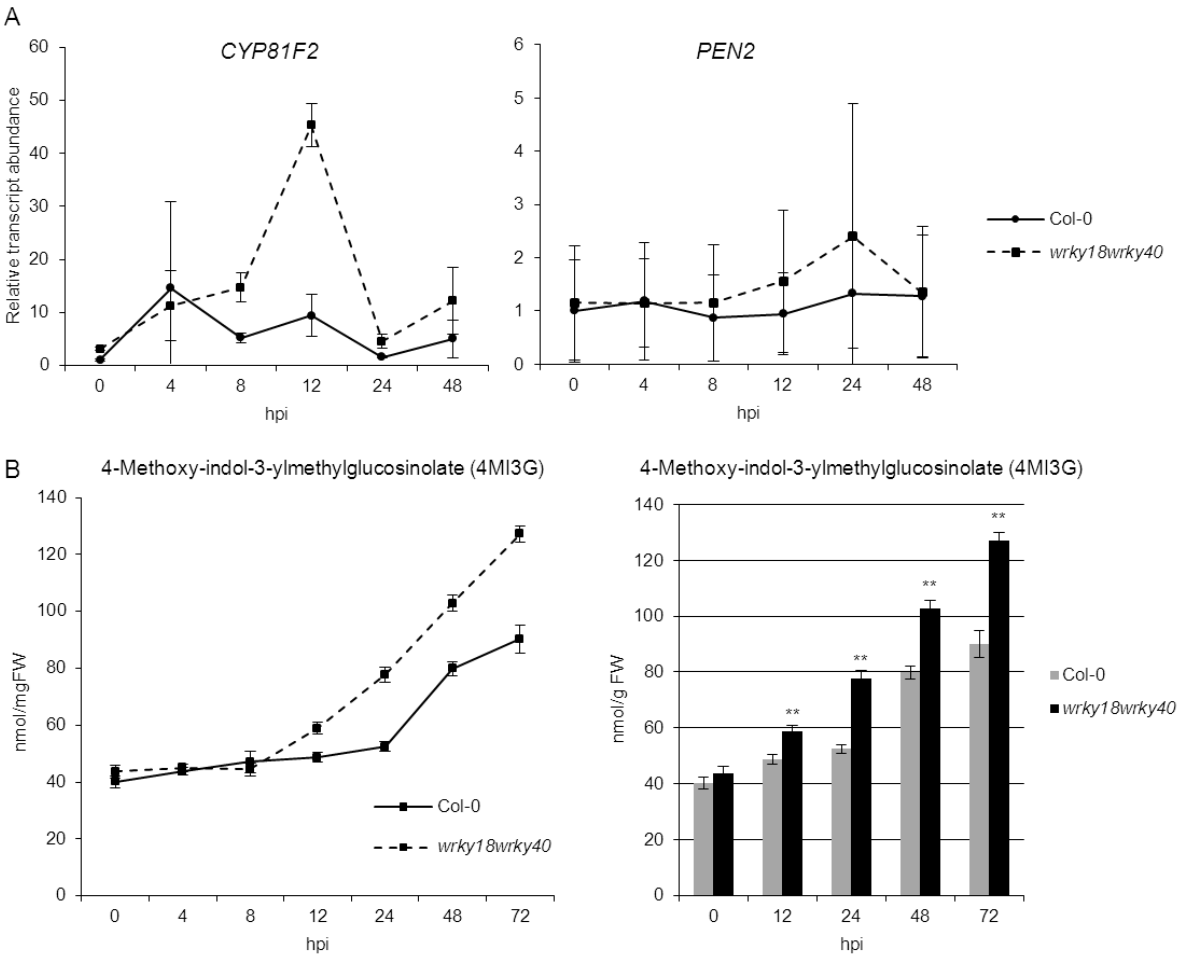


Figure 6: Activation of the glucosinolate pathway during early *G. orontii* infection. (A) qPCR anaylsis of the the expression of 4MI3G biosynthesis gene *CYP81F2* and the 4MI3G activating gene *PEN2* at indicated time points upon *G. orontii* infection in susceptible Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants. Fold changes are relative to non-challenged Col-0 samples set to 1. Error bars represent SD (n=3). **(B)** Accumulation of the antifungal glucosinolate 4MI3G in susceptible Col-0 and resistant *wrky18wrky40* plants during the first 72 h of *G .orontii* infection at indicated time points. Asterisks (**) indicate p-value < 0.01 based on Student's *t*-test.

2.5. *G. orontii* infection of *wrky18* and *wrky40* single mutant plants

The two TFs WRKY18 and WRKY40 are thought to have partly redundant functions in plant defense. This is corroborated by the observation that only the double mutant *wrky18wrky40* exhibits a resistant phenotype towards *G. orontii* infection, whereas individual mutations of *WRKY18* or *WRKY40* are not sufficient to establish resistance (Pandey et al., 2010; Shen et al., 2007). On the other hand, constitutive expression of *WRKY18* leads to an increased resistance against the bacteria *Pto* DC3000 that has not been shown for *WRKY40* overexpressing plants (Chen and Chen, 2002; Xu et al., 2006). In the same line, increased susceptibility towards infections with the necrotrophic fungus *B. cinerea* was observed for

WRKY40 but not for *WRKY18* overexpressing plants (R. Birkenbihl, MPIPZ Cologne, unpublished).

To further elucidate functions of both WRKY18 and WRKY40, a global expression analysis using the Affymetrix AGRONOMICS tiling array was performed. In addition to genes differentially regulated in uninduced state due to the lack of WRKY18 or WRKY40, genes that are differentially expressed between both genotypes upon pathogen challenge are of particular interest to dissect roles of WRKY18 and WRKY40. It was previously shown that *WRKY18* and *WRKY40* expression is rapidly induced already 4 h after infection and transcriptional reprogramming by these TFs occurs at early stages of the infection process (Pandey et al., 2010). To cover major early events of the fungal infection process, 4 weeks old wildtype Col-0 plants as well as *wrky18* and *wrky40* mutants were infected with the powdery mildew fungus *G. orontii* and leaf material was harvested prior to inoculation (0 hpi), during the penetration event (6 hpi) and during haustorium formation (14 hpi) in three independent replicates. Transcript accumulation was analyzed compared to wildtype Col-0 plants and genes with a fold-change ≥ 1.5 or ≤ 0.66 with and a p-value ≤ 0.05 were included into the analyses. Numbers of up- and down-regulated genes at 0, 6 and 14 hpi in *wrky18* and *wrky40* mutants are depicted in Figure 7 A. Overall, 473 genes in *wrky18* and 441 genes in *wrky40* mutants are differentially regulated prior to infection. A large part of the genes were down-regulated, with *wrky18* mutants showing >50% down-regulated genes. At 6 hpi, however, more than 3/4 of differentially regulated genes in *wrky18* mutants were up-regulated, whereas the opposite was observed for *wrky40* mutant plants upon *G. orontii* infection. In total, 92 out of 387 genes were down-regulated in *wrky18* plants, whereas the majority of genes were up-regulated. This is in contrast to only 72 out of 303 up-regulated and 231 down-regulated genes in *wrky40* plants compared to wildtype Col-0 plants at the same time point. This discrepancy between the two genotypes is outweighed at 14 hpi, with a total of 245 and 247 misregulated genes in *wrky18* and *wrky40* mutants, respectively, and similar ratios of up- and down-regulated genes. At this stage, ~1/3 of the differentially regulated genes is down-regulated in both genotypes compared to wildtype plants at the same time point upon *G. orontii* infection. Overlaps between differentially regulated genes in *wrky18* and *wrky40* mutants are illustrated by Venn-diagrams in Figure 7 B. A total of 109 genes are differentially regulated in both genotypes prior to infection, whereas only 20 genes are shared upon *G. orontii* infection 6 hpi. At the latest time point 14 hpi, ~20% of the misregulated genes are in common between *wrky18* and *wrky40* mutants.

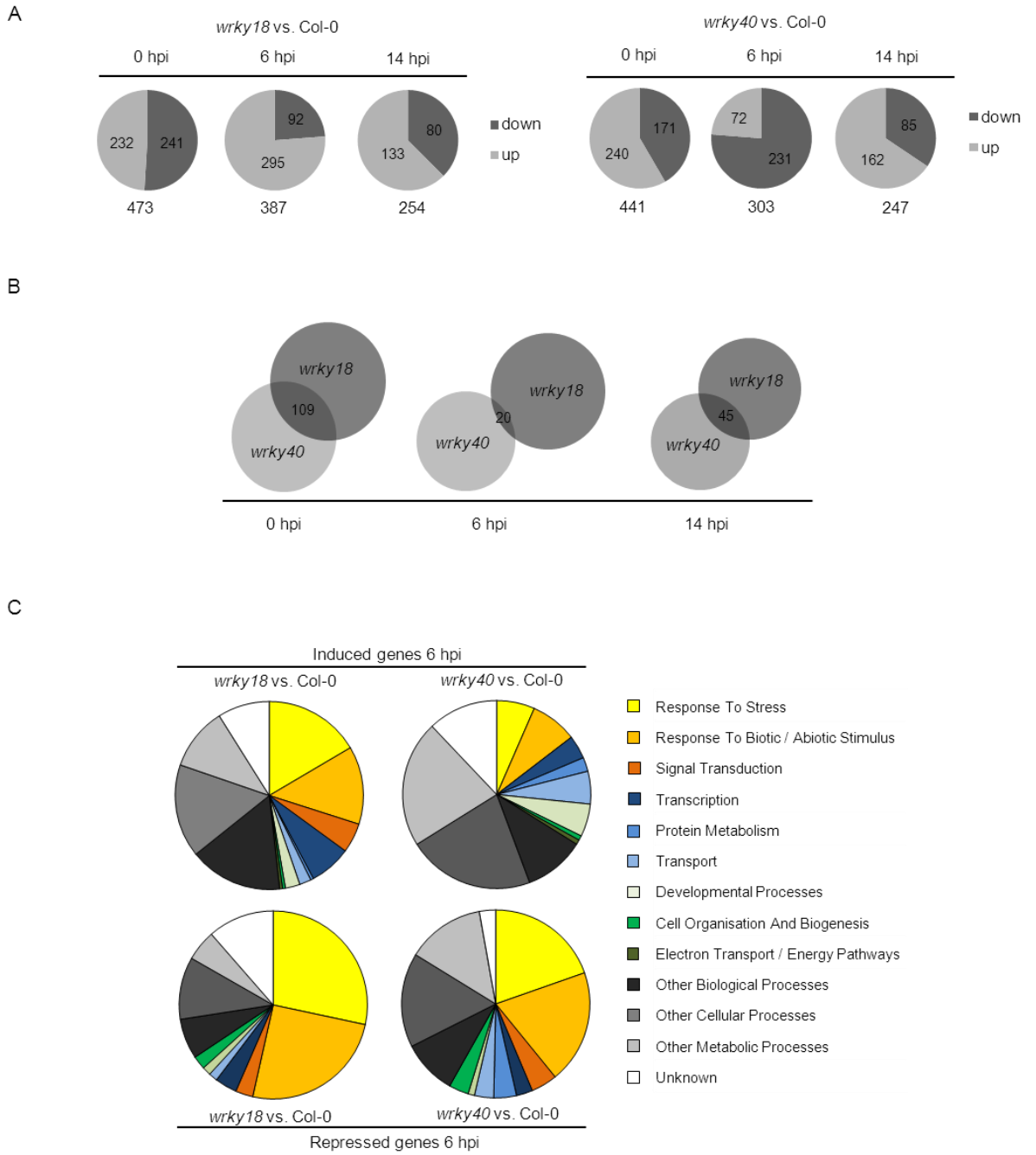


Figure 7: Analysis of differentially regulated genes in *wrky18* and *wrky40* mutants upon *G. orontii* infection. Whole rosette leaves of 4 weeks old plants were harvested prior to infection (0 hpi), or 6 and 14 h after *G. orontii* infection for comparative transcriptome analysis of *wrky18* and *wrky40* plants using the AGRONOMICS microarray. All samples were analyzed in triplicates and fold-changes calculated relative to wildtype Col-0 samples at the same time point. Only genes ≥ 1.5-fold up or ≤ 0.66-fold down with p-value ≤ 0.05 were included into the analysis. **(A)** Comparison of up- and down-regulated genes in *wrky18* and *wrky40* plants relative to Col-0 plants at the same indicated time points. Total number of misregulated at the given time point are presented below. **(B)** Venn-diagrams displaying overlaps of differentially regulated genes between *wrky18* and *wrky40* at the indicated time points. **(C)** Functional classification of the 50 most highly induced or repressed genes in *wrky18* and *wrky40* plants upon *G. orontii* infection 6 hpi according to Gene Ontology classification (TAIR).

This data illustrates considerable differences in the misregulation of genes depending on the absence of either WRKY18 or WRKY40 compared to wildtype plants already in an uninduced situation and even more pronounced during the infection process 6 and 14 hpi. However, no considerable overlap between misregulated genes in both genotypes was observed.

Functional classifications based on their biological functions of the 50 most up- or down-regulated genes 6 hpi in *wrky18* and *wrky40* plants revealed a predominant association with defense-related processes. In contrast to *wrky4*0 plants at 6 hpi, a large part of the genes induced in *wrky18* mutants are related to stress, biotic and abiotic stimuli, transcription, and signal transduction (Figure 7 C). Nevertheless, within the 50 strongest repressed genes at 6 hpi, more than 50% are related to response to stress, biotic and abiotic stimuli, signal transduction and transcription in both genotypes compared to wildtype plants, indicating a direct or indirect positive effect of WRKY18 and WRKY40 on these processes. However, within the strongest up-regulated genes in the *wrky18* mutant, a noticeable multitude of JA/ET-related genes are induced (Table 1). Besides several members of the JAZ family of

Table 1: Selection of genes most strikingly induced (p-value ≤ 0.05) in *wrky18* plants 6 hpi *G. orontii* infection. Genes were selected based on fold-changes relative to Col-0 6 hpi. Total number of W-boxes (T/C TGAC T/C) and W-box-like motifs (TGAC T/C) in the promoter of each gene was counted and set in relationship to the promoter size, respectively.

			FC relative to Col-0 6 hpi		
			wrky18	# W-boxes	W-boxes/1000bp
1	AT1G74930	ORA47	24,53	3	1,80
2	AT2G34600	JAZ7	13,09	3	1,29
3	AT5G48850	ATSDI1	6,18	20	5,05
4	AT4G17500	ERF-1	5,57	21	3,76
5	AT4G13395	ROTUNDIFOLIA like 12	4,82	6	4,36
6	AT5G47220	ERF2	4,59	9	4,90
7	AT5G61590	Integrase-type DNA-binding superfamily protein	4,45	6	4,33
8	AT1G27730	STZ/ZAT10	4,41	17	2,95
9	AT4G37610	BT5	4,20	8	3,63
10	AT5G19120	Eukaryotic aspartyl protease family protein	3,98	1	0,71
	…				…
20	AT3G49570	response to low sulfur 3	3,01	7	2,82
21	AT3G44260	ATCAF1A	3,00	5	4,55
22	AT1G19180	JAZ1	2,94	10	2,33
23	AT1G37537	transposable element gene	2,89	13	3,33
24	AT1G17380	JAZ5	2,87	5	1,28
25	AT1G60960	iron regulated transporter 3	2,87	4	1,39
26	AT1G72920	TIR domain family protein	2,79	3	2,86
27	AT1G80440	Galactose oxidase/kelch repeat superfamily protein	2,76	11	2,82
28	AT3G48360	BT2	2,65	7	1,84
29	AT1G28480	GRX480	2,62	8	4,04
30	AT5G61600	ERF104	2,61	2	1,60

JA-signaling repressors or the transcriptional repressor STZ/ZAT10, also positive regulators of JA- and ET-signaling (e.g. ORA47) are represented. As these genes are potential direct target genes of WRKY18, the number of W-box and W-box-like motifs in the promoter region of these genes was analyzed and is depicted in Table 1. Moreover, the number of W-box and W-box-like sequences relative to the promoter size is shown. From the most up-regulated genes, a sulphate deficiency-induced gene, *ATSDI1*, contained more than 5 W-box or W-box-like motifs per 1000 bp. Also JA/ET signaling related genes *ERF-1* and *ERF2* revealed over-representations of WRKY *cis*-regulatory elements in their promoter regions, whereas the strongest up-regulated gene ORA47 did not reveal many W-box or W-box-like sequences in its promoter.

To confirm the altered expression levels of these genes in the *wrky18* mutant during *G. orontii* infection, a subset of the genes were analyzed by qPCR during the temporal progression of infection to account for transcript levels variations by potentially asynchronous infection in the microarray experiments. For this, 4 weeks old plants were infected with *G. orontii* and whole leaf samples were taken at 0, 4, 8, 12 and 24 hpi. All tested genes revealed strongly increased transcript abundance in *wrky18* plants relative to Col-0 samples, although the timing and magnitude differed to the results observed on the microarrays (Figure 8). This indicates that the synchronicity of the infection process resulting in changes in gene expression was slightly altered in this set of experiments compared to those of the microarray experiments.

However, all tested genes showed elevated transcript levels already at 0 h or 4 h after infection, whereas only *JAZ5* was also up-regulated at 8 hpi.

Essentially, all tested genes were induced in the *wrky18* mutant compared to Col-0 samples upon *G. orontii* infection, thus verifying the microarray data. Moreover, over-representation of W-box and W-box-like sequences in promoter regions of some of these genes make them good candidates for direct WRKY18 targeting. To test by chromatin immunoprecipitation (ChIP)-qPCR whether these genes are de facto direct targets of WRKY18, an epitope-tagged *WRKY18* complementation line was established enabling detection of WRKY18-HA protein in ChIP experiments.

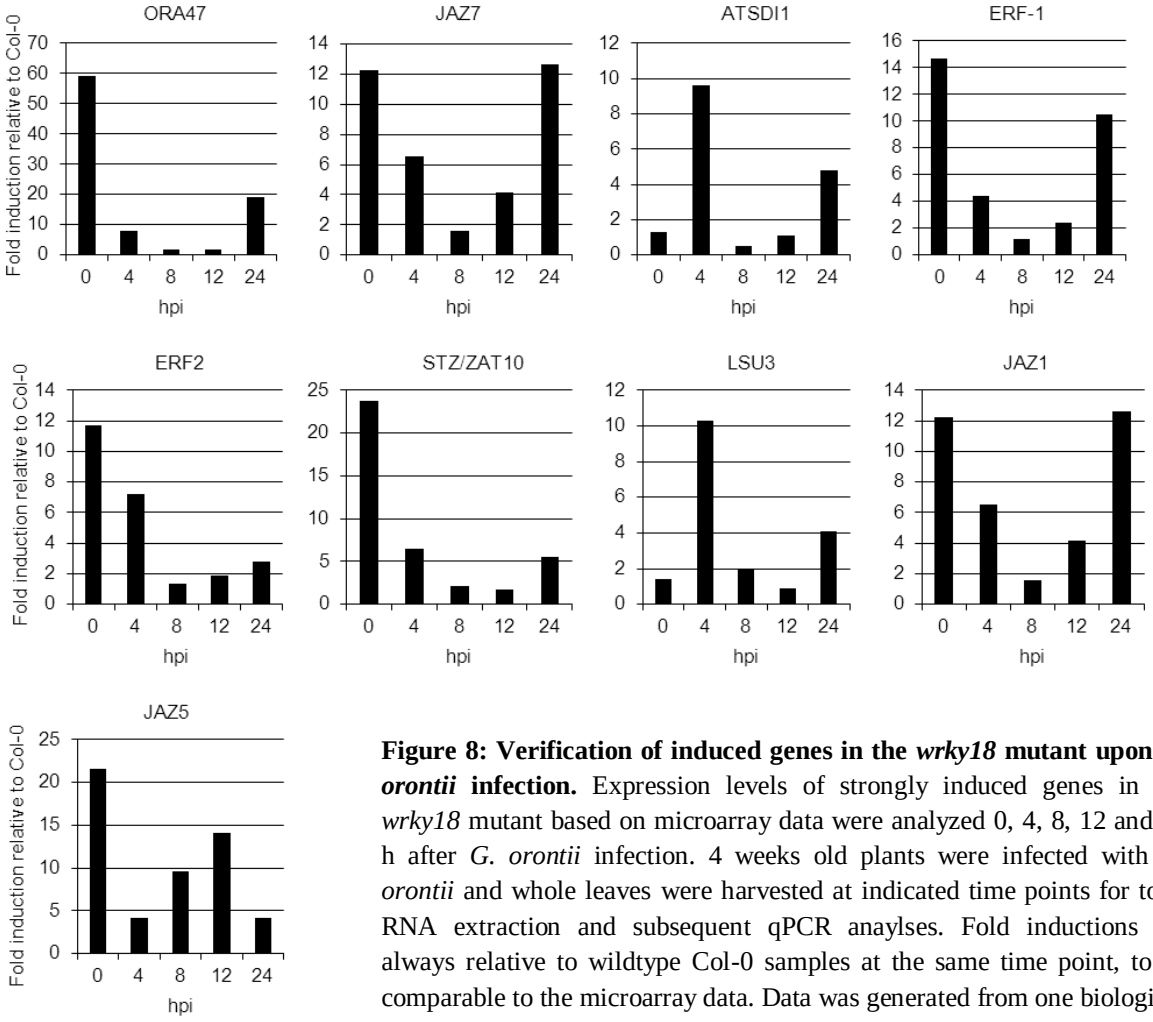


Figure 8: Verification of induced genes in the *wrky18* mutant upon *G. orontii* infection. Expression levels of strongly induced genes in the *wrky18* mutant based on microarray data were analyzed 0, 4, 8, 12 and 24 h after *G. orontii* infection. 4 weeks old plants were infected with *G. orontii* and whole leaves were harvested at indicated time points for total RNA extraction and subsequent qPCR anaylses. Fold inductions are always relative to wildtype Col-0 samples at the same time point, to be comparable to the microarray data. Data was generated from one biological replicate to confirm microarray data.

2.6. Characterization of the *WRKY18* complementation line

Microarray data revealed a broad transcriptional reprogramming in *WRKY18* mutant plants upon infection with *G. orontii*. W-box sequences in promoter regions of several of the up-regulated genes were found to be over-represented, which makes them into good potential candidates for direct targeting by WRKY18. Apart from binding of *NPR1* and *WRKY18* gene promoters in EMSA analyses of *WRKY18* overexpressing lines (Chen and Chen, 2002), association of WRKY18 with regulatory regions of potential target genes has not been previously described. In fact, lack of specific WRKY18 antibodies hampered the identification of direct target genes and created the need for an epitope-tagged version of WRKY18. Moreover, overexpression lines often do not mimic native situations regarding protein levels and binding behavior.

Therefore, the genomic region -4428 bp to +1514 bp relative to the start codon, including the native *WRKY18* promoter, was cloned, fused to a 3xHA-epitope tag and stably transformed into *wrky18wrky40* double mutant plants.

A prerequisite for further use of this complementation line was the demonstration of functional complementation of the infection phenotype, pathogen-inducibility of *WRKY18* expression and WRKY18-HA protein abundance (Figure 9). No obvious altered growth phenotypes were observed for the tested complementation line. Four weeks old plants were infected with *G. orontii* and the infection phenotype was macroscopically determined 9 dpi

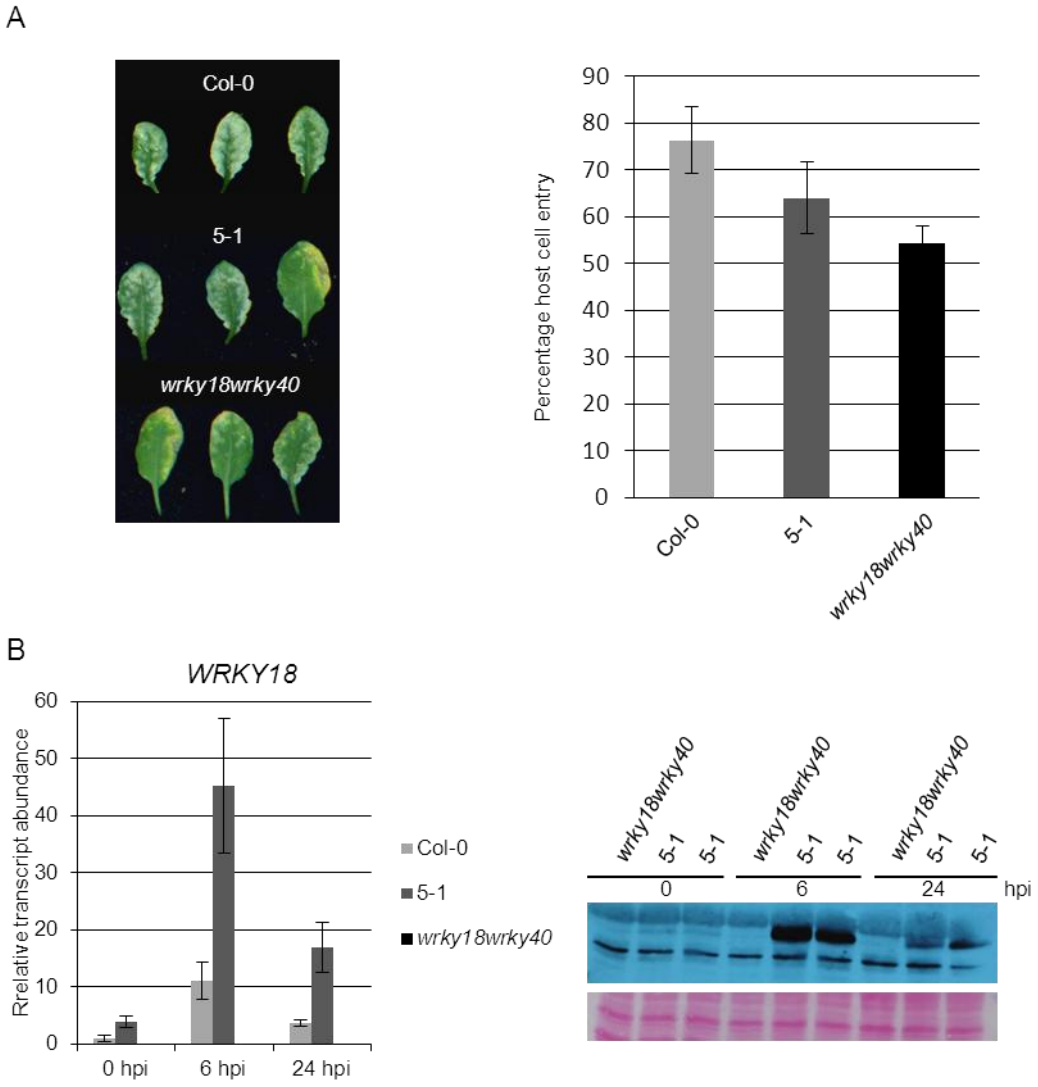


Figure 9: Characterization of the epitope-tagged *WRKY18* complementation line. (A) Phenotypic characterization of *WRKY18* complementation line 5-1 after *G. orontii* infection compared to susceptible Col-0 and resistant *wrky18 wrky40* plants. Pictures were taken 9 days after infection. Rate of host cell entry was determined 48 h after infection. **(B)** qPCR analysis of *WRKY18* expression in the *WRKY18* complementation line 5-1 compared to Col-0 0, 6 and 24 h after *G. orontii* infection, and corresponding protein levels determined using an HA antibody.

(Figure 9 A). Transformation of resistant *wrky18wrky40* plants with the epitope-tagged *WRKY18*-construct reconstituted the susceptible wildtype phenotype, demonstrating the functionality of this construct. This observation was additionally supported by an increase in fungal host cell entry rates observed 48 hpi for this complementation line compared to resistant *wrky18wrky40* plants (Figure 9 B). To test pathogen-dependent inducibility of the respective construct, total RNA was isolated from wildtype Col-0 plants and *WRKY18* complementation line 5-1 prior to, or 6 h and 24 after infection with *G. orontii*. Expression analysis revealed a ~3-fold higher transcript abundance already in the uninfected 5-1 line (0 hpi), possibly indicating multiple insertions or position effects of the transformed DNA construct (Figure 9 B). However, expression of *WRKY18* was strongly induced in both genotypes 6 hpi followed by a decrease in transcript abundance 24 hpi. Although ~3-fold more transcripts were consistently observed for the complementation line at all time points, this data demonstrates pathogen-responsiveness of the respective construct. As transcript abundance does not necessarily correlate with protein levels and to test accessibility of the epitope tag for the antibody, protein abundance was tested upon *G. orontii* infection at 0, 6 and 24 hpi. Similar to that observed for *WRKY18* transcript, protein levels were strongly induced 6 hpi and subsequently declined at 24 hpi. This data demonstrates that the transformed *WRKY18* construct is functional *in planta* and is inducible upon *G. orontii* infection, making the tested complementation line 5-1 suitable for further investigations.

2.7. WRKY18-HA protein binds to the *WRKY18* and *WRKY40* promoters

Feedback regulation is a common feature in the highly connected transcriptional network of WRKY transcription factors (Eulgem and Somssich, 2007). In fact, *in vitro* analysis suggested binding of WRKY18 to W-boxes in its own promoter (Chen and Chen, 2002). To confirm these results *in vivo* and to test whether *WRKY18* expression during *G. orontii* infection is possibly regulated in a (negative) feedback-loop, ChIP-qPCR was performed on cross-linked leaf material harvested 2 and 6 h after *G. orontii* infection. Different primer sets spanning regions containing W-box clusters upstream of the *WRKY18* start codon were employed for qPCR amplification of chromatin immunoprecipitated material (IP) with input DNA (IN) serving as control. Four weeks old plants of complementation line 5-1 (5-1) and *wrky18wrky40* (KO) mutants were used for ChIP experiments. A schematic illustration of the *WRKY18* promoter showing also position and orientation of W-box (T/C TGAC T/C) and W-box-like sequences (TGAC T/C) is depicted in Figure 10 A. A region ~1000 bp upstream of the *WRKY18* start codon harboring 3 clustered W-boxes was strongly enriched in the

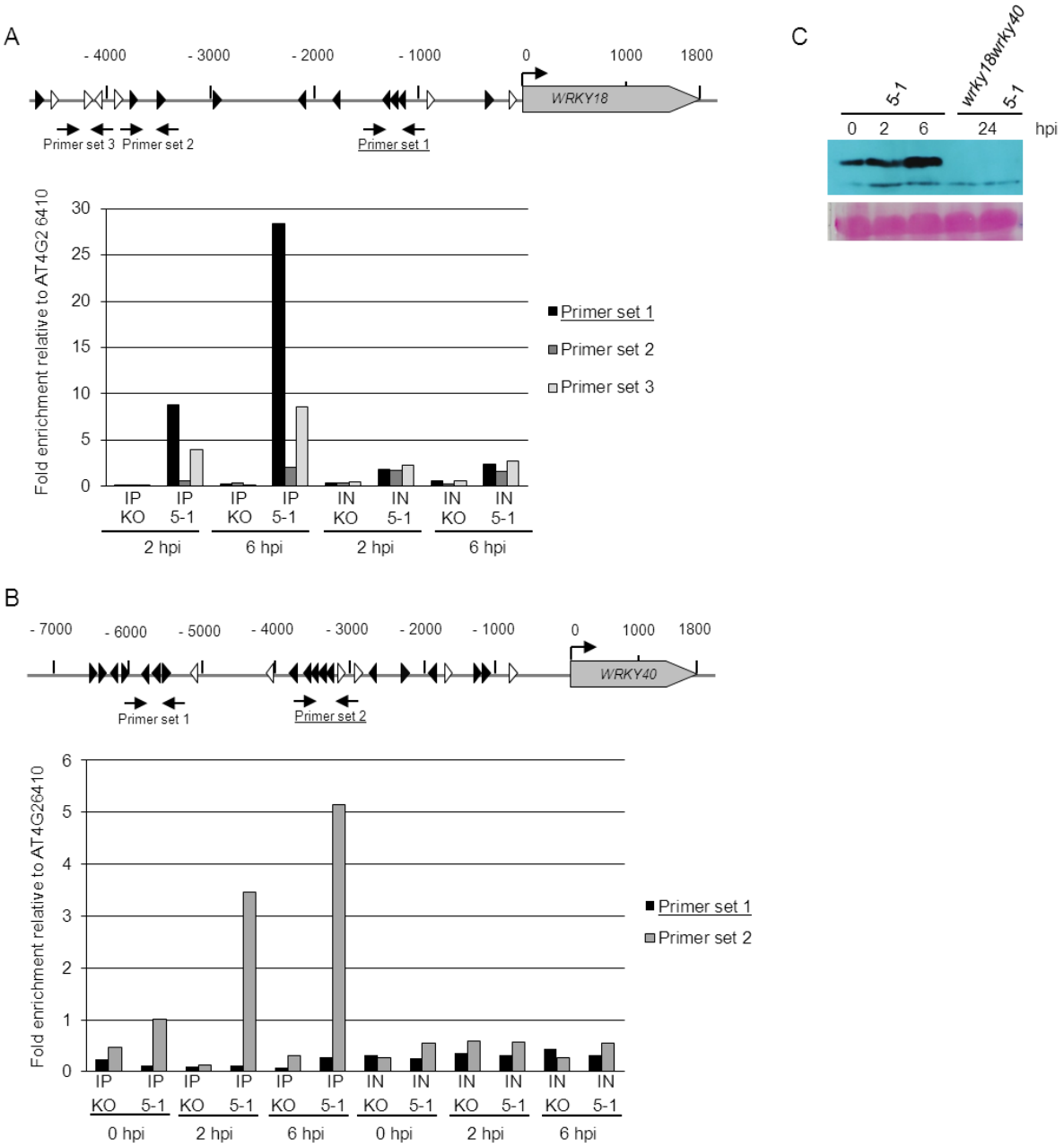


Figure 10: Direct binding of WRKY18-HA protein to the *WRKY18* and *WRKY40* promoters.
Schematic illustration of W-box and W-box-like motifs in the **(A)** *WRKY18* promoter and **(B)** *WRKY40* promoter. W-boxes (T/C TGAC T/C) are represented by black and W-box-like sequences (TGAC T/C) by white triangles at indicated positions. Triangle direction illustrates the orientation of the motifs. Open reading frames of the respective genes are depicted as large grey arrows. Primer sets used for ChIP-qPCR are located as shown. The scale is in bp relative to the translation start site (black arrow). For ChIP-qPCR, 4 weeks old *wrky18wrky40* mutant plants (KO) and *WRKY18* complementation line 5-1 plants were infected with *G. orontii* and leaves harvested 0 and/or 2 and 6 hpi. Input DNA (IN) before co-immunoprecipitation and co-immunoprecipitated DNA using anti-HA antibodies (IP) were analysed by qPCR employing the indicated primer sets. Fold enrichment is depicted relative to a DNA fragment of AT4G26410 (Expressed protein). ChIP experiments were repeated at least twice with similar results. **(C)** Protein accumulation in the *WRKY18* complementation line 5-1 at 0, 2, 6 and 24 h after *G. orontii* infection.

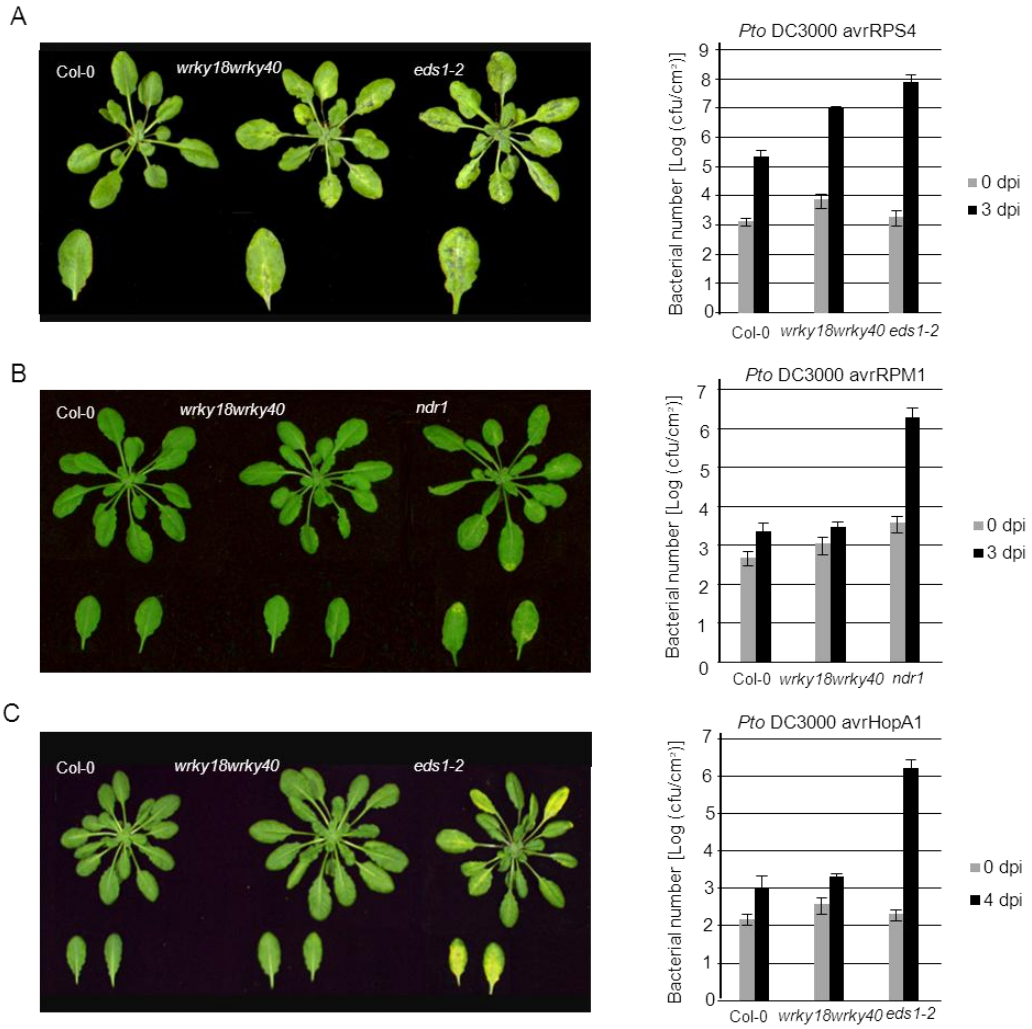
chromatin immunoprecipitated material of complementation line 5-1 using the anti-HA antibody at 6 hpi relative to a DNA fragment of AT4G26410 (Figure 10 A).

Already 2 hpi, a relative enrichment of ~8-fold was observed for this region. Moreover, a second region ~4000 bp upstream of the *WRKY18* start codon containing 2 W-box-like motifs revealed ~4-fold enrichment 2 hpi and ~8-fold enrichment 6 hpi, whereas a third primer set and the input control did not show any significant enrichments.

Also the *WRKY40* promoter contains several regions of W-box and W-box-like clusters as illustrated in Figure 10 B. Since *WRKY18* and *WRKY40* are co-expressed during *G. orontii* infection and are thought to act in a partially redundant manner (Pandey et al., 2010), mutual regulation is also conceivable. To test whether WRKY18 might also be involved in regulating *WRKY40* expression, different primer sets spanning regions encompassing the W-box and W-box-like motifs were used for ChIP-qPCR analyses. Indeed, binding of WRKY18 to a region ~5500 bp upstream of the *WRKY40* start codon could be clearly demonstrated, whereas no significant binding was found for the control input DNA and for a second region ~3000 bp upstream of the *WRKY40* start codon (Figure 10 B). Moreover, no binding of WRKY18 to the *WRKY40* promoter was observed prior to *G. orontii* infection (0h), although low amounts of WRKY18-HA was already detectable in the uninfected 5-1 line (Figure 10 C). As was the case for the *WRKY40* promoter, binding of WRKY18 to its own promoter was also dependent on *G. orontii* infection, indicating that binding of WRKY18 protein to both promoters at 2 and 6 hpi requires de novo expression of *WRKY18-HA* upon pathogen challenge. This data suggest that WRKY18 is involved in regulating the expression of its own gene as well as that of *WRKY40* in a pathogen dependent manner.

2.8. WRKY18 and WRKY40 are positive regulators of RPS4-mediated resistance

Negative regulation of resistance against pathogens is a common phenomenon of the *WRKY* gene family (Peng et al., 2008; Xing et al., 2008). Apart from negative regulation of resistance towards the biotrophic powdery mildew fungus *G. orontii* there is only sparse knowledge about resistance or susceptibility of *wrky18wrky40* double mutants towards other pathogens. Xu et. al (2006) found a reduction in susceptibility against the hemibiotrophic bacterium *P. syringae* DC3000, although the observed effect was rather weak. In contrast, an increase in fungal biomass in infected *wrky18wrky40* tissue after treatment with *B. cinerea* was described. So far, reports about infection of *wrky18wrky40* plants with other *P. syringae* (*Pto*) strains are missing. Therefore, different *Pto* strains were used for infection of



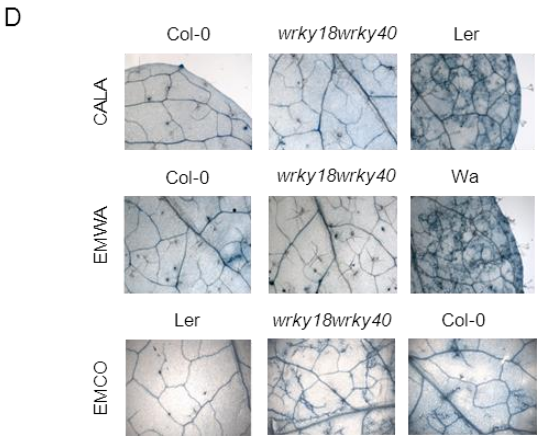


Figure 11: *wrky18wrky40* plants show a *Pto* DC3000 AvrRPS4-specific phenotype. Phenotype and bacterial colony counts of 4 weeks old plants spray-infected (O.D. = 0.2) with **(A)** *Pto* AvrRPS4, **(B)** *Pto* AvrRPM1 and **(C)** *Pto* HopA1. *Eds1-2* and *ndr1* mutants were included as susceptible controls. Pictures were taken 3 dpi (AvrRPS4 and AvrRPM1) and 4 dpi (HopA1). Bacterial colony counts were performed 0 and 3 dpi (AvrRPS4 and AvrRPM1) and 0 and 4 dpi for HopA1. **(D)** Microscopic characterization of 12 days old wildtype Col-0 plants and *wrky18wrky40* mutants upon spray-infection ($4x10^4$ spores /ml) with different *Hpa* isolates. Samples were taken 7 dpi and stained with trypan blue. Appropriate susceptible *Arabidopsis* ecotypes (Ler, Wa, Col-0) controls were included.

wrky18wrky40 plants (Figure 11). The bacterial strain *Pto* DC3000 AvrRPS4 is avirulent on *Arabidopsis* Col-0 plants. Secretion of the avirulence effector protein AvrRPS4 into the plant cell via the TTSS is recognized by the plant's TIR-NB-LRR-type R-protein RPS4. EDS1 signaling downstream of receptor activation is a prerequisite step for RPS4 resistance and TIR-NB-LRR-mediated resistance in general, whereas CC-NB-LRR mediated resistance is EDS1-independent and instead requires functional NDR1 (Aarts et al., 1998). After infection of *wrky18wrky40* plants with *Pto* AvrRPS4, mutant plants revealed increased susceptibility, accompanied with the formation of water-soaked lesions and yellow, necrotic areas, which were absent in resistant wildtype plants (Figure 11 A). The macroscopic phenotype was confirmed by increased bacterial numbers (colony forming units, cfu) observed in *wrky18wrky40* leaves. Bacteria were more successful in colonizing *wrky18wrky40* plants compared to wildtype plants 3 dpi. To test, whether *wrky18wrky40* plants are generally impaired in ETI, mutants were infected with another *Pto* strain namely *Pto* AvrRPM1. Resistance of wildtype Col-0 plants is based on the intercellular CC-NB-LRR-type R-protein RPM1 that acts EDS1 independently. However, neither a phenotypic difference was observed for wildtype plants compared to *wrky18wrky40* mutants upon *Pto* AvrRPM1 infection, nor did bacterial titer reveal a significant increase in bacterial colonization (Figure 11 B). Recently, Pandey et al. (2010) demonstrated binding of WRKY40 protein to a promoter region of *EDS1*. It is conceivable, that mutations in both *WRKY18* and/or *WRKY40* affect the EDS1 signaling pathway. Therefore, *wrky18wrky40* plants were challenged with additional pathogens for which resistance is known to depend on functional EDS1 (Figure 11 D). Resistances against *Hyaloperonospora arabidopsidis* (*Hpa*) isolates EMWA and CALA is mediated by TIR-NB-LRR-type R-proteins and thus are EDS1-dependent (Slusarenko and Schlaich, 2003). Infection with none of these two isolates revealed differences in susceptibility between wildtype and *wrky18wrky40* plants. Also, *wrky18wrky40* mutants were as susceptible as wildtype plants when challenged with the compatible *Hpa* isolate EMCO (Figure 11 D).

Together, this data demonstrates that increased susceptibility of *wrky18wrky40* towards *Pto* AvrRPS4 appears to be highly specific and is not due to a general breakdown of EDS1 function. To test whether *WRKY18* and *WRKY40* are specifically involved in the TIR-NB-LRR signaling pathway during plant-*Pto* interactions, a second avirulent *Pto* strain HopA1 was used for infection of *wrky18wrky40* mutant and Col-0 wildtype plants. It has been shown that resistance of wildtype Col-0 plants towards *Pto* HopA1 is mediated by a TIR-NB-LRR-type R-protein (Kim et al., 2009). However, infection of both genotypes did not reveal

differences in phenotype and bacterial colonization (Figure 11 C), pointing to a rather specific phenotype of *wrky18wrky40* double mutants upon *Pto* AvrRPS4 infection and an opposite phenotypic effect of loss-of-*WRKY18* and -*WRKY40* function in this plant-pathogen interaction compared to *G. orontii* infection. A summary of all tested pathogen infections involving the *wrky18wrky40* mutant is presented in the following section.

2.8.1. Additional *wrky18wrky40* plant-pathogen interactions

Three powdery mildews of the same genus are able to successfully infect *Arabidopsis* Col-0 plants. However, only robust resistance of *wrky18wrky40* against *G. orontii* was described so far (Shen et al., 2007), raising the question of a whether the *wrky18wrky40* double mutant confers broad-spectrum resistance towards all three powdery mildews, as has been reported for *mlo2mlo6* mutant plants (Consonni et al., 2006). To test for susceptible or resistant phenotypes of *wrky18wrky40* pants, different biotrophic or hemibiotrophic and necrotrophic pathogens were used for infection of *wrky18wrky40* and control wildtype Col-0 plants (Figure 12).

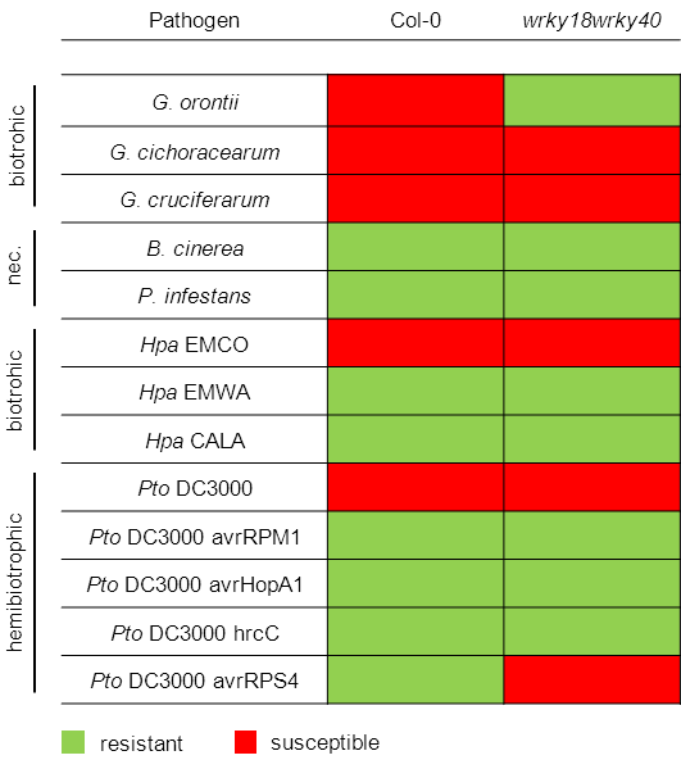


Figure 12: Testing *wrky18wrky40* plants with other pathogens. Four weeks old plants were infected with the indicated pathogens. Macroscopic characterization of phenotype was performed for all pathogens. Microscopic measurements were done for powdery mildews *G. orontii* (rate of host cell entry), *G. cichoracearum* and *G. cruciferarum* (conidiophore counts). Bacterial colony counts were performed for *Pto* using spray-infections (O.D.=0.2). *B. cinerea* was droplet-infected ($5x10^5$ spores/ml) and leaf lesion sizes were macroscopically compared. Susceptibilty to *P. infestans* was determined by trypan blue staining of droplet-infected leaves (500 spores/µl) and *Hpa* infections were performed by spray infection ($4x10^4$ spores/ml) of 10 days old seedlings and susceptibilty was determined by trypan blue staining. All infections were repeated at least twice with similar results.

Interestingly, *wrky18wrky40* plants did not show increased resistance against biotrophic powdery mildews *G. cichoracearum* and *G. cruciferarum*. Both, wildtype and *wrky18wrky40* plants revealed no differences in conidiophore numbers (Supplementary Figure 4), indicating that these fungi can proliferate on both genotypes equally, which was further supported by macroscopic analyses. Conversely, but similar in both genotypes, resistance of wildtype plants against the necrotrophic fungus *B.cinerea* isolate 2100 and the oomycete *P. infestans* was not altered in *wrky18wrky40* double mutants. Moreover, wildtype Col-0 and *wrky18wrky40* plants infected with the hemibiotrophic bacterium *P.syringae* DC3000 showed comparably strong susceptibility whereas infection with the disarmed *Pto* strain hrcC, being impaired in effector delivery, resulted in a uniform resistance of both genotypes.

To summarize, *wrky18wrky40* double mutants do not exhibit a broad-spectrum resistance against powdery mildews. Besides *G. orontii* infection, the only phenotypic difference between wildtype and *wrky18wrky40* plants was observed for infections with *Pto* AvrRPS4, apparently constituting two very specific interactions.

In this context it is conceivable that these pathogens differentially affect *wrky18wrky40* plants leading to susceptibility towards *Pto* AvrRPS4 and resistance to *G. orontii* infections. Both pathogens likely secrete a substantial repertoire of effectors into the plant cell during infection that alter the plants immune response (Lindeberg et al., 2009; Lindeberg et al., 2012; Spanu et al., 2010), possibly involving WRKY18 and/or WRKY40.

The sequencing of the barley powdery mildew *Bgh* genome in 2010 (Spanu et al., 2010), and the draft genome assemblies of *G. orontii*, revealed a large set of highly species-specific secreted effector candidates (OECs, *orontii* effector candidate). Several of the identified OECs were shown to interfere with the plant's immune response in 'effector detector vector system'-mediated infections, leading to increased susceptibility of *Arabidopsis* Col-0 plants towards *Pto* strains secreting single OECs (Sohn et al., 2007). In *G. orontii*, however, almost all tested OECs are expressed at early stages of the infection process (0-12 hpi), coinciding with *WRKY18* and *WRKY40* induction in plants upon *G. orontii* infection. Moreover, localization studies demonstrated nucleo-cytoplasmic localization of most of these OECs in plants, turning them into potential interaction partners of WRKY18 and WRKY40. To test interaction of OECs with WRKY18 and WRKY40, yeast 2-hybrid analyses were performed (Supplementary Figure 5). Mutual co-transformation of WRKY proteins and OECs did not reveal any interaction in yeast, suggesting that the tested OECs probably do not directly interact with WRKY18 or WRKY40.

In the case of *Pto* AvrRPS4, recent work of Heidrich et. al (2011) showed direct physical association of the R-protein RPS4 and EDS1 as well as the secreted effector AvrRPS4 and EDS1 in the plant cell nucleus, demonstrating that RPS4 is involved in protein-protein interactions. Moreover, interaction between a R-protein and WRKY TFs is the basis for MLA-mediated resistance in barley upon *Bgh* infection (Shen et al., 2007).

Therefore, interaction of WRKY18 and WRKY40 with RPS4 was tested in a yeast 2-hybrid approach. For this, full-length (aa 1-1218) and truncated versions containing only the N-terminal TIR-domain (aa 1-185) of RPS4 were co-transformed into yeast. Additionally, the atypical R-protein RRS1, harboring a WRKY domain at the C-terminus, was included as a full-length (aa 1-1288) and as a truncated form containing only the N-terminal TIR-domain (aa 1-155). It has been shown that *rrs1* mutants are as susceptible towards *Pto* AvrRPS4 infection as *RPS4* mutants (Birker et al., 2009), making RRS1 a potential partner for WRKY18 and WRKY40. However, no interaction was found between WRKY18 and/or WRKY40 with RPS4 or RRS1 in yeast 2-hybrid co-transformations (Supplementary Figure 6 A).

To overcome constraints of the yeast 2-hybrid system in terms of proper protein folding, protein modifications or subcellular localization, interaction of RPS4 and RRS1 full-length proteins were tested by acceptor photo bleaching (APB) in *N. benthamiana* epidermal cells. Apart from co-localization of WRKY18, WRKY40, RRS1 and RPS4 to the plant cell nucleus (Supplementary Figure 6 B), no direct interaction was detectable. However, it is still very likely that WRKY18 and/or WRKY40 are involved in still undefined protein interactions upon pathogen challenge.

2.9. Yeast 2-hybrid screen for interaction partners of WRKY18 and WRKY40

Belonging to class IIa of WRKY TFs, WRKY18 and WRKY40 contain an additional leucine-zipper motif in the N-terminus of the protein, required for homo-and heterodimerization. Recently, it has been shown that WRKY18 and WRKY40 interact with a putative ABA-receptor at the chloroplast membrane upon ABA treatment (Shang et al., 2010). Apart from that, no direct protein interaction partners of WRKY18 and WRKY40 are known. To elucidate potential new interaction partners, a yeast 2-hybrid screen using full-length *WRKY18* and *WRKY40* bait constructs was performed.

On the one hand, WRKY18 and WRKY40 have been shown to negatively regulate resistance to *G. orontii*, on the other hand they are required for efficient regulation of ETI during *Pto*

AvrRPS4 challenge and the observed phenotype appeared to be RPS4-specific. However, no direct interaction of WRKY18 or WRKY40 with RPS4 was found, raising the question whether other *P.syringae* induced proteins interact with WRKY18 and/or WRKY40.

A cDNA library (kindly provided by B. Kemmerling, Tübingen) constructed from *Arabidopsis* leaf material infected with different virulent and avirulent *P.syringae* strains and harvested at various time points after infection was used for the screening.

In yeast, protein abundance of WRKY18 was significantly higher compared to WRKY40 (Figure 13 A). Both proteins showed no autoactivity (did not activate the reporter construct alone) and thus could be included into the screening. In total, ~330.000 clones with a mating efficiency of ~3.3% were screened for WRKY18, whereas a mating efficiency of ~1.8% and ~165.000 screened clones for WRKY40 were considerably lower. Two most promising candidates that showed interaction with WRKY18 and WRKY40 were selected for further analyses. A truncated version of a 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (Oxidoreductase, At2G38240) and FIBRILLIN4 (FIB4, At3G23400) were found to interact with WRKY18 and WRKY40 in yeast. For both candidates the truncated version and a full-length cDNA clone were included in the verification in yeast. Exemplary, verification of interaction by yeast co-transformation with WRKY18 is depicted in Figure 13 B. SV40 T-antigen and murine p53 proteins were included as positive controls, known to interact strongly in yeast (Li and Fields, 1993). Besides co-transformation with WRKY18 and WRKY40, candidates were also co-transformed with another nuclear-localized protein, SPL3, serving as negative control. No interaction of WRKY18 and WRKY40 was found for full-length Oxidoreductase and FIBRILLIN4 (data not shown). However, interaction could be verified for the truncated version of both proteins, whereas no interaction was found with SPL3 (Figure 13 B). Similar results were obtained for WRKY40 (data not shown).

A prerequisite for direct protein interaction is the co-localization in the plant cell. To test whether candidate proteins co-localize with WRKY18 and WRKY40 *in planta*, YFP- and CFP-fusion proteins were cloned, transformed into *A .tumefaciens* and infiltrated into *N. benthamiana* leaves. Results of co-infiltration into tobacco epidermal cells are shown in (Figure 13 C a-c). As expected, WRKY18 and WRKY40 were localized to the plant cell nucleus. Indeed, also the truncated and full-length Oxidoreductase and FIBRILLIN4 proteins localized to the nucleus. Moreover, traces of truncated Oxidoreductase and truncated FIBRILLIN4 were also detected in the cytoplasm (Figure 13 a-d). Notably, truncated Oxidoreductase could only be localized when infiltrated alone (Figure 13 d). Although

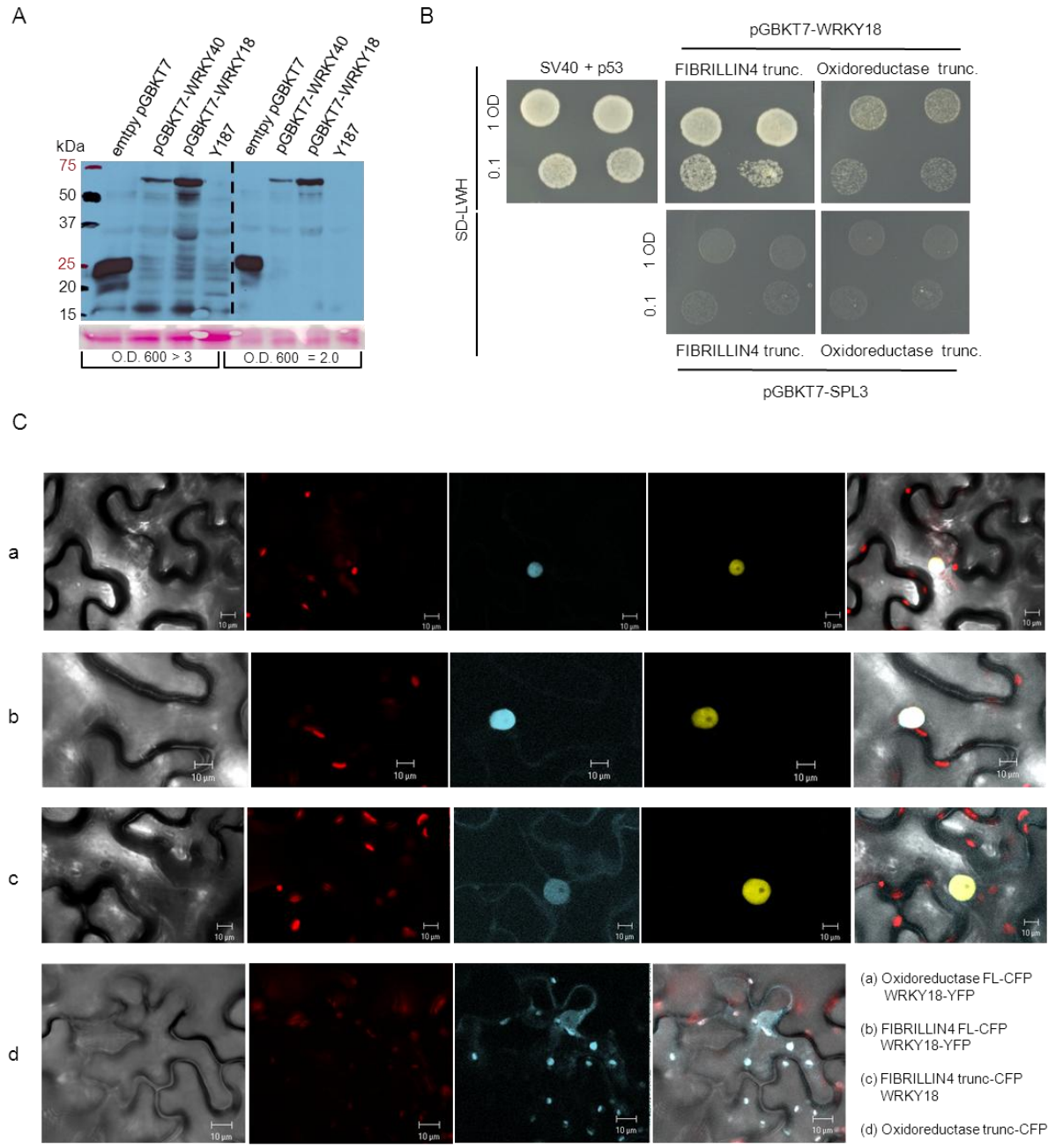


Figure 13: Localization of potential protein interaction partners identified from a yeast 2-hybrid screen with WRKY18 and WRKY40. (A) WRKY18 and WRKY40 protein abundance in yeast. Two different optical densities were used for protein extraction as indicated. Empty vector (pGBKT7) and untransformed yeast strain Y187 were included as controls. **(B)** Potential WRKY18 interaction partners were co-transformed into yeast and plated on SD-LWH selection media. Pictures were taken from undiluted and 1:10-diluted log-phase growing yeast after 2 days of incubation at 30°C. As positive control, the human SV40 T-antigen (T) and murine p35 protein (p35) (Clontech, Mountain View, USA) were included. Co-transformation of candidates with another nuclear-localized protein (SPL3) served as negative controls. **(C)** (Co-) localization of potential WRKY18 interaction partners transiently expressed in tobacco leaves. Pictures were taken 2 days post co-infiltration. Co-localization of YFP-tagged WRKY18 and CFP-tagged candidate proteins are shown in (a-c). Cellular localization of CFP-tagged truncated Oxidoreductase protein is depicted in (d). The scale bar represents 10 µm.

repeated several times, no signal was detectable when co-infiltrated with WRKY18 or WRKY40.

Together, all tested proteins localized to the nucleus, enabling them to potentially interact with WRKY18 and WRKY40 *in vivo*. To overcome restrictions of the yeast 2-hybrid system in terms of protein folding, protein modifications or localization, candidate proteins were employed for BiFC-analysis using a Split-YFP-system. Although WRKY18 and WRKY40 co-localize with the candidate proteins, no interactions could be detected in BiFC analyses (data not shown).

2.9.1. Characterization of interaction candidate mutants

To test whether *Oxidoreductase* and/or *Fibrillin4* are involved in defense, T-DNA mutant lines were obtained, homozygous lines generated and characterized and used for infection with *G. orontii* and *Pto* DC3000. Interestingly, when grown under controlled short-day conditions, *fibrillin4* (*fib4*) mutants exhibited a severe growth phenotype, observable already 7 days after sowing (Figure 14 A). *Fibrillin4* seedlings showed an elongated hypocotyl compared to wildtype Col-0 plants grown under same conditions. At later stages, *fibrillin4* plants developed elongated petioles and smaller leaves compared to Col-0 plants of the same age (4 weeks) (Figure 14 B).

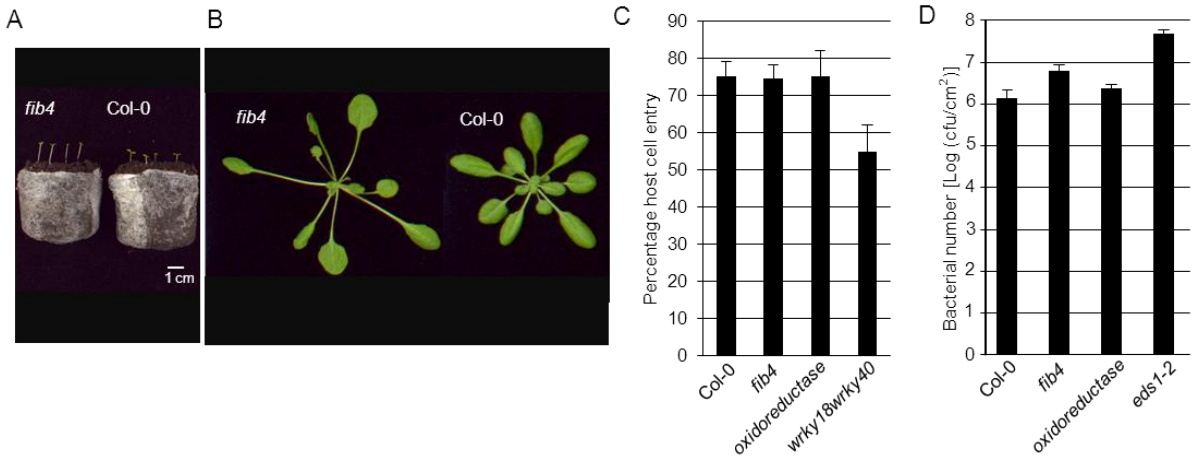


Figure 14: Phenotypic characterization of candidate interaction partners of WRKY18 and WRKY40. Growth phenotype of **(A)** 7 days old and **(B)** 4 weeks old *FIBRILLIN4* mutant plants (*fib4*) compared to wildtype Col-0 plants. Plants were grown under controlled short-day conditions on Jiffy-pots (Jiffy, Ryomgaard, Denmark). **(C)** Four weeks old *FIBRILLIN4* and *Oxidoreductase* mutants were infected with *G. orontii* and rate of host cell entry of fungal structures was determined 48 hpi. **(D)** Infection with bacterial strain *P.syringae* DC3000 *pv.* tomato was performed by spraying bacteria (O.D.600 = 0.2) on 4 weeks old plants. To determine bacterial numbers for each genotype, samples were taken 3 dpi. Hypersusceptible *eds1-2* plants were included as control.

Infection of *oxidoreductase* and *fibrillin4* mutants with *G. orontii* did not reveal any increase in resistance or susceptibility compared to susceptible wildtype Col-0 or resistant *wrky18wrky40* plants, respectively (Figure 14 C).

Additionally, no observable altered phenotype was found for *Oxidoreductase* mutant plants after infection with *Pto* DC3000. *Fibrillin4* mutants, however, displayed an increased susceptibility towards *Pto* DC3000, with enhanced bacterial colonization compared to wildtype Col-0 plants (Figure 14).

3. Discussion

3.1. Induction of different *Arabidopsis* defense hormone pathways upon early *G. orontii* infection

Plant hormones play essential roles in the regulation of plant immune responses and defense against biotic and abiotic stresses (Glazebrook, 2005). Incompatible plant-microbe interactions display the majority of biotic stresses and plants have evolved powerful mechanisms to counteract constant threats from the environment. However, some pathogens are able to circumvent the plant immune system and establish a compatible interaction with their host. Previous data has shown that the obligate biotrophic powdery mildew fungus *G. orontii* colonizes wildtype *Arabidopsis* Col-0 plants and elicits a strong transcriptional reprogramming already during the first 8 hours after infection (Pandey et al., 2010). Moreover, *G. orontii* induces a large number of transcription factor coding genes including two members of the *WRKY* gene family, *WRKY18* and *WRKY40* (Supplementary Figure 1).

Shen and colleagues demonstrated that *wrky18wrky40* plants were resistant towards infection with the otherwise virulent *G. orontii* fungus (Shen et al., 2007) but the underlying mechanisms leading to resistance remain unknown. Comparative microarray analyses revealed a large set of genes that show constitutively elevated transcript levels under non-challenged conditions in *wrky18wrky40* compared to wildtype plants, underlining a negative regulatory role of WRKY18 and WRKY40 in plant immunity (Pandey et al., 2010). Additionally, differential regulation of several hormone-associated genes during the early plant-pathogen interaction was also observed, leading to the assumption that plant hormones may play an essential role for the formation of the resistant phenotype observed in *wrky18wrky40* plants.

3.1.1. WRKY18 and WRKY40 negatively regulate *JAZ* gene expression

Induction of five members of the *JAZ* family of JA signaling repressors *(JAZ1, -5, -7, -8* and *-10*) in untreated *wrky18wrky40* mutant plants already suggested a direct or indirect regulation of *JAZ* genes by WRKY18 and/or WRKY40 (Figure 2). *JAZ* gene expression has recently been analyzed upon application of different stimuli. Several members of the *JAZ* gene family are induced upon mechanical wounding, herbivory attack, JA-treatment or bacterial infection (Chini et al., 2009; Chung et al., 2008; Demianski et al., 2012; Thines et al., 2007). However, temporal expression studies upon challenge with the obligate biotrophic powdery mildew fungus *G. orontii* have never been investigated. This thesis provides first insights into the differential regulation of all *JAZ* family members in the course of early *G. orontii* infection.

JAZ1, *-5*, *-7* and *-8* showed elevated transcript levels already in non-challenged *wrky18wrky40* compared to wildtype plants (Figure 2). Promoter analysis of these genes revealed the presence of W-box and W-box-like motifs, the cognate binding sites for WRKY transcription factors, pointing to a direct or indirect negative regulation of these genes by WRKY18 and/or WRKY40. Indeed, we could previously demonstrate protein binding of ectopically expressed WRKY40-HA protein to the *JAZ8* promoter (Pandey et al., 2010). Additionally, those *JAZ* genes that are induced upon *G. orontii* infection show higher transcript levels in the mutant compared to wildtype plants. This was significantly observed for *JAZ1*, *-5*, *-7*, *-8* and *-9* showing similar expression patterns in wildtype and *wrky18wrky40* plants, but much higher relative fold-changes in the mutant upon infection (Figure 2). Hence, the *wrky18wrky40* double mutant appears to lack efficient regulation of those *JAZ* genes, which is further supported by the presence of several W-box elements in the promoters of these genes, suggesting that numerous *JAZ* genes are directly targeted by WRKY transcription factors. Induced expression of JAZ genes in *wrky18wrky40* plants might be an immediate consequence due to lack of direct repression by WRKY18 and WRKY40 or due to a more indirect effect by misregulation of other transcription factors, including other WRKY proteins, that require WRKY18 and/or WRKY40 for proper spatial and temporal action. However, direct evidence for a role of pathogen-triggered W-box mediated expression of *JAZ* genes remains to be demonstrated.

In contrast, no differences in fold-induction or in their expression kinetics between susceptible wildtype and resistant *wrky18wrky40* plants were observed for *JAZ3*, *-4*, *-6*, *-11* and *-12* during the first 48 hours of infection, indicating no effect of loss-of *WRKY18* and *WRKY40* function for the regulation of these genes (Figure 2). Moreover, expression levels of *JAZ3*, *JAZ4*, *JAZ6*, *JAZ11* and *JAZ12* were considerably low, which is consistent with previous observations (Chung et al., 2008; Demianski et al., 2012). Interestingly, low transcript levels and unresponsiveness of *JAZ4* and *JAZ11* gene expression were similarly observed upon wounding, herbivory attack and bacterial and fungal infection (Chung et al., 2008; Demianski et al., 2012), probably constituting a general characteristic of these *JAZ* gene family members or these two genes may have other functions unrelated to abiotic or biotic stress. Nevertheless, differences in gene expression between different stimuli were found for other *JAZ* genes (Chung et al., 2008; Demianski et al., 2012). In the case of *JAZ6*, enhanced expression was not observed during early *G. orontii* infection, whereas it revealed a strong increase in transcript abundance upon *Pto* DC3000 infection and a rapid induction upon herbivory attack and wounding. In contrast, *JAZ7* was strongly induced during the *G. orontii*

infection process and after wounding, but only moderate expression was detected upon *Pto* or herbivory challenge. Significantly strong upregulation of gene expression was observed for *JAZ9*, being the most up-regulated *JAZ* gene upon *Pto* infection and herbivory attack, but also showing high transcript levels after wounding and *G. orontii* infection (Chung et al., 2008; Demianski et al., 2012). This illustrates that some *JAZ* genes are similarly responsive to various treatments, whereas others are specifically induced only upon certain stimuli. Whether these differences affect the resulting response to different challenges or constitute insignificant differences due to post-transcriptional- and –translational regulation or functional redundancy between *JAZ* genes remains to be investigated.

The current model of JA signaling suggests that *JAZ* genes are induced in response to JA or JA-inducing stimuli in order to replenish the pool of JAZ proteins degraded by the SCF^{COI1}-complex and thus to attenuate signaling (Chico et al., 2008; Demianski et al., 2012). Based on this model it is conceivable that the induction of specific *JAZ* genes upon certain stimuli reflect the degradation of the respective JAZ protein, implicating that this protein is involved in regulating JA responses to the specific stimulus. Moreover, extensive co-expression observed upon wounding, *G. orontii* or *Pto* infection and the fact that several *JAZ* genes in *Arabidopsis* are the result of genomic duplication events support the idea of partial functional redundancy within the *JAZ* family members (Demianski et al., 2012; Vanholme et al., 2007). Analysis of mutants lacking multiple *JAZ* genes upon different challenges would contribute to further dissect the roles of individual *JAZ* genes.

Recent identifications of JAZ-interacting proteins regulating various aspects of the JA pathway demonstrated that JAZ repressors indeed have overlapping, but finely separated functions in JA signaling and are also involved in crosstalk between JA and other hormone signaling pathways (Kazan and Manners, 2012; Niu et al., 2011). It has been shown in yeast 2-hybrid experiments that JAZ proteins form homo-and heterodimers through interaction of their characteristic ZIM-domain (Chini et al., 2009). Although the biological relevance of dimerization has not yet been demonstrated, expression kinetics revealed co-regulated *JAZ* genes during early powdery mildew infection. *JAZ1* and *JAZ5* show elevated transcript levels at 4 hpi in the *wrky18wrky40* double mutant and the same expression pattern can be observed in wildtype samples, although much weaker (Figure 2). Moreover, expression of *JAZ9* was also induced at 4 hpi together with *JAZ1* and Chini and colleagues demonstrated heterodimerization of these two JAZ proteins in yeast 2-hybrid assays (Chini et al., 2009). The same holds true for *JAZ3* and *JAZ9* with both being simultaneously up-regulated at 12 hpi, supporting the idea of JAZ proteins acting as heterodimers. Almost all members of the

JAZ gene family showed elevated levels at 0 hpi in *wrky18wrky40* plants, with significant increases observed for *JAZ1*, *JAZ5*, *JAZ7* and *JAZ8* (Figure 2). As it has recently been shown that plants ectopically expressing *JAZ10* are insensitive to MeJA treatment, a root growth assay was performed testing *wrky18wrky40* plants for MeJA insensitivity (Yan et al., 2009). However, no root growth phenotypes could be observed for the tested *WRKY* double mutants (Figure 2). Either expression levels of induced *JAZ* genes in *wrky18wrky40* mutants may not be sufficient to effectively suppress JA responses although several *JAZ* genes were up-regulated or the applied assay was not appropriate to detect a subtle suppression effect. One must note that in this study only changes in expression levels were studied. Thus, it cannot be excluded that these RNA transcript levels do not significantly alter JAZ protein levels. To date, no specific antibodies are available to monitor changes of individual JAZ protein amounts. This is a crucial issue that needs to be addressed in the future since nearly all current conclusions rely on extrapolations derived from expression profiling studies.

If up-regulation of *JAZ* genes can alter responses to JA but also influences other plant hormone signaling pathways (Kazan and Manners, 2012), overexpression and mutations of *JAZ* genes may also have an effect on the infection with powdery mildew. Indeed, a role for JAZ repressors in mediating JA-SA antagonistic crosstalk was proposed (Chen et al., 2009; Kazan and Manners, 2012). The essential SA biosynthesis gene *SID2* is repressed by the TFs ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE-LIKE1 (EIL1), which are in turn negatively regulated by JAZ proteins (Chen et al., 2009). Thus, JA-induced JAZ degradation might lead to a de-repression of *EIN3* and *EIL1* resulting in repression of SA biosynthesis (Kazan and Manners, 2012). Alternatively, pathogen-induced *JAZ* gene induction as observed in *wrky18wrky40* leads to an activation of SA biosynthesis and signaling. Unfortunately, loss-of-function mutants for most *JAZ* genes are currently not available. Two available transgenic *JAZ* mutants were analyzed upon infection with *G. orontii*. No significant differences in penetration rate or fungal growth were observed between wildtype, *jaz7* and *JAZ10ox*-lines. Either different JAZ proteins can compensate for loss-of and/or gain-of JAZ functions due to redundancy or disruption of the JA signaling pathway is insufficient to generate an observable phenotype.

3.1.2. Resistant *wrky18wrky40* mutants exhibit exaggerated responses to JA

To further investigate the role of JA during *G. orontii* infection and to test whether differences in *JAZ* expression result in altered JA-responses, JA levels and JA-responsive genes were analyzed with regard to differences between susceptible wildtype and resistant *wrky18wrky40* plants during early *G. orontii* infection.

JA accumulation was biphasic in wildtype plants peaking at 4 and 24 hpi, whereas *wrky18wrky40* did not show the second increase in JA at 24 hpi (Figure 3). Similar increases in wildtype and *wrky18wrky40* plants at 4 hpi suggest no direct influence of elevated transcript levels of *JAZ1*, *5* and *9* at 4 hpi on the accumulation of JA. However, induction of JA levels may occur temporally before JAZ proteins can negatively affect JA biosynthesis. By this means reduced JA levels in *wrky18wrky40* at 24 hpi might be caused by the strong upregulation of *JAZ* genes at 12 hpi resulting in decreased JA biosynthesis in the double mutant. Although only slight differences in the expression of the JA biosynthesis gene *LOX2* were observed (Figure 3), weak induction in wildtype plants together with stronger upregulation of *JAZ* genes in *wrky18wrky40* plants may result in the observed difference in JA accumulation 24 hours after *G. orontii* infection. Moreover, Chung and colleagues proposed MYC2 as direct regulator of *JAZ* genes and of its own gene but not of *LOX2* (Chung et al., 2008). This also implies a direct role of JAZ proteins in the regulation of *MYC2* and *JAZ* genes, but only an indirect role in the regulation of *LOX2*. Although JA responses are partly regulated by JAZ proteins, it is difficult to draw concrete conclusions from the expression of *JAZ* genes as negative regulators of JA responses and the expression of JA-responsive genes. Transcript abundance levels do not necessarily reflect the final amount of proteins being translated and biological active and functional redundancy may occur. The fact that *myc2* mutants are not fully JA-insensitive (Lorenzo et al., 2004) also supports the idea of further transcription factors being involved in the regulation of JA-induced defense genes, which may also include WRKY transcription factors. Recent work demonstrated that, besides MYC2, other TFs directly interact with JAZ proteins involving MYC2-related proteins MYC3 and MYC4 (Niu et al., 2011) but also members of the MYB TF family (Qi et al., 2011), which also showed significant de-regulation in *wrky18wrky40* plants upon *G. orontii* infection (Pandey et al., 2010).

In general, only a fraction of produced JA is converted into its bioactive derivative JA-Ile. Wildtype plants accumulated ~6-fold more JA-Ile than *wrky18wrky40* 24 hpi, although JA levels differed <2-fold between wildtype and *wrky18wrky40* plants at this respective time point. Thus, conversion of JA to JA-Ile is more efficient in wildtype plants compared to the double mutant or regulatory mechanisms of JA-conversion are influenced by WRKY18 and/or WRKY40. Although overall JA levels in wildtype and *wrky18wrky40* plants were relatively low compared to JA levels accumulating after wound responses or infection with necrotrophic pathogens (Zhang and Turner, 2008), the outcomes of JA accumulation especially with regard to interactions with the SA hormone pathway depend on the relative

concentration of each hormone (Mur et al., 2006). Thus, also subtle differences or changes in hormone levels may affect the outcome of an infection.

Despite rather similar JA levels, JA-responsive genes behaved significantly different in both genotypes upon *G. orontii* infection. The early JA-responsive gene *VSP2*, which is reported to be under direct regulation by MYC2 (Pieterse et al., 2009), was strongly induced 8 hpi in *wrky18wrky40* plants whereas no *JAZ* gene expression was significantly up-regulated at this time point, enabling MYC2 to positively influence *VSP2* expression (Figure 3). In contrast, *VSP2* expression in wildtype plants was found to be remarkably moderate. Moreover, only weak induction of the JA-responsive gene *PDF1.2*, which is under negative control of MYC2 (Pieterse et al., 2009), in wildtype plants (<10-fold) is in clear contrast to a very strong and abrupt transcript accumulation observed in the resistant double mutant 24 hpi (> 100-fold) (Figure 3). It is conceivable that *wrky18wrky40* mutants exhibit an exaggerated response to JA characterized by the significant induction of early and late JA-responsive genes, indicating that WRKY18 and WRKY40 participate in the repression and/or fine-tuning of JA-responses. This is in agreement with WRKY18 and WRKY40 generally being regarded as transcriptional repressors (Pandey et al., 2010; Shen et al., 2007). Additionally, microarray analysis of *wrky18* single mutants upon *G. orontii* infection further support this hypothesis, as several early JA-responsive transcriptional activators and repressors are strongly induced 6 h after *G. orontii* infection (Table 1).

Interestingly, *Arabidopsis* non-host powdery mildew *Bgh* triggers significantly stronger *PDF1.2* expression compared to plants infected with the adapted powdery mildew *G. cichoracearum* (Zimmerli et al., 2004). Moreover, induction of defensins was correlated with non-host resistance, as also the oomycete non-host potato late blight pathogen clearly enhanced *PDF1.2* expression in wildtype plants, whereas the virulent *Arabidopsis* downy mildew pathogen did not (Zimmerli et al., 2004). It was concluded that biotrophic host pathogens, like *G. orontii,* must either fail to elicit or suppress the JA signaling transduction pathway in *Arabidopsis* wildtype plants (Wen et al., 2011; Zimmerli et al., 2004). Assuming the latter, it is possible that repression of JA signal transduction triggered by *G. orontii* is compromised due to loss-of *WRKY18* and *WRKY40,* leading to a de-repression of JA signaling and a strong increase in *PDF1.2* expression in *wrky18wrky40* double mutants. Notably, ectopic activation of the JA signaling pathway protected *Arabidopsis* Col-0 plants against *G. cichoracearum* (Ellis and Turner, 2001; Zimmerli et al., 2004), suggesting that enhanced activation of JA signaling in *wrky18wrky40* mutants may contribute to the resistant phenotype towards *G. orontii*. This would imply a potential role of JA signaling during the

very first stages of fungal invasion, showing *VSP2* and *PDF1.2* induction during the establishment of a fungal penetration peg (~5 hpi), subsequent penetration of the epidermal cell layer (~5-12 hpi) and haustorium formation (24 hpi) (Figure 1; Figure 3). Thus, it can also be hypothesized that repression of JA signal transduction in wildtype plants is mediated by haustorial-secreted fungal effectors that directly or indirectly interfere with WRKY18 and/or WRKY40 function affecting the JA signaling pathway. At least it is conceivable that the early induction of JA-response marker genes may also be caused or enhanced by wounding responses due to the penetration of the epidermal cell layer, as wound responses can rapidly induce expression of these genes (Koo et al., 2009; Suza and Staswick, 2008). However, based on studies using the poorly adapted powdery mildew isolate *G. cichoracearum* UMSG1, Wen and colleagues proposed only minor contributions of the JA pathway to post-invasion resistance, as *Arabidopsis* JA pathway mutants were not impaired in post-invasive defenses (Wen et al., 2011). This fungal isolate overcomes pre-invasive defenses of wildtype *Arabidopsis* plants but its post-invasive proliferation is restricted by callosic encasement of the haustorial complex and the execution of HR, both appeared to be controlled by SA-dependent and –independent defense pathways with *sid2pad4* mutants becoming almost fully susceptible to *G. cichoracearum* isolate UMSG1. Thus it can be hypothesized that the loss-of *WRKY18*- and *WRKY40*-dependent de-repression of JA signaling accounts for the observed reduction of fungal entry rate in *wrky18wrky40* mutants compared to wildtype plants.

Obviously, the effect of JA accumulation and signal transduction on the outcome of infection with *G. orontii* has to be genetically investigated by the analysis of JA-deficient mutants and the generation of appropriate triple mutants impaired in *WRKY18* and *WRKY40* expression and JA biosynthesis or perception, respectively. Along this line, JA perception mutant *coi1* revealed a significant increase in susceptibility towards adapted powdery mildew *G. cichoracearum* compared to wildtype plants (Ellis et al., 2002; Ellis and Turner, 2001), underlining the necessity of those analyses. Whether the host pathogen *G. orontii* suppresses or fails to elicit JA signaling in wildtype plants in contrast to *wrky18wrky40* mutants remains elusive, as appropriate fungal mutants are so far missing.

In summary, strong differences in the expression of *JAZ* genes prior to and during *G. orontii* infection suggest a role of WRKY18 and/or WRKY40 in regulating members of the *JAZ* gene family. So far, binding of WRKY40 to the *JAZ8* promoter has been demonstrated (Pandey et al., 2010). The presence of numerous W-box motifs in promoter regions of other *JAZ* genes together with substantial differences in transcript levels point to a more general role of

WRKY18 and WRKY40 in the regulation of *JAZ* genes. Whether this regulation occurs in a direct or indirect way remains unclear. Chromatin-immunoprecipitation (ChIP) followed by qPCR analysis (ChIP-PCR) or massive parallel sequencing (ChIP-Seq) should contribute to answering this question. Moreover, exaggerated responses even to low JA levels in the *wrky18wrky40* mutant compared to wildtype plants together with a considerable number of JA-related genes up-regulated in *wrky18* single mutants provide further evidence of WRKY18 being involved in regulating JA signaling events. Alternatively, loss-of *WRKY18* and *WRKY40* activates pathogen-dependent JA signaling or overcomes *G. orontii*-mediated suppression of JA signal transduction, leading to enhanced downstream JA marker gene expression. In this respect, *wrky18wrky40* JA responses resemble those of non-host resistance against non-adapted powdery mildews and thus may be involved in mediating pre-invasion resistance towards *G. orontii*.

3.1.3. SA is indispensable for resistance of *wrky18wrky40* mutants

G. orontii infection induces SA-dependent defense responses in *Arabidopsis*, with mutants in SA biosynthesis and signaling exhibiting enhanced susceptibility to this fungus (Dewdney et al., 2000; Reuber et al., 1998). Moreover, exogenous treatment with the SA analog BTH was found to protect susceptible plants against fungal infection (Fabro et al., 2008; Maleck et al., 2002). This indicates that SA contributes to active defense against powdery mildews to limit the extent of infection. As JA and SA mostly act antagonistically (Leon-Reyes et al.; Liu et al., 2006; Vlot et al., 2008), one may have expected differences in SA levels and SA responses during *G. orontii* infection to account for the susceptible wildtype and resistant *wrky18wrky40* phenotypes. Unexpectedly, both susceptible wildtype and resistant *wrky18wrky40* plants revealed equal pathogen-induced accumulation of SA during the first 72 h after *G. orontii* infection (Figure 4). Also downstream marker genes of SA signaling were similarly induced 48 hpi in both genotypes (Figure 4), demonstrating undisturbed correlations between SA accumulation and marker gene induction independent of WRKY18 and WRKY40. Thus, the SA signaling pathway appears to be activated at later stages of powdery mildew infection in both genotypes, starting 48 hours after the inoculation. At this stage the fungus begins to establish secondary hyphae and starts to invade adjacent epidermal cells (Figure 1). Previous studies have shown that the SA signaling pathway of the host plays a more important role at later infection stages that is after fungal penetration (Dewdney et al., 2000; Reuber et al., 1998). This observation is consistent with a detailed study by Chandran et al. (2009) demonstrating the SA dependency of later stage infection processes but SA independency of early host responses. Also infection with the powdery mildew *G.*

cichoracearum leads to clearly elevated SA levels 48 hpi (Fabro et al., 2008), as it was observed for infection with *G. orontii*.

Apparently, induction of SA biosynthesis, SA accumulation and downstream signaling is insufficient to effectively restrict fungal proliferation in wildtype plants and was similarly observed in resistant *wrky18wrky40* mutant plants. If SA accumulation and responses did not differ between susceptible wildtype and resistant *wrky18wrky40* plants in the course of *G. orontii* infection, SA might be dispensable for the outcome of infection. To test this, the *wrky18wrky40sid2* triple mutant impaired in SA biosynthesis was infected with *G. orontii*. Somewhat surprisingly, *wrky18wrky40sid2* plants reconstituted pre- and post-invasive wildtype-like phenotypes (Figure 5), clearly demonstrating that resistance mediated by loss-of *WRKY18* and *WRKY40* depend on SA biosynthesis. Interestingly, triple mutants re-constituted an almost wildtype-like pre-invasive phenotype with slightly reduced penetration rates compared to wildtype plants. This is in contrast to infection studies with non-host powdery mildews demonstrating only marginal effects of SA biosynthesis on pre-invasive defense in *Arabidopsis* (Lipka et al., 2005; Zimmerli et al., 2004). However, penetration rates are determined 48 h after fungal infection, representing the stage of secondary hyphae formation (Figure 1). At this time point coincident induction of SA levels and *EDS1* expression was observed in *wrky18wrky40* mutants pointing to a role of the SA and/or EDS1 signaling pathway also for pre-invasive defense in a loss-of *WRKY18*- and *WRKY40*-dependent manner.

Besides increased penetration rates, triple mutants revealed wild-type like fungal proliferation 9 dpi, demonstrating a crucial role of SA for post-invasive resistance of *wrky18wrky40* mutants. This is in agreement with recent studies reporting that *SID2* and *PAD4* are effectively required for post-invasion resistance in *Arabidopsis* (Wen et al., 2011). Moreover, previous studies associated the SA signaling pathway with late responses to adapted powdery mildews and found important roles of SA biosynthesis and signaling for host defense (Chandran et al., 2009; Dewdney et al., 2000; Lipka et al., 2005; Reuber et al., 1998; Wen et al., 2011).

The resistant phenotype of *wrky18wrky40* double mutants was accompanied by cell-death restricted to infection sites, which were not observed in wildtype plants upon infection with *G. orontii* (Figure 5). The fact that blocking SA biosynthesis suppresses the cell-death phenotype of *wrky18wrky40* plants indicates that SA is essential for cell-death execution in *wrky18wrky40* double mutants and thus essential for resistance. This also implies a role for WRKY18 and WRKY40 in negatively regulating cell-death induction, as SA is similarly

induced in susceptible wildtype plants without showing comparable cell-death formation upon *G. orontii* infection.

Additionally, the SA-related EDS1 signaling pathway is not considerably induced in wildtype plants during *G. orontii* infection. In contrast, *wrky18wrky40* plants accumulate *EDS1*, *PAD4* and *NPR1* transcripts 48 hpi (Figure 4). The expression pattern of these genes is strikingly similar to the dynamics of SA accumulation, indicating that this signaling pathway is effectively activated in resistant *wrky18wrky40* plants, which is in agreement with negative regulation of *EDS1* by WRKY40 (Pandey et al., 2010). Moreover, acting upstream of SA, EDS1 as a key regulator of immune responses to various virulent biotrophic and hemi-biotrophic pathogens (Falk et al., 1999; Feys et al., 2001; Lipka et al., 2005) is required for resistance of *wrky18wrky40* plants. Recent analysis of *G. orontii* infected *wrky18wrky40eds1* triple mutants demonstrated that loss-of *EDS1* in *wrky18wrky40* double mutants restores a wildtype-like pre- and post-invasive phenotype (A. Töller, MPIPZ Köln, unpublished). This may also explain full impairment in penetration and post-invasion resistance of *wrky18wrky40sid2* mutants, as EDS1 and SA are also involved in a positive feedback loop important for defense (Wiermer et al., 2005).

On the other hand, the REDOX-RESPONSIVE-TRANSCRIPTION-FACTOR1 (RRTF1), which was integrated into a co-expression network upon redox-stress involving WRKY33 and EDS1 (Khandelwal et al., 2008) and shown to be directly regulated by WRKY40 (Pandey et al., 2010), is dispensable for resistance (Supplementary Figure 3). Thus, resistance of *wrky18wrky40* double mutants towards *G. orontii* is dependent on SA and *EDS1*, although the role of WRKY18 and WRKY40 in affecting pathogen-triggered cell-death remains to be elucidated.

3.1.4. WRKY18 and WRKY40 negatively regulate 4MI3G biosynthesis

Strong up-regulation of glucosinolate biosynthesis gene *CYP81F2* in microarray analysis suggested the activation of this defense-related pathway also in *wrky18wrky40* upon *G. orontii* infection (Pandey et al., 2010). Indeed, detailed qPCR analysis revealed a strong increase in *CYP81F2* transcript in the resistant double mutant during early *G. orontii* infection (Figure 6). This was reflected by enhanced levels of the respective product 4MI3G in these plants and a significant difference of ~25% compared to susceptible wildtype plants 72 hpi (Figure 6). The promoter region of *CYP81F2* harbors 19 W-box and W-box-like motifs partly clustering in the 4000 bp upstream region of the gene, pointing to a direct regulation of this gene by WRKY TFs. However, preliminary ChIP-PCR analyses using the generated *WRKY18*

complementation line could not detect direct association of WRKY18 to the *CYP81F2* promoter (data not shown).

Following the conventional biosynthesis route, 4MI3G requires activation by the atypical myrosinase enzyme PEN2 for antifungal activity (Bednarek et al., 2009). However, *PEN2* expression was not significantly induced and revealed no obvious difference between the two genotypes during *G. orontii* infection (Figure 6). This is in agreement with publicly available expression data demonstrating no remarkable *PEN2* transcript induction upon various treatments (Genevestigator, V4), indicating a post-translational regulation of PEN2 activity.

Interestingly, no 4MI3G increase was observed by Bednarek and colleagues (2009) in wildtype Col-0 plants 16 h after infection with the non-adapted powdery mildew *Bgh*. Similarly, Col-0 plants initially began to accumulate 4MI3G 12-24 h after infection with the adapted powdery mildew *G. orontii*, whereas loss-of *WRKY18 WRKY40* mutants revealed an increase of this compound already 8 hpi (Figure 6). This suggests a pathogen-induced de-repression of 4MI3G biosynthesis dependent on loss-of *WRKY18* and *WRKY40*. Although no direct WRKY18 association to the *CYP81F2* promoter was found, other regulatory proteins, e.g. WRKY40, may be involved in mediating WRKY18 binding to the promoter or other WRKY TFs regulate expression of this gene.

A direct role of 4MI3G in antifungal defense has not yet been demonstrated. 4MI3G itself may have a yet unknown signaling function required for resistance of *wrky18wrky40* mutants, as it was hypothesized to act as a signaling molecule for MAMP-induced callose formation (Clay et al., 2009). Alternatively, altered 4MI3G levels may result in additional activated compounds that may affect the outcome of the infection. In this context, PEN2 and CYP81F2 activities were demonstrated to be required for effective defense against non-adapted powdery mildews and other fungal pathogens such as *P. infestans* or *Magnaporthe oryzae* (Bednarek et al., 2009; Maeda et al., 2009; Sanchez-Vallet et al., 2010). Sanchez-Vallet and colleagues (2010) demonstrated a crucial role of *CYP81F2* and *PEN2* for non-host resistance against the adapted necrotrophic fungal isolate *Plectospharella cucumerina* (*P. cucumerina*) BMM, whereas resistance responses to non-adapted isolates were unaffected in *cyp81f2* and *pen2* mutants. On the other hand, mutations in genes *CYP79B1/2* acting upstream of *CYP81F2* and *PEN2* in the Trp-derived indole-glucosinolate biosynthesis pathway revealed increased susceptibility also towards non-adapted isolates (Sanchez-Vallet et al., 2010). This is in contrast to work by Bednarek et al (2009) showing increased susceptibility towards non-adapted powdery mildews for all of these mutants, strongly suggesting additional, *PEN2*-independent, products with antifungal activity (Sanchez-Vallet et al., 2010). Nevertheless,

both adapted and non-adapted *P.cucumerina* isolates similarly trigger 4MI3G accumulation (Sanchez-Vallet et al., 2010). Along the same line, an essential role for 4MI3G or 4MI3G-derived yet unknown compounds was demonstrated by the analysis of *wrky18wrky40cyp81f2* triple mutants reconstituting a susceptible, wildtype-like, phenotype after *G. orontii* infection (A. Töller, MPIPZ Köln, unpublished). Host cell entry rates of this triple mutant were restored to wildtype levels, suggesting a role of 4MI3G in pre-invasive defense against the powdery mildew fungus. This is further confirmed by successful fungal proliferation on triple mutant plants, underlining an important role of 4MI3G or 4MI3G-derived compounds also in post-invasive defense, implicating that observed accumulation of 4MI3G is effectively important for resistance of *wrky18wrky40* plants. In this context, analysis of *wrky18wrky40pen2* triple mutants would contribute to further dissect a direct or indirect role of 4MI3G in defense against *G. orontii*.

3.2. Dissecting WRKY18 and WRKY40 functions

It has been proposed that *WRKY18* and *WRKY40* act at least partially redundant (Pandey et al., 2010; Shen et al., 2007). This holds true for defense against powdery mildew *G. orontii*, as *wrky18* and *wrky40* single mutants are both susceptible towards this fungal pathogen, whereas *wrky18wrky40* double mutants are resistant (Shen et al., 2007). Thus, WRKY18 and WRKY40 regulate common processes that render the plant susceptible. Similarly, positive regulation of resistance against bacterial strain *Pto* DC3000 AvrRPS4 appears to be mediated in a redundant manner, because preliminary results showed no enhanced susceptibility of *wrky18* or *wrky40* single mutants (data not shown). On the other hand, overexpression of *WRKY18* leads to an increased resistance against virulent *Pto* DC3000 that was not observed for *WRKY40* overexpressing plants (Xu et al., 2006). In contrast, these plants were more susceptible against *B.cinerea* infection, whereas *WRKY18* overexpression did not alter the wildtype-like resistant phenotype (R. Birkenbihl, MPIPZ Köln, unpublished). This data supports functional redundancy in defense responses for some pathogen challenges and diverse functions for others.

3.2.1. WRKY18 and WRKY40 are functionally but not genetically redundant

To further elucidate commonalities and differences of transcriptional regulation by WRKY18 and WRKY40, global expression analyses upon *G. orontii* infection were performed. Interestingly, already in the unchallenged state, lack of WRKY18 or WRKY40 results in a tremendous transcriptional de-regulation with similar proportions of up- and down-regulated genes between both genotypes (Figure 7). Almost one-third of the significantly de-regulated

genes, with a two-fold difference in transcript abundance compared to wildtype plants at the same timepoint (p-value ≤ 0.05), were shared by the two TFs. This changed drastically during the course of infection. Most strikingly, *wrky18* mutants showed an up-regulation of a majority of genes 6 h after *G. orontii* infection, whereas *wrky40* plants revealed the opposite effect. Moreover, only a fractional amount of affected genes are commonly de-regulated in both genotypes (Figure 7). This indicates that upon pathogen attack WRKY18 and WRKY40 affect distinct subsets of genes, with WRKY18 acting rather as a repressor and WRKY40 as an activator of expression. Notably, this observation is based on infection with the biotrophic powdery mildew *G. orontii* and may be different upon infection with other pathogens. Additionally, it is difficult to conclude a general repressive or activating role for these TFs, as repression of genes might be an indirect effect of positively regulating expression of other negative transcriptional regulators.

Obviously, a large number of genes up-regulated 6 h after infection in *wrky18* mutants were related to the JA signaling pathway or otherwise related to biotic or abiotic stresses, whereas *wrky40* plants lack this over-representation (Figure 7). Furthermore, W-box motifs in promoter regions of up-regulated genes in *wrky18* were highly abundant, pointing to an involvement of WRKY TFs in the regulation of these genes (Table 1).

Recently, *WRKY18* was shown to be inducible by MeJA treatment and overexpression induces *VSP2* expression (Wang et al., 2008). The fact that *wrky18wrky40* plants also showed up-regulation of *VSP2* upon *G. orontii* infection may indicate that *VSP2* is not a direct target of WRKY18, as suggested by Wang and colleagues (Wang et al., 2008), but rather indirectly affects *VSP2* expression by influencing other regulators of JA signaling. In fact, *wrky18* mutants showed significant up-regulation of positive (e.g. *ORA47*, *ERF2*) and negative regulators (e.g. *ZAT10*, *JAZ7*) of the JA signaling pathway (Pauwels and Goossens, 2008) upon early *G. orontii* infection, pointing to a complex de-regulation of JA signaling whose outcome might also depend on the applied biotic or abiotic stress. Preliminary *in vivo* binding studies utilizing the *WRKY18* complementation line did not reveal direct binding of the *ZAT10*, *ERF2* or *JAZ5* promoter by WRKY18, which might still be due to unresolved technical problems. Alternatively, regulation of these genes is mediated indirectly by WRKY18, involves other WRKY TFs or absence of WRKY40 influences binding affinity, as demonstrated earlier (Xu et al., 2006).

However, WRKY18 has been suggested to positively regulate JA-mediated signaling upon wounding (Wang et al., 2008) but also has a positive role in SA signaling in a NPR1-dependent manner (Wang et al., 2006). Moreover, recent data point to a role in sensing and

signal transduction of bacterial volatile compounds, as *wrky18* mutants were less susceptible to growth-inhibiting volatiles of pathogenic rhizobacteria (Wenke et al., 2012). Thus WRKY18 appears to be an important mediator of various signaling pathways, in some cases acting functional redundant with WRKY40 but also regulating a significantly different set of genes. Based on the findings that WRKY18 and WRKY40 can form heterodimers and reveal differences in binding activity dependent on the presence of each other (Xu et al., 2006) it can be hypothesized that the transcriptional de-regulation found in *wrky18* and *wrky40* mutants is different to that found in *wrky18wrky40* mutants upon *G. orontii* infection. This may imply that WRKY18 and WRKY40 individually regulate diverse sets of genes also varying in an activating or repressing role and heterodimers may even regulate a completely different set of genes. This may explain why overlaps between the *wrky18* and *wrky40* data sets are strikingly different (Figure 7) and phenotypic outcomes of infection studies are so diverse (Pandey et al., 2010; Wenke et al., 2012; Xu et al., 2006). Obviously, one can not rule out a role for WRKY60 in these regulatory processes, as it was shown that WRKY60 is also involved in heterodimerization with WRKY18 and WRKY40 (Xu et al., 2006). However, WRKY60 appears to have only a minor role in transcriptional regulation since mutants and overexpressing lines did not show significant phenotypes upon the tested pathogen infections (Xu et al., 2006).

Notably, also *wrky18wrky40* double mutants were included in the global expression analysis upon *G. orontii* infection (data not shown). Unexpectedly, comparison of the new data set to already published data (Pandey et al., 2010) revealed only weak overlaps of de-regulated genes, although the same mutant line and the same fungal pathogen were employed. Obviously, different time points, growing conditions and the fitness of the fungus may influence plant defense responses, but also the fact that a different microarray technology (AGRONOMICS tilling array (Rehrauer et al., 2010)) was used may account for the observed differences of the two experiments. Almost no genes that were reproducibly induced in detailed infection kinetics by qPCR analysis of *wrky18wrky40* mutants were also induced in the newly obtained *wrky18wrky40* data set. This discrepancy obviously requires further detailed clarification, and thus will not be further discussed here.

To summarize, WRKY18 and WRKY40 regulate an apparent diverse set of genes, especially in the course of *G. orontii* infection. Potential direct target genes of WRKY18 will be investigated in further analyses and should help to place WRKY18 into specific (defense) signaling pathways.

3.2.2. WRKY18 is involved in feedback regulation of its own gene and *WRKY40*

Positive and negative feedback regulation is a common phenomenon in the wired web of WRKY TFs (Eulgem and Somssich, 2007). Auto-regulation or cross-regulation by WRKY TFs has been demonstrated in various previous reports (Hu et al., 2012; Rushton et al., 1996; Turck et al., 2004). Well-studied is the function of MAMP-induced parsley *PcWRKY1* which was shown to be regulated by a conserved arrangement of three synergistically acting W-boxes in its native promoter (Turck et al., 2004). Further analyses could demonstrate that *in vivo* binding of *Pc*WRKY1 to its own promoter results in a down-regulation of *PcWRKY1* expression, thus indicating a negative feedback regulation by *Pc*WRKY1 (Eulgem and Somssich, 2007; Turck et al., 2004). Concomitantly, *Pc*WRKY1 induction leads to the activation of *PcPR1* gene expression, showing that this factor simultaneously acts as a repressor on its own gene but as an activator of another gene. Similarly, auto- or cross-regulation was suggested for *Arabidopsis* WRKY18 and binding of recombinant, overexpressed WRKY18 to three contiguous W-box motifs of its native promoter was demonstrated *in vitro* (Chen and Chen, 2002). The same motifs were now shown to be occupied by WRKY18 upon *G. orontii* infection *in vivo* (Figure 10), thus confirming that WRKY18 indeed binds to its own promoter. Moreover, *WRKY18* transcript abundance and binding are clearly induced at early time points in response to pathogen challenge, and expression declined at later stages (Figure 9; Figure 10), suggesting that WRKY18 might be negatively regulating the expression of its own gene to avoid exaggerated defense responses. This is in agreement with previous studies demonstrating that overexpression of *WRKY18* leads to retarded plant growth (Chen and Chen, 2002), possibly due to a detrimental de-regulation of defense responses. This indicates that tight regulation of pathogen-induced *WRKY18* is essential for a balanced transcriptional reprogramming. Additionally, WRKY18 protein was found to bind to W-box rich regions of the *WRKY40* promoter in a pathogen-dependent manner (Figure 10). In the uninduced state, WRKY18 was not found associated with the *WRKY40* promoter, indicating that upon *G. orontii* infection, WRKY18 protein is recruited to the promoter for transcriptional regulation. Whether binding results in an activation or repression of *WRKY40* expression remains to be determined. However, expression of *WRKY40* in the *wrky18* single mutant is more strongly induced compared to wildtype plants (data not shown), pointing to a repressive function of WRKY18 on *WRKY40* expression, which may again protect the plant against transcriptional imbalances. A pathogen-induced repressive function of WRKY18 can easily be squared with data obtained from global expression analysis of *wrky18* single mutants revealing a majority of up-regulated

genes 6 h after *G. orontii* infection (Table 1). Nevertheless, it is conceivable that other WRKY TFs are involved in fine-tuning *WRKY18* and *WRKY40* expression. For *PcWRKY1*, promoter regions were constitutively occupied with other WRKY TFs and binding of *Pc*WRKY1 to its own promoter occurred at later stages after elicitation (Turck et al., 2004). Whether this is the case for WRKY18 and WRKY40 might be clarified by ChIP experiments using an anti-all-WRKY antibody that recognizes the majority of the WRKY protein family. Notably, binding studies were performed in the *wrky18wrky40* double mutant background to ensure functional complementation of the recombinant WRKY18 protein but also implying the possibility that absence of WRKY40 influences binding behavior of WRKY18. As both WRKY18 and WRKY40 can form homo- and heterodimer (Xu et al., 2006), their function may depend on or be influenced by these associations. Homodimers and heterodimers might be involved in regulating different sets of genes, or act as activators in one case and repressors in the other case. This hypothesis is supported by *in vitro* analyses of Xu and colleagues (Xu et al., 2006), demonstrating that efficient WRKY18 and WRKY40 binding activity to an artificial W-box containing DNA probe indeed depended on the presence of each other. Also the binding affinity of WRKY18 was significantly weaker compared to WRKY40, which might be due to differences in W-box adjacent nucleotides (Ciolkowski et al., 2008) or underline a crucial role of WRKY40 for binding activity.

Therefore, simultaneous complementation of *wrky18wrky40* double mutants with recombinant WRKY18 and WRKY40 protein might give new insights into the role of dimerization of these WRKY TFs and their individual roles in plant defense.

3.2.3. A working model for WRKY18 WRKY40-mediated susceptibility

Mutations affecting *WRKY18* and *WRKY40* render *Arabidopsis* plants resistant towards the otherwise compatible biotrophic powdery mildew *G. orontii*, demonstrating a negative regulation of resistance by these two WRKY TFs (Pandey et al., 2010; Shen et al., 2007).

In wildtype plants, *WRKY18 WRKY40* transcript and protein levels are rapidly induced within the first 4-6 h after *G. orontii* infection (Supplementary Figure 1). It is noteworthy that the powdery mildew fungus *G. orontii* only penetrates and infects epidermal cells and the expression analyses reported in this study were performed using total leaf extracts. The observed alteration in transcript levels may therefore only represent a diluted expression intensity of both infected and uninfected host cells. So far, little is known about signaling initiating from the region of fungal penetration in epidermal cells to sub-epidermal mesophyll layers within the entire leaf. However, as this induction occurs prior to fungal penetration, the elicitation signal is likely derived from a MAMP stimulus or a so far unknown trigger.

Analysis of *wrky18wrky40cerk1* triple mutants may contribute to the elucidation of the elicitation signal, although chitin is not solely responsible for CERK1 activation (Gimenez-Ibanez et al., 2009; Petutschnig et al., 2010). Additionally, tissue-specific promoters restricting WRKY18 or WRKY40 expression either to the epidermal or the mesophyll cell-

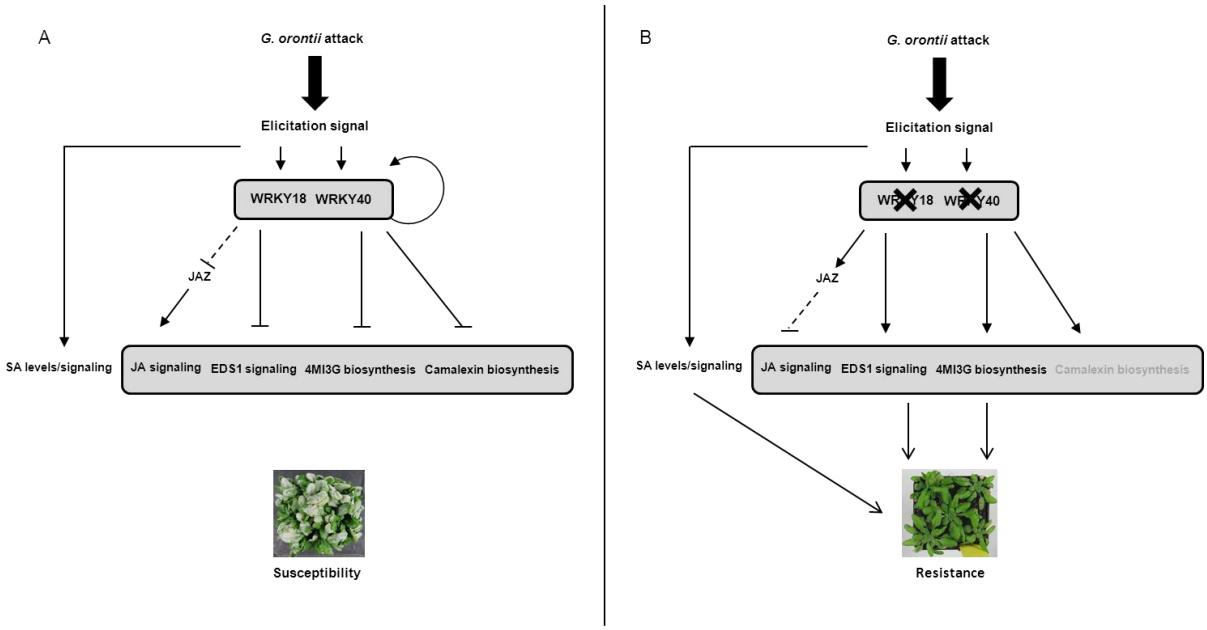


Figure 15: A hypothetical working model of WRKY18 WRKY40-mediated responses to *G. orontii* infection. **(A)** In susceptible wildtype plants, *G. orontii* attack triggers an elicitation signal leading to the induction of *WRKY18* and *WRKY40* expression, accumulation of SA and the activation of the SA signaling pathway. WRKY18 and /or WRKY40 positively regulate JA signaling by repressing negative regulators of JA-signaling. Binding of the EDS1 promoter down-regulates EDS1 signaling. Presence of *WRKY18* and *WRKY40* negatively influences 4MI3G and camalexin biosynthesis**,** culminating in successful fungal proliferation and a susceptible phenotype. **(B)** In plants simultaneously mutated in *WRKY18* and *WRKY40*, *G. orontii* attack triggers an elicitation signal leading to a *WRKY18 WRKY40* independent accumulation of SA and the activation of the SA signaling pathway. Knockdown of *WRKY18* and *WRKY40* results in an activation of *JAZ* gene expression and a misregulation of the JA signaling pathway. Activation of 4MI3G biosynthesis and accumulation of the respective product contributes to the resistant phenotype of *wrky18wrky40* plants, whereas induced camalexin accumulation appears dispensable for *wrky18wrky40* resistance.

layer would contribute to decipher their cell autonomous or non-autonomous requirement for resistance. However, transcriptional induction of *WRKY18* and *WRKY40* implies a direct or indirect role of WRKY18 and/or WRKY40 in such processes in this specific plant-pathogen interaction.

In both susceptible wildtype and resistant *wrky18wrky40* plants *G. orontii* infection is accompanied by the accumulation of SA and the activation of the respective signaling pathway (Figure 4). Although induced in both genotypes, SA plays a crucial role for resistance in the double mutant, as wildtype-like susceptibility is reconstituted in the

wrky18wrky40sid2 triple mutant impaired in SA biosynthesis (Figure 5). In the course of infection, binding of WRKY18 protein to promoter regions of WRKY18 and WRKY40 suggests a pathogen-dependent activation of a feedback-loop to regulate the expression of *WRKY18* and *WRKY40*. Presumably, negative-feedback regulation should keep expression of these transcriptional regulators in a balanced level. Moreover, our recent findings demonstrating direct binding of WRKY40 to the promoter of *JAZ8* suggests a positive regulation of the JA signaling pathway by WRKY40 and/or WRKY18 through repression of negative regulators of JA signaling (Pandey et al., 2010). That WRKY18 is also involved in a misregulation of the JA signaling pathway is further supported by microarray analyses of *wrky18* single mutants upon *G. orontii* infections, revealing upregulation of several JA-related transcriptional repressors but also activators 6 h after infection (Figure 7). As a key component of basal resistance, EDS1 is crucial for resistance towards many pathogens, including fungi (Wiermer et al., 2005). It has been proposed that direct binding demonstrated for WRKY40 (Pandey et al., 2010) and WRKY18 (R. Birkenbihl, MPIPZ Köln, personal communication) to the *EDS1* promoter mediates a repression of EDS1 signaling, thereby hampering a key node of defense against fungal pathogens (Pandey et al., 2010). Requirement of EDS1 signaling for successful defense against *G. orontii* in *wrky18wrky40* plants was shown by the analysis of *wrky18wrky40eds1* triple mutants, being as susceptible as wildtype plants (A. Töller, MPIPZ Köln, unpublished). Furthermore, up-regulation of indole-glucosinolate biosynthesis gene *CYP81F2* was found in *wrky18wrky40* double mutants giving rise to a stronger accumulation of 4MI3G in resistant *wrky18wrky40* compared to wildtype plants (Figure 6). We recently also revealed an induction of the camalexin biosynthesis gene *PAD3* in resistant *wrky18wrky40* mutants, resulting in a significant accumulation of this main phytoalexin (Pandey et al., 2010). Unaltered resistance of *wrky18wrky40pad3* triple mutants demonstrated dispensability of camalexin accumulation for post-invasive resistance of *wrky18wrky40* plants towards *G. orontii* infection, but revealed an important role in pre-invasive resistance, as *wrky18wrky40pad3* plants reconstituted wildtype-like penetration rates (A. Töller, MPIPZ Köln, unpublished).

To summarize, susceptibility of wildtype plants is partly the consequence of *G. orontii*-mediated induction of the transcriptional regulators WRKY18 and WRKY40, leading to a repression of negative regulators of JA signaling and the EDS1 signaling pathway. Additionally, accumulation of the indole-glucosinolate 4MI3G is repressed, culminating in a susceptible phenotype of wildtype plants. In the absence of *WRKY18* and *WRKY40*, *JAZ* genes are induced resulting in a misregulation of JA signaling and strong induction of *PDF1.2*

expression. De-repression of EDS1 signaling and 4MI3G biosynthesis leads to a robust resistance towards *G. orontii* infection. Although pathogen induced SA accumulation in both genotypes is independent of *WRKY18* and *WRKY40*, there is a clear requirement for SA in ultimately establishing the resistance observed in *wrky18wrky40* plants.

However, if these alterations in key defense signaling pathways in *wrky18wrky40* plants have a general influence on resistance towards powdery mildew fungi, one would expect to observe broad-spectrum resistance towards other adapted powdery mildews. Notwithstanding, neither (increased) resistance towards *G. cichoracearum* nor *G. cruciferarum* was observed (Supplementary Figure 4). Based on these findings, it is conceivable that specific *G. orontii* effectors manipulate the host cell possibly by directly or indirectly targeting WRKY18 and/or WRKY40. In this case, WRKY18 and WRKY40 may be regarded as host susceptibility factors.

3.3. WRKY18 and WRKY40 in other plant-pathogen interactions

Differences in the expression of various defense-related signaling pathway genes between wildtype and *wrky18wrky40* mutants indicate an activation of defense mechanisms that are effective against *G. orontii*. The fact that loss-of *WRKY18* and *WRKY40* renders *Arabidopsis* plants resistant against *G. orontii* infection raises the question of whether plant resistance or susceptibility towards other biotrophic, but also hemi-biotrophic and necrotrophic pathogens is also affected in *wrky18wrky40* mutants.

3.3.1. WRKY18 and WRKY40 are positive regulators of RPS4-mediated resistance

Infections of *wrky18wrky40* mutants with different virulent and avirulent bacterial strains of *Pto* DC3000 revealed a highly specific increase in susceptibility of *wrky18wrky40* plants towards the otherwise avirulent strain *Pto* AvrRPS4. Resistance towards this strain is mediated via the TIR-NB-LRR R-protein RPS4 that forms a complex with EDS1 and thus mediates recognition of the bacterial effector AvrRPS4 (Heidrich et al., 2011). The RPS4-dependent specificity of the phenotype was clearly demonstrated by the finding that not only infection with *Pto* AvrRPM1, which is mediated by a CC-NB-LRR R-protein und thus EDS1-independent, but also by infection with pathogens, where resistance is known to be EDS1-dependent, did not show any differences between wildtype and *wrky18wrky40* plants (Figure 11). Most strikingly, no increase in susceptibility of *wrky18wrky40* plants was observed upon infection with the avirulent bacterial strain *Pto* HopA1, which is thought to require similar components for resistance as *Pto* AvrRPS4 infection (Bhattacharjee et al., 2011).

Data presented by Heidrich and colleagues illustrate that the R-protein RPS4 is involved in direct physical interactions with other proteins (Heidrich et al., 2011). Moreover, the WRKY18 and WRKY40 barley homologs *Hv*WRKY1 and *Hv*WRKY2 have been shown to directly interact with the isolate-specific R-protein MLA and this interaction is crucial for resistance towards *Bgh* (Shen et al., 2007). Additionally, RPS4 and the WRKY domain containing TIR-NB-LRR receptor RRS1 act cooperatively in AvrRPS4-triggered immunity (Narusaka et al., 2009). The presence of a WRKY domain and a NLS motif in RRS1 suggests that this R-protein is involved in perception of pathogens and might also play a direct or indirect role in transcriptional regulation of defense responses. This fusion protein of a TIR-NB-LRR structure with a WRKY domain is indicative for a physical interaction of the proteins linked by such a domain-fusion (Lahaye, 2002). Based on such findings it is conceivable that WRKY18 and/or WRKY40 may actually be additional components of the EDS1/PAD4, RPS4, RRS1 regulatory complex required for efficient defense against this bacterial strain.

However, various experiments employing full length versions of the proteins or truncated versions, which in the case of barley *Hv*WRKY1 and *Hv*WRKY2 with the CC-domain of MLA were sufficient to promote interaction (Shen et al., 2007), failed to detect physical interactions with WRKY18 and WRKY40 (Supplementary Figure 5; Supplementary Figure 6). Since such interactions may require a post-recognition event dependent on the presence of the respective effector, possibly involving conformational changes and thus activation of the R-protein, as postulated for other NB-LRR proteins (Rairdan and Moffett, 2006; Shen et al., 2007), FRAP analyses were also performed in tobacco leaves co-infiltrated with one of the WRKY TFs, RPS4 and/or RRS1 and the bacterial effector AvrRPS4. Although nuclear co-localization was found for WRKY18, WRKY40, RPS4 and RRS1 as expected (Supplementary Figure 6), no direct physical interaction could be detected. Thus WRKY18 and/or WRKY40 do not appear to directly interact with RPS4 or RRS1 irrespective of effector presence, or physical interaction requires additional, yet still unknown, components. It is still conceivable that EDS1 forms a sort of platform for protein-protein interactions/associations involving WRKY18 and/or WRKY40 and thus it would be interesting to test WRKY18 and WRKY40 interaction also with EDS1.

Recently, Heidrich and colleagues could separate a requirement of nuclear EDS1 and RPS4 accumulation for bacterial growth restriction from the nucleo-cytoplasmic coordination that is essential for programmed cell death and transcriptional resistance reinforcement upon *Pto* AvrRPS4 infection (Heidrich et al., 2011). On the basis of this knowledge, experiments

employing the avirulent bacterial strain *Pseudomonas fluorescens* (*Pfo*) transgenically expressing *AvrRPS4* were initiated but preliminary results revealed no differences in cell-death formation between *wrky18wrky40* mutants and wildtype Col-0 plants upon infection (A. A. Töller, MPIPZ Köln, personal communication). This indicates that WRKY18 and WRKY40, most likely, affect nuclear processes involved in resistance. Whether these processes involve protein interactions therefore remains unclear. Certainly, comparative transcriptional analyses of *wrky18wrky40* mutants upon infection with *Pto* AvrRPS4 or by the avirulent strain *Pto* HopA1 should further help to unravel differences in transcriptional reprogramming leading to the observed different phenotypes.

3.3.2. *wrky18wrky40* mutants do not exhibit a broad-spectrum resistance towards powdery mildews

As described in the previous section, infection of *wrky18wrky40* mutants with *G. orontii* results in a complex de-regulation of defense related signaling pathways, culminating in resistance towards this obligate biotrophic fungal pathogen. Besides *G. orontii*, two additional powdery mildew pathogens from the same clade are able to successfully penetrate and colonize *Arabidopsis* Col-0 plants (Eichmann and Huckelhoven, 2008).

Thus the question arose, if loss-of *WRKY18* and *WRKY40* results in a general broad-spectrum resistance towards all three adapted powdery mildews, as it was reported for *mlo2mlo6* mutant plants (Consonni et al., 2006; Nishimura et al., 2003). This however was not the case since infections with *G. cichoracearum* and *G. cruciferarum* did not reveal any significant reductions in fungal colonization of *wrky18wrky40* compared to wildtype Col-0 plants (Supplementary Figure 4). This clearly separates resistance mediated by loss-of *WRKY18* and *WRKY40* from other reported powdery mildew resistances. Whereas resistance towards a wide range of powdery mildews is conferred ecotype specifically by the *RPW8* locus, *Arabidopsis* Col-0 plants lack this monogenetic trait (Xiao et al., 2005; Xiao et al., 2001; Xiao et al., 2004). Additionally, it was proposed that progressive differentiation of fungal infection structures may require diverse host target molecules (Panstruga, 2003). These compatibility factors, if absent in the plant, lead to a durable resistance against all pathogen species, without exhibiting constitutive defense activation. In this way, simultaneous mutations in *Arabidopsis MLO2* and *MLO6* confer broad-spectrum resistance towards *G. cichoracearum* and *G. orontii* characterized by early termination of fungal infection due to failed host cell invasion that is linked to host cell wall remodeling (Consonni et al., 2006). Penetration rates of *mlo2mlo6* mutants are reduced to ~30% compared to ~80% in wildtype and ~60% in *wrky18wrky40* mutant plants and resistance is obviously not accompanied by extensive host cell-death, as

observed for *wrky18wrky40* plants. Moreover, resistant *mlo* mutants additionally impaired in SA biosynthesis do not reconstitute a susceptible phenotype comparable to that of *wrky18wrky40sid2* triple mutants (Consonni et al., 2006). Thus the role of SA for loss-of *MLO* resistance is considerably low, whereas it is indispensable for *wrky18wrky40* resistance. Along the same line, mutations in POWDERY MILDEW RESISTANT 4 (*PMR4*), encoding a callose synthase, leads to a SA-dependent broad-spectrum resistance associated with cell-death execution at infection sites (Nishimura et al., 2003) and thereby resembles *wrky18wrky40* resistance. However, resistance of *pmr4* mutants is hypothesized to be based on enhanced activation of the SA signaling pathway and successive downregulation of JA signaling compared to wildtype plants, which is not observed for *wrky18wrky40* resistance. Thus, noticeable differences exist between *mlo2mlo6* and *pmr4* compared to *wrky18wrky40* resistance. Nevertheless, it will be important in future studies to define whether these mutations affect some common nodes within the genetic network or not. The generation and analysis of *wrky18wrky40mlo* triple mutants may provide new insights into potentially important and distinct layers of defense that may lead to additive effects on powdery mildew resistance. Last but not least, it is quite evident that a major difference between these resistant mutants is the involvement of MLO and PMR4 in the composition of cell-wall components, whereas WRKY18 and WRKY40 are TFs directly involved in transcriptional reprogramming of defense responses. Whether WRKY18 and WRKY40 are involved in (negatively) mediating morphological alterations through transcriptional regulation or direct physical interactions with other proteins leading to enhanced resistance remains to be experimentally addressed.

Another mutant conferring resistance towards the host adapted powdery mildew *G. cichoracearum* is *enhanced disease resistance 1* (*edr1*) (Frye and Innes, 1998). Coding for a protein kinase with homology to mitogen activated protein kinase kinase kinases (MPAKKK), *EDR1* negatively regulates cell-death in a SA-dependent manner upon pathogen infection (Christiansen et al., 2011; Frye et al., 2001). Upon infection with *G. cichoracearum*, *edr1* plants reveal no differences in spore germination compared to wildtype Col-0 plants but exhibit enhanced cell-death formation and increased post-invasive resistance (Frye and Innes, 1998), thereby macroscopically resembling the *wrky18wrky40* phenotype. Infection is accompanied by the induction defense-related genes, including the SA related genes *PAD4* and the downstream marker gene *PR1* (Christiansen et al., 2011; Frye and Innes, 1998). Interestingly, global expression analysis of *edr1* mutants upon powdery mildew infection revealed a significant number of *WRKY* genes being up-regulated during *G. orontii* infection

and many of the 5' regulatory regions of up-regulated genes in the *edr1* mutant were enriched in W-box motifs (Christiansen et al., 2011), strongly suggesting that WRKY TFs also play important roles in powdery mildew resistance mediated by loss-of *EDR1*. Notably, WRKY18 and WRKY40 were not among the up-regulated *WRKY* genes and it can be hypothesized that either these two TFs do not play a role in *edr1*-mediated resistance or that WRKY18 and WRKY40 act downstream of *EDR1* or in a different (signaling) pathway. Interestingly, *edr1* mutants do not show *PDF1.2* induction upon powdery mildew infection and do not reveal an increased pre-invasive defense (Christiansen et al., 2011; Frye and Innes, 1998). This is in contrast to *wrky18wrky40* mutants and underlines the hypothesis that early JA signaling leading *to PDF1.2* induction may contribute to pre-invasive defenses in *wrky18wrky40* plants. Based on the finding that fractions of EDR1 protein were detected in the nucleus, Christiansen and colleagues suggested a potential interaction of EDR1 with TFs within the nucleus (Lipka et al., 2005), which may also involve WRKYs. Thus, analysis of *wrky18wrky40edr1* mutants may further contribute to the dissection of the underlying resistance mechanisms.

Together, *wrky18wrky40* resistance towards *G. orontii* appears rather specific for this plant-pathogen interaction. Interestingly, *WRKY18* and *WRKY40* barley homologs *HvWRKY1* and *HvWRKY2* are involved in isolate specific R-gene mediated resistance towards barley powdery mildew *Bgh* (Shen et al., 2007). However, co-evolution of *Arabidopsis* and *G. orontii* is regarded as too short for the establishment of a gene-for-gene resistance system. Thus it is conceivable that secreted fungal effectors manipulate *Arabidopsis* host defenses to the benefit of the fungus and that this involves *WRKY18* and *WRKY40*. A multitude of potentially secreted effector candidates (OEC) were recently identified in the genome of *G. orontii* (Spanu et al., 2010) that may interfere with *WRKY18* and *WRKY40* function to alter plant's immune response. Several of them, pre-selected by their virulence function, were tested for direct physical interaction with WRKY18 and WRKY40. Up to now however the tested OECs did not reveal any interaction (Supplementary Figure 5).

3.3.3. WRKY18 and WRKY40 interact with pathogen-induced proteins in yeast and co-localize *in planta*

Homo- and hetero-dimerization of WRKY18 and WRKY40 in the plant cell nucleus was demonstrated several years ago (Xu et al., 2006). Since then, no reports about WRKY18- or WRKY40-protein interactions were published. However, a recent report suggests a role of WRKY18 and WRKY40 outside of the plant cell nucleus (Shang et al., 2010). Upon ABA induction, nuclear WRKY40 is recruited to the cytosol and thought to interact with the

potential ABA receptor, ABAR, at the chloroplast membrane. Cytoplasmic relocation of WRKY40 leads to a de-repression of the ABA-responsive transcription factor ABI5, resulting in the induction of ABA responsive genes. Although the proposed model is highly controversial (Antoni et al., 2011; Tsuzuki et al., 2011), a cytosolic interaction of ABAR and WRKY40 cannot be completely excluded since several independent molecular biology techniques were originally employed and yielded consistent results (Shang et al., 2010).

The yeast 2-hybrid analyses to detect potential WRKY18 and WRKY40 interactors performed in this study resulted in a very limited number of candidates.

Based on yeast studies it could be verified that WRKY18 and WRKY40 interact with truncated versions of FIB4 and a yet uncharacterized Oxidoreductase (AT2G38240) (Figure 13). However, interactions were neither detected with full-length proteins in yeast nor *in planta,* which might be due to conformational alterations of the fusion-proteins. Though, co-localization to the plant cell nucleus was demonstrated for all tested full-length proteins (Figure 13) and co-expression analysis revealed that the tested genes are simultaneously induced upon pathogen challenge, wounding or abiotic stress, although *FIB4* shows only moderate responsiveness to those challenges (Genevestigator, V4). Nevertheless, this indicates that the temporal and spatial occurrence of the tested proteins would potentially enable physical interaction in the nucleus.

Notably, FIB4 and the Oxidoreductase (AT2G38240) lack any known NLS motifs but truncated versions also partly localized to the cytosol, indicating a less efficient nuclear import of the truncated proteins. Oxidoreductases are common in plants with ~100 members identified in the *Arabidopsis* genome and are involved in the biosynthesis of many metabolites including flavonoids, gibberellins, and ethylene (Wilmouth et al., 2002).

Infection of *oxidoreductase* mutant plants with the biotrophic powdery mildew *G. orontii* and the hemi-biotrophic bacteria *Pto* DC3000 did not reveal any increased susceptible or resistant phenotype, thus it is currently difficult to hypothesize a role of this Oxidoreductase in plant defense. However, publicly available data demonstrates that this Oxidoreductase is induced upon biotic and abiotic stresses (Genevestigator, V4). Moreover, wound-induced expression of Oxidoreductase in wildtype Col-0 plants was shown to be JA-dependent and MeJA treatment strongly induced Oxidoreductase expression, but an overexpression line did not reveal a JA-insensitive phenotype (Yan et al., 2007). The *DOWNY MILDEW RESISTANT 6* (*DMR6*) gene encodes for a putative 2OG-Fe(II) oxygenase of unknown function, but was shown to be required for susceptibility to downy mildew infection (van Damme et al., 2008). Interestingly, no phenotype of *dmr6* plants was found upon infection with *G. orontii* and *Pto*

DC3000, in this sense resembling observations made with *Oxidoreductase* mutant plants. This and the fact that Oxidoreductase is highly responsive to JA support the necessity for further analyses of *oxidoreductase* mutants. Even more, interaction of Oxidoreductase with WRKY18 and WRKY40 by co-immunoprecipitation (Co-IP) followed by mass- spectrometry (MS) analysis using the established WRKY18-HA complementation line may now become feasible due to the increasing sensitivity of the current methodology.

Obviously, additional proteins responsible for bridging the interaction may be involved or the protein requires modifications or a specific trigger. This is conceivable for FIB4, which was already suggested to be involved in plant disease resistance responses (Jones et al., 2006; Sanabria and Dubery, 2006). It was demonstrated that MAMP treatment stimulates its phosphorylation (Jones et al., 2006; Singh et al., 2010) and MAMP application induces the expression of its ortholog in tobacco, which was further shown to interact with the virulence protein HrpN of the potato fire blight pathogen *Erwinia amylovora* (Sanabria and Dubery, 2006; Singh et al., 2010). Pathogen infections of *fib4* revealed no differences between mutant and wildtype Col-0 plants upon biotrophic powdery mildew infection but confirmed the increased susceptibility towards hemi-biotrophic bacterial strain *Pto* DC3000 (Figure 14) (Singh et al., 2010). FIB4 was previously found to associate with the PSII light-harvesting complex and locate to plastoglobules, lipoprotein bodies attached to the thylakoids that store lipids, including antioxidants, carotenes and plastoquinones (Singh et al., 2010). Singh and colleagues demonstrated alterations in plastoglobule ultrastructure and increased sensitivity to oxidative stress in *fib4* plants. Thus, the authors hypothesized a correlation between enhanced sensitivity to oxidative stress and increased susceptibility towards *Pto* infection, as *Pto* is known to induce ROS accumulation in host tissue via the phytotoxic virulence factor coronatine (Ishiga et al., 2009; Singh et al., 2010). Alternatively, the observed morphological phenotype of *fib4* mutants found in this study, which was not yet described previously, may account for the observed susceptibility. The fact that the severe growth phenotype was not reported by Singh and colleagues may result from different growing conditions used in their study. This may point to a role of FIB4 in the perception of and/or response to different light conditions, which could be easily tested in further analyses. The presence of a conserved lipocalin signature in FIB4 further indicates a function in transport of lipophilic antioxidants upon light stresses (Singh et al., 2010). Notably, chloroplasts are location of lipid-derived JA biosynthesis. The committed step of JA biosynthesis is catalyzed by AOS, which is also associated with plastid membranes and plastoglobules (Schaller and Stintzi, 2009). Recently, correlation between JA insensitivity, light conditions and growth phenotypes involving

elongated hypocotyls were demonstrated (Kazan and Manners, 2011; Robson et al., 2010). Furthermore, Youssef and colleagues reported that a fibrillin subfamily (FIB1a, -1b, -2) conditions jasmonate production under photosynthetic stress, mediated by plastoglobule accumulation as the potential site for initiating chloroplast stress-related JA biosynthesis (Youssef et al., 2010). Whether *fib4* mutants are affected in JA biosynthesis or signaling is highly speculative but can readily be analyzed both for hormone measurements and responses in *fib4* mutants along with appropriate double mutants. However, the role of nuclear localization of FIB4 and a potential interaction with WRKY TFs remains elusive.

3.4. Concluding remarks

In conclusion this study has provided several novel insights into the role of WRKY18 and WRKY40 in regulating diverse responses related to plant immunity. Although valuable information has been gained that should certainly advance our knowledge of what these two transcription factors influence within the host, we are still far from fully understanding the specific molecular components with which they interact or the specific signaling pathways/subpathways they are involved in. In particular we are not yet at a stage to pinpoint key factors that depend on WRKY18 and/or WRKY40 functions and that are critical for the outcome of the interaction with *G. orontii* or with the bacteria *Pto* AvrRPS4. The fact that WRKY18 and WRKY40 play an important role in plant immunity towards *Pto* AvrRPS4 bacteria may partly explain why they are indispensable for the host despite the fact that loss of these two TF genes confers strong resistance towards the powdery mildew fungus *G. orontii*.

Up-regulation of numerous defense-related positive and negative regulators of defense signaling and the exaggerated activation of downstream maker genes in *wrky18wrky40* mutants upon pathogen challenge also points to a role of WRKY18 and WRKY40 in balancing and/or fine-tuning of defense responses.

By focusing in the future on elucidating the direct targets of WRKY18 and WRKY40 during the infection with *G. orontii* and *Pto* AvrRPS4 we may hope to uncover some of the still missing vital components that are essential in determining the distinct outcomes observed in the host. Moreover, it is obvious that we must position these transcription factors into the increasing complex regulatory network of the host in order to understand how their functions influence, both positively and negatively, host cell responses.

4. Material and Methods

The material and methods section is subdivided into two parts, listing all materials used within this work in the first part (4.1). This includes the list of oligonucleotides, plant lines, bacterial strains, pathogens, buffers and chemicals, media, solutions and enzymes. The second part (4.2) describes the methods applied in this work.

4.1. Material

4.1.1. Oligonucleotides

Table 2: Oligonucleotides used in this study

Gene	Locus	Forward primer (5' - 3')	Reverse primer (5' - 3')	Purpose
CYP81F2	AT5G57220	CCACGTTCTCCATCTGAAGATC	GCACCAGGACACGTTCTTCG	qPCR
EDS1	AT3G48090	AAGCATGATCCGCACTCG	CGAAGACACAGGGCCGTA	qPCR
Expr. Protein	AT4G26410	GAGCTGAAGTGGCTTCCATGAC	GGTCCGACATACCCATGATCC	qPCR
ICS1	AT1G74710	CATTGATCTATGCGGGGACAG	TGGACAAAAGCTCGTACCTGAG	qPCR
PAD4	AT3G52430	GGTTCTGTTCGTCTGATGTTT	GTTCCTCGGTGTTTTGAGTT	qPCR
NPR1	AT1G64280	AGGCACTTGACTCGGATGATATTG	CTTCACATTGCAATATGCAACAGC	qPCR
PR1	AT2G14610	TTCTTCCCTCGAAAGCTCAA	AAGGCCCACCAGAGTGTATG	qPCR
JAZ1	AT1G19180	TATATTCTACGCCGGGCAAGT	TGCGATAGTAGCGATGTTGC	qPCR
JAZ2	AT1G74950	CGGTAAACTTCGAGCCTGTC	AACCCCAGAAGCAAACATTG	qPCR
JAZ3	AT3G17860	AGGCAAAGGCGATAATGTTG	CGACAGAGGCACGAGTATGA	qPCR
JAZ4	AT1G48500	TTCCCCCTCAGTTGACAATC	CGGTTTAGCATGAGGTCCAT	qPCR
JAZ5	AT1G17380	CACCGTCTGATTTGATATGGG	TCATCGTTATCCTCCCAAGC	qPCR
JAZ6	AT1G72450	GCAACATCAGGTTGTGGAACG	CAACCATCTCTGGCTTGGGAG	qPCR
JAZ7	AT2G34600	GATCCTCCAACAATCCCAAA	TGGTAAGGGGAAGTTGCTTG	qPCR
JAZ8	AT1G30135	CGATCGCAAGCAGAGAAATG	GATCCGACCCGTTTGAGGAT	qPCR
JAZ9	AT1G70700	CAATGCAGCTCCTCGTAACA	GGATTCTCCGGTCGACAAAT	qPCR
JAZ10	AT5G13220	CGAGTCGTCGATGGAGACAG	CTCGAGAAAACGTTGCAGTG	qPCR
JAZ11	AT3G43440	GCCTTCCGTTGTTGTACGAT	AAAGAGGAGGTGCGAGATGA	qPCR
JAZ12	AT5G20900	GCACATCTAATGTGGCATCACC	CCAATCTGTCCCGTCTTTTC	qPCR
LOX2	AT3G45140	TGAATTGCAAGCTGTTGCTC	GCAGAAGCTACAAGACCACC	qPCR
OPR3	AT2G06050	CGGCTATAGATCACTTGGACG	GCGAGCTTTGAGCCATTAACAC	qPCR
PDF1.2	AT5G44420	ACGCACCGGCAATGGTGGAA	TGCATGATCCATGTTTGGCTC	qPCR
PEN2	AT2G44490	CTTCGAGAGATTTGGGGACA	CAGCAACACTAGCGCCATTA	qPCR
VSP2	AT5G24770	ACCGTTGGAAGTTGTGGAAG	CCAAATCAGCCCATTGATCT	qPCR
WRKY18	AT4G31800	AAGGGACGCATAACCACTTG	CCTTTCGTTTTTCTCCAACG	qPCR
WRKY40	AT1G80840	CTTGACTGTGCCGGTGACTA	GAAGAAGCCATTTGCTCCAC	qPCR
WRKY18	AT4G31800	GATTTTTCATTTTCGTTAAAGC CAAATAGTAAGGGAAACTAGGA GCTTGACTCATCATCAACTTAAG	GTCCGACTGAATGAGAAGTTC AGAAAAATTTGGACGTCATAC GTTATGATAGTTTTAGGTCAAATG	ChIP Set 1 ChIP Set 2 ChIP Set 3
WRKY40	AT1G80840	CGATGGTATCGTCAATTTGTC GGAAAGAATAAAGTTGTTTTCAC CTGTTAGAAAGATTAAATCAAAGG	CGTGCTTAATTAGGAAGTTTAATA TGGTAGAAGACTTGAGTACCTTAG TCTTAGTTATCATTTTTCTCCG	ChIP Set 1 ChIP Set 2 ChIP Set 3
JAZ7 (WiscDsLox7H11)	AT2G34600	AAATGCGACTTGGAACTTCG AAATGCGACTTGGAACTTCG TGGTAAGGGGAAGTTGCTTG	TGGTAAGGGGAAGTTGCTTG AACGTCCGCAATGTGTTATTAAGTTGTC CATCAAAAACTGCGACAAGCC	LP/RP RP/LB expression
JAZ10ox	AT5G13220	ATGTCGAAAGCTACCATAGAAC	CTCGAGAAAACGTTGCAGTG	expression
FIBRILLIN4 (SALK_122950)	AT3G23400	TTCTTTAGCAGCCACTTCAGC ACGGCAAATGTTCACGTAATC	ACGGCAAATGTTCACGTAATC GATCTCAGGGGAAAACGAAAC	LP/RP RP/LB
Oxidoreductase (SAIL_268_B05)	AT2G38240	GTGAGAAAACTGTGCGAAAGG GTGAGAAAACTGTGCGAAAGG ATGGCTACATGCTGGCCTGAG	TGGCCAACCATAATTTCATTG TAGCATCTGAATTTCATAACCAATCTCGATACAC AGAACGGTACTCGTCGAACC	LP/RP RP/LB expression

WRKY18 (GABI_328G03)	AT4G31800	CGGATTTCGTCTGATCCATT CGGATTTCGTCTGATCCATT	CGATTCATTTCGATGCAAAG ATATTGACCATCATACTCATTGC	LP/RP RP/LB
WRKY40 (SLAT collection)	AT1G80840	attB1-ACATGGATCAGTACTCATCCTCTTTGG attB1-ACATGGATCAGTACTCATCCTCTTTGG	TCCACCAAAGCACTTGTCTG GTTTTGGCCGACACTCCTTACC	LP/RP RP/LB
FIBRILLIN4	AT3G23400	attB1-ccATGGCGACTTCTTCTAC attB1-ccATGGCGACTTCTTCTAC attB1-cCATAGCAGAGGTGGAATTAG attB1-cCATAGCAGAGGTGGAATTAG	attB2-TTAAGCAATGACGAATACCC attB2-AGCAATGACGAATACCCTAAG attB2-TTAAGCAATGACGAATACCC attB2-AGCAATGACGAATACCCTAAG	FL Donor FL Donor no Stop Trunc. Donor Trunc. Donor no Stop
Oxidoreductase	AT2G38240	attB1-ccAACCCTTCCAAGTGGCC attB1-ccAACCCTTCCAAGTGGCC attB1-atATGGCTACATGCTGGCCTG attB1-atATGGCTACATGCTGGCCTG	attB2-TTATCTAGTTAATAACAGTGAGTCG attB2-TCTAGTTAATAACAGTGAGTCGAC attB2-TTATCTAGTTAATAACAGTGAGTCG attB2-TCTAGTTAATAACAGTGAGTCGAC	FL Donor FL Donor no Stop Trunc. Donor Trunc. Donor no Stop
SPL3	AT2G33810	attB1-ccATGAGTATGAGAAGAAGC attB1-ccATGAGTATGAGAAGAAGC	attB2-TTAGTCAGTTGTGCTTTTCC attB2-GTCAGTTGTGCTTTTCC	FL Donor FL Donor no Stop
WRKY18	AT4G31800	attB1-tcATGGACGGTTCTTCGTTTCTCG	attB2-TCATGTTCTAGATTGCTCC	FL Donor
WRKY40	AT1G80840	attB1-taATGGATCAGTACTCATCCTCTTTGGTCG	attB2-CTATTTCTCGGTATGATTCTGTTGATAC	FL Donor
pDNOR207		TCGCGTTAACGCTAGCATGGATCTC	GTAACATCAGAGATTTTGAGACAC	Insert sequencing
pGADT7		TCATCGGAAGAGAGTAGT	TTTTCGTTTTAAAACCTAAGAGTC	Insert amplification
pGBKT7		AGATGGTGCACGATGCACAG	TAATACGACTCACTATAGGG	Insert amplification

4.1.2. Plant material

All experiments were performed using *Arabidopsis thaliana* Columbia-0 (Col-0) wildtype plants or mutants in the Col-0 background. The *WRKY18 WRKY40* double mutant was generated by crossing the homozygous *wrky18* (GABI_328G03) and *wrky40* SLAT-line as described earlier (Shen et al., 2007; Tissier et al., 1999). *JAZ7* mutants were ordered from Wisconsin mutant collection (Wisc_7H11) and genotyped to identify homozygous mutant plants. Such plants were checked for the absence of *JAZ7* transcript 1 h after wounding. Homozygous plants were used for further analysis. *JAZ10* overexpressor (*JAZ10*ox) plants (Yan et al., 2007) were kindly provided by Dr. Edward E. Farmer (University of Lausanne) and checked by PCR for constitutive *JAZ10* expression. *FIBRILLIN4* mutants were ordered from SALK mutant collection (SALK_122950) and homozygous plants were identified. This line was already shown to be a null mutant (Singh et al., 2010). Mutants for *At2g38240* (Oxidoreductase) were ordered from NASC (SAIL_268_B05) and homozygous mutant plants were identified. Absence of transcript was confirmed by PCR. Triple mutants were generated by crossing *wrky18wrky40* homozygous plants with *sid2* or *rrtf1* homozygous plants. Triple mutants were identified by PCR and used for further experiments.

4.1.2.1. Mutant *Arabidopsis* lines used in this study

Table 3: *Arabidopsis* mutants used in this study

gene	accession	reference/source
wrky18wrky40	Col-0	Shen et al., 2007
wrky18	Col-0	Shen et al., 2007
wrky40	Col-0	Shen et al., 2007
eds1-2	Col-0 (Ler-0)[a]	Bartsch et al., 2006
sid2-1	Col-0	Wildermuth et al., 2001
jar1-1	Col-0	Staswick et al., 2002
ndr1-1	Col-0	Century et al., 1995
jaz10ox	Col-0	Yan et al., 2007
jaz7	Col-0	this study
fibrillin4	Col-0	this study
At2g38240 (*oxidoreductase*)	Col-0	this study

[a] Ler eds1-2 allele introgressed into Col-0 genetic background (8th backcrossed generation)

4.1.2.2. *Arabidopsis* complementation lines used in this study

To generate the *WRKY18* 3xHA-tagged complementation line, the genomic region from -4428 bp to 1514 bp relative to the start codon of *WRKY18* was PCR amplified and cloned into a binary pPAM vector (GenBank accession AY027531) derivative carrying a hygromycin resistance gene (M. Roccaro, unpublished). In a second step, the stop codon was removed and substituted by an in-frame 3xHA-sequence. This construct was stably transformed to *wrky18wrky40* double mutant *Arabidopsis* plants.

4.1.3. Bacterial strains

Bacterial strains used in this study are listed in Table 4. In addition the precise bacterial strain and genotype are stated.

Table 4: Bacterial strains used in this study

name	strain	Genotype
Escherichia coli	DH5α	F^- f80lacZDM15 D(lacZYA-argF) U169 deoR endA1 recA1 hsdR17 (r_k^-,m_k) sup44 thi-1 gyrA96 relA1 phoA
Escherichia coli	DB3.1	F^- gyrA462 endA D(sr1-recA) mcrB mrr hsdS20 (r_B^-, m_B^-) supE44 ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Dleu mtl1
Agrobacterium tumefaciens	GV3101	PMP90RK Gm^R Km^R Rif^R

4.1.4. Yeast strains and cDNA library

All yeast 2-hybrid experiments were performed using strains AH109 and Y187 (Clontech; Mountain View, Germany). Detailed descriptions of both strains are given blow.

Table 5: Yeast strains used in this study

strain	genotype	reference/source
AH109	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2 : : GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3 : : MEL1$_{UAS}$-MEL1 $_{TATA}$-lacZ*	James *et al.*, 1996; A. Holtz, unpublished
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met–, gal80Δ, URA3 : : GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ*	Harper *et al.*, 1993

The cDNA library was kindly provided by Birgit Kemmerling (Center For Plant Molecular Biology, University of Thübingen). In addition, Gateway-system (Invitrogen) compatible bait and prey vectors pGBKT7 (pGBKT7-GW) and pGADT7 (pGADT7-GW) were provided (frame B). cDNA library was constructed from pathogen challenged plants. 6 weeks old *Arabidopsis* Col-0 plants were infiltrated with $1x10^8$ cfu/ml of *Pseudomonas syringae* pv *tomato* DC3000, hrcC, AvrRPM1 and *Pseudomonas syringae* pv *phaseolicola*. Plant material was harvested 2, 6 and 24 h after infiltration. Library construction was performed according

to Clontech Matchmaker Library Construction and Screening Kit. cDNA was cloned into pGADT7-GW and transformed into yeast strain AH109. Transformation efficiency was determined ($3x10^5$ cfu/3 µg pGADT7; expected according to Clontech $1x10^6$ cfu/3 µg pGADT7). Number of clones in the library was calculated with $5.2x10^6$.

4.1.5. Pathogens

All pathogens employed in this study are depicted in Table 6.In addition, source or reference is stated.

Table 6: Pathogens used in this study

Name	reference/source
Golovinomyces orontii	MPIPZ[a]
Golovinomyces cichoracearum	V. Lipka[b]
Golovinomyces cruciferarum	V. Lipka[b]
Pseudomonas syringae pv. *tomato DC3000*	R. Innes[c]
Pseudomonas syringae pv. *tomato DC3000 AvrRPS4*	K. Heidrich[a]
Pseudomonas syringae pv. *tomato DC3000 AvrRPM1*	K. Heidrich[a]
Pseudomonas syringae pv. *tomato DC3000 HopA1*	K. Heidrich[a]
Pseudomonas syringae pv. *tomato DC3000 hrcC*	K. Shirasu[d]
Hyaloperonospora arabidopsidis EMCO	J. Parker[a]
Hyaloperonospora arabidopsidis EMWA	J. Parker[a]
Hyaloperonospora arabidopsidis CALA	J. Parker[a]
Phytophtora infestans	D. Scheel[e]
Botrytis cinerea (isolate 2100)	R.Birkenbihl[a] / Spanish Type Culture[f]

[a] Max-Planck Institute for Plant Breeding Research, Cologne, Germany
[b] Georg-August-University, Göttingen. Germany
[c] Indiana University, Bloomington, Indian, USA
[d] The Sainsbury Laboratory, John Innes Centre, Norwich, UK
[e] Leibnitz Institute for Plant Biochemistry, Halle Germany
[f] CollectionUniversidad de Valencia, Burjassot, Spain

4.1.6. Vectors

Vectors used in this study are listed in Table 7. In additional a reference or the source of the construct is stated.

Table 7: Vectors used in this study

Name	reference/source
pDONR207	Invitrogen; Heidelberg, Germany
pGBKT7	Clontech; Mountain View, USA
pGADT7	Clontech; Mountain View, USA
pUC-SPYCE	Walter et al., 2004
pUC-SPYNE	Walter et al., 2004

4.1.7. Antibodies and Enzymes

Primary and secondary antibodies used in this study are listed in Table 8 and Table 9. Additionally, the source, dilution of use, reference and the purpose the antibodies were used for are stated.

4.1.7.1. Primary and secondary antibodies

Table 8: List of primary antibodies used in this study

name	source	dilution	reference/source	purpose
α-HA	rabbit (polycl.)	1:5000 in 5% milk	Sigma	ChIP
α-HA	rat (monocl.)	1:5000 in 5% milk	Roche	Western
α-myc	mouse (monocl.)	1:500 in 5% milk	Santa Cruz Biotechnology	Western

4.1.7.2. Secondary antibodies

Table 9: List of secondary antibodies used in this study

name	label	dilution	reference/source	purpose
α-rat IgG	HRP[a]	1:10.000 in 5% milk	Sigma	Western
α-mouse IgG	HRP[a]	1:5.000 in 5% milk	ECL (GE Healthcare)	Western

[a] horseradish peroxidase

4.1.7.3. Enzymes

Restriction enzymes were purchased from New England Biolabs (NEB, Frankfurt am Main, Germany) and Fermentas (St. Leon-Rot, Germany). Restriction digestion was performed according to the manufacture's protocol. *Taq*-Polymerase used for standard PCR- reactions was ordered from Ampliqon (Odense, Denmark) and used according to the manufacturer's instructions. High accuracy *Taq*-Polymerase from *TaKaRa* (Clontech, Mountain View, USA) was used for generating DNA fragments for cloning according to the manufacture's protocol. BP- and LR-clonase II enzyme mixes were purchased from Invitrogen (Karlsruhe, Germany) and used following the manufacturer's instructions.

4.1.8. Antibiotics and Chemicals

4.1.8.1. Antibiotics

Antibiotic stock solutions (1000x) were prepared as indicated and stored at -20°C.

Table 10: List of antibiotics used in this study

name	concentration	source
Ampicilin	50 mg/ml in H_20	Roth
Kanamycin	50 mg/ml in H_20	Sigma
Gentamycin	25 mg/ml in H_20	Sigma
Carbencilin	50 mg/ml in H_20	Sigma
Rifampicin	100 mg/ml in DMSO	Sigma

4.1.8.2. Chemicals

Laboratory grade chemicals and reagents were purchased from Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma-Aldrich (München, Germany) unless stated otherwise.

Pierce ProteinA agarose for ChIP-experiments and Pierce Western substrate were purchased from Thermo Fisher Scientific (Rockford, USA).

4.1.9. Media, buffer and solutions

4.1.9.1. Media

Sterilized media was used for growing bacteria, yeast or *Arabidopsis* plants *in vitro*. For sterilization, media was autoclaved for 20 min at 121°C and cooled down prior adding heat instable antibiotics or other supplements. Heat instable compounds were filter-sterilized before use. Agar for agar plate preparation was purchased from Becton (Franklin Lakes, USA) and MS medium was ordered from Duchefa (Haarlem, Netherlands) or Sigma.

Escherichia coli medium	Luria-Bertani (LB) broth or agar plates
Agrobacterium tumefaciens medium	YEB broth or agar plates
Pseudomonas syringae medium	NYGA broth or agar plates
Arabidopsis thaliana medium	½ Murashige-Skoog (MS) medium including vitamins and 0.5% sucrose
Saccharomyces cerevisiae (yeast) medium	Prepared according to Clontech Matchmaker Library Construction and Screening Kit User Manual (Clontech, Mountain View, USA)

4.1.9.2. Buffers

3xSB buffer:
150 mM Tris 6.8 (3 ml 1M)
6 mM EDTA (240 µl 0,5 M)
3% SDS (6 ml 10% SDS)
3% β-mercaptoethanol (β-MOH) (600 µl)
24% glycerol (4,8 ml)
0.075%
5,31 ml H_20

Edward solution:
200 mM Tris 7,5 pH
250 mM NaCl
25 mM EDTA
0.5 % SDS

PBS:
80 g NaCl
2 g KCl
14.4 g Na_2HPO_4
2.4 KH_2PO_4
pH 7.4 (HCl)

Ponceau S:
ATX Ponceau S (Fulka) 1:5 in dH_20

3x Protein loading buffer:
150 mM TRIS
3% SDS
6 mM EDTA
3% β-mercaptoethanol
24% glycerol
0.0075% bromphenol blue
resolved in dH_2O

10x running buffer:
250 mM Tris (30.25 g)
1.92 M glycin (144.29 g)
1% SDS (10 g)
ad 1 l H_20

10x transfer buffer:
5.8 g TRIS
29 g glycine
10 ml 10% SDS (1g SDS)
200 ml MeOH (methanol)
ad 1 l H_20

Yeast transformation buffer:
8 ml PEG3350 (50%)
1 ml TE (10x)
1 ml LiAc (10x)

TE-buffer:
10 mM TRIS
1mM EDTA
pH 8.0 (HCl)

4.2. Methods

4.2.1. Maintenance and cultivation of *Arabidopsis* plants

Arabidopsis thaliana seeds were sowed out on 42 mm Jiffy-7 pots (Jiffy Products, Stange, Norway) soaked in four liters of water containing 0.1% Wuxal fertilizer (Manna, Germany), to avoid unspecified pathogen infections derived from garden soil. Plants were grown for 4-5 weeks under short-day conditions (10 hours light at 23°C/14 hours darkness at 20°C and 60% humidity) in closed cabinets (Schneijder chamber) until use. For pathogen infection plants were transferred to designated growth-chambers for the respective pathogens. See the 'Pathogen infections'-section for more details.

4.2.1.1. Stable *Arabidopsis* transformation

Stable transformation of *Arabidopsis* plants was performed using the floral dip method as described previously (Bent, 2006). Transformants were selected on MS agar plates containing the appropriate antibiotics.

4.2.1.2. *Agrobacterium tumefaciens* transformation by electroporation

A. tumefaciens strain GV3101 (pMP90 RK) was used for transformation. 50 µl of competent cells were mixed with 0.2-0-5 µg of plasmid DNA and transferred to a chilled 2 mm (electrode distance) electroporation cuvette and kept on ice. The BioRad Gene Pulse apparatus was set to 25 µF, 2.5 kV and 400 Ω. The cells were pulsed once at the above setting and 1 ml YEB was added to the cuvette. Cells were transferred to a 1.5 ml reaction tube and incubated for 2 h at 28°C shaking at 600 rpm. A 10 µl fraction of the transformation mixture was subsequently plated onto YEB agar plates containing the appropriate antibiotics and incubated at 28°C for 2 days.

4.2.1.3. Transient expression in *Nicotiana benthamiana*

For transient expression of CFP- and/or YFP-tagged constructs in *N. benthamiana*, fresh *A. tumefaciens* strains expressing the construct of interest were grown on YEB agar plates containing the appropriate antibiotics for 2 days at 28°C. Bacteria were harvested from plate and diluted to OD_{600} 0.7 in infiltration medium (10 mM MES pH 6.5 and 10 mM $MgCl_2$). Bacterial suspension was infiltrated into abaxial *N.benthamiana* leaves using a needle-less syringe. For localization studies, 1 ml of bacterial suspension was infiltrated, whereas for co-localization, 0.5 ml of each construct were mixed and infiltrated subsequently. Two days after infiltration, plant leaves were prepared for confocal microscopy.

Split-YFP constructs were infiltrated as described earlier by Walter et. al (2004).

4.2.2. Pathogen infections

4.2.2.1 Powdery mildew infections

Golovinomyces orontii was propagated on *pad4sag101* double mutant *Arabidopsis* plants in closed cabinets (Percival chamber) under short-day conditions (10 h light/14 h darkness) with 70% humidity and 22°C. Four weeks old plants were used for infections. All infections were performed by dusting without touching the plants to avoid any wound responses. A brush was used to spread spores ("powder") over the plant. Infected plants were further kept under short-day conditions in closed cabinets. Control samples (0 hpi) were always taken before inoculation with the fungus.

Golovinomyces cichoracearum and *Golovinomyces cruciferarum* were propagated on their natural host plants zucchini (Gold rush) and *Brassica juncea* under short-day conditions (8 h light/16 h darkness) at 80% humidity and 22 °C. Different fungal pathogens were kept in individual growth chambers to avoid cross-contaminations. Infection with *G. cichoracearum* and *G. cruciferarum* were performed by spreading spores evenly onto 4 weeks old *Arabidopsis* plants without touching the plant. Conidiophore counts were performed 7 dpi. Experiments with *G. cichoracearum* and *G.cruciferarum* were performed by C. Roth at the University of Göttingen, Germany.

4.2.2.2. Penetration counts

Infections were achieved by dusting spores over the plants. Leaf samples were taken 48 hpi and chlorophyll was destained in ethanol:acetic acid solution (3:1) for at least 24 hours. To visualize epiphytic fungal growth, 4 leaves of one biological replicate were stained in Coomassie Brilliant Blue solution (45% MeOH, 10% acetic acid, 0.05% Coomassie R250) and prepared for microscopy. Four biological replicates (individual plants) were analyzed per experiment and each experiment was repeated at least twice. For fungal host cell entry rates, the ratio of penetrating spores that formed secondary hyphae to all spores that formed an appressorium was calculated. Values represent means ± SD of one representative experiment comprising four individual plants (n=4).

4.2.2.3. Conidiophore counts

For conidiophore counts, at least nine leaves of three individual plants (n=3) were harvested at 7 dpi and destained in 80% EtOH for several days. To visualize fungal structures, destained leaves were stained in 0.6% Coomassie Brilliant Blue solution (in 100% EtOH) and prepared for microscopy. Microscopy was performed using a Leica epiflourescence microscope (Leica

GmbH, Wetzlar, Germany). Transmitted light and filter A (BP 340-380) was used for conidiophore counting. This was done by determining the number of conidiophores of single sporulated spores at 7 dpi.

4.2.2.4. *Pseudomonas syringae* infections

For *Pseudomonas syringae* infections, bacteria were plated from glycerol stocks on fresh NYGA agar plates containing the appropriate antibiotics. Bacteria were grown 2 days at 28°C and plated again on fresh NYGA agar plates containing the appropriate antibiotics and incubated over night at 28 °C. Grown bacteria were harvested from plate and resuspended in 10 mM $MgCl_2$. Bacterial suspension was diluted to $O.D._{600}$ = 0.2 in 10 mM $MgCl_2$ containing 0.04% Silwet L-77. Plants were covered with sealed hoods that were sprayed with water to increase humidity and induce stomata opening at least 3 h before infection. Plants were sprayed evenly using a micro diffuser (Roth) until leaves were covered with bacterial suspension. The plants were covered with a sealed hood for 1 h, afterwards the hood was removed until leaf surface was dry. 0 dpi samples were subsequently harvested by detaching three leaves. Leaves were washed in 70% ethanol, followed by two washing steps in H_20. After drying the leaves on a paper towel, one 0.7mm leaf-disc was punched from each leaf and collected in 2 ml reaction tubes containing 0.5 ml 10 mM $MgCl_2$ supplemented with 0.01% Silwet L-77. Reaction tubes were incubated shaking at 600 rpm for 1 h at 28°C. For each sample, a dilution series (undiluted, 10^{-1} – 10^{-6}) was plated on NYGA agar plates containing the appropriate antibiotics. 20 µl were plated of each dilution and incubated 2 days at 28°C. Colonies were counted and bacterial number was calculated. Similarly, after 3 days under controlled short-day conditions (10 h light at 23°C / 14 h darkness at 22°C and 65% humidity), 3 dpi samples were harvested and leaf discs shaked in 1.5 ml $MgCl_2$ containing 0.01% Silwet L-77 for 1 h at 600 rpm at 28°C. For 3 dpi samples, 10 µl of each dilution was plated on NYGA agar plates containing the appropriate antibiotics and colonies were counted after 2 days incubation at 28°C. Samples taken 4 dpi were treated similarly. Values represent means ± SD of a representative experiment comprising four individual plants (n=4).

4.2.2.5 *Hyaloperonospora arabidopsisdis* infections

Hyaloperonospora arabidopsidis were propagated on *Arabidopsis* Col-0 *eds1-2* plants. A concentration of $4x10^4$ spores/ml in H_2O was used for infection of 10 days old plants. Infected plants were incubated in sealed trays under short-day conditions (10 h light/14 h darkness, 22 °C, 70% humidity) for 7 days. Afterwards, 20-30 leaves were harvested into 10 ml reaction

tubes and transferred to 1:1 trypan blue – EtOH solution for staining of oomycete structures. Leaf samples were boiled for 5 min (with open lids) and trypan blue/EtOH solution was removed and exchanged by chloralhydrate for destaining plant leaves. Samples were incubated on a rotating shaker overnight at RT. Chloralhydrate was exchanged and samples were incubated another night under conditions described above. Afterwards, chloralhydrate was removed and 75% glycine was added to the samples. Leaf samples were prepared for microscopy on glass slides and pictures were taken using transmitted light microscopy. Infections were at least repeated twice with similar results.

4.2.2.6. *Phytophtora infestans* infections

Phytophtora infections were carried out with two different concentrations. Four weeks old plants were infected with 500 spores/µl in H_20 or 50 spores/µl in H_20. 10 µl were used for drop-infection of plant leaves. Leaf samples were harvested 3 days after inoculation and stained with trypan blue as described by Lipka et. al (2005). After destaining of chlorophyll, leaves infected with high spore-concentrations were scanned and trypan blue staining intensities at inoculation sites were determined using the image processing software ImageJ (http://rsbweb.nih.gov/ij/). Leaves infected with low spore-concentration were investigated using transmitted light microscopy. Infections were repeated twice with similar results. *Phytophtora* experiments were performed by L. Westphal at the Leibnitz Institute of Plant Biochemistry (IPB) in Halle, Germany.

4.2.2.7. *Botrytis cinerea* infections

For *B. cinerea* infections, isolate 2100 was used, which was cultivated on potato dextrose plates for 10 days at 22°C. Spores were collected from plate, washed and stored in 0.8% NaCl at a concentration of 10^7 spores/ml at -80°C until use. For drop-inoculation of 5 weeks old *Arabidopsis* plants, spores were diluted in Vogelbuffer (in 1l: 15 g sucrose, 3 g Na-citrate, 5 g K_2HPO_4, 0.2 g $MgSO_4$ $7H_2O$, 0.1 g $CaCl_2$ 2H2O, 2 g NH_4NO_3) to $5x10^5$ spores/ml. For drop infections, 5 µl of diluted spores were applied to single leaves. Mock infections were performed using only Vogelbuffer. Plants were kept prior to and during the infection under sealed hoods with high humidity. *B. cinerea* infections were performed by R. Birkenbihl at the MPIPZ in Cologne, Germany.

4.2.3. Molecular biological methods

4.2.3.1. *Escherichia coli* transformation

Chemical transformation was carried out for transformation of *E.coli* cells. 10 to 100 ng plasmid DNA or 3 µl of BP or LR reactions (Invitrogen) were mixed with 50 µl of chemically competent *E.coli* cells and incubated on ice for 30 min. After incubation, cells were heat shocked by incubation for 45 sec at 42°C and subsequently put on ice for additional 2 min. 300 µl pre-warmed LB liquid medium was added to the cells and incubated for 1 h at 37°C shaking at 700 rpm. Afterwards, cells were centrifuged for 1 min at 5000 rpm and pellet was resuspended in 100 µl LB medium. 10 and 50 µl were plated on LB agar plates containing the appropriate antibiotics and incubated overnight at 37°C.

4.2.3.2. DNA isolation

For genotyping, 10 mg plant leaf material was collected into 96-well Collection Microtubes (Qiagen, Hilden, Germany) containing10-15 1 mm Zirconia Beads (BioSpec). Samples were frozen in liquid nitrogen and homogenized using a TissueLyser (Qiagen, Hilden, Germany) for 30 sec at 30 strokes/sec. 400 µl extraction buffer (1% SDS, 0,5 M NaCl) was added to the homogenized samples and shaked again in the TissueLyser for 10 sec at 30 strokes/sec. The following DNA extraction was performed according to Kotchoni et. al (Kotchoni and Gachomo, 2009). 2 µl of extracted DNA was used for subsequent PCR analysis.
Isolation of high quality plasmid DNA was performed using the Machery Nagel (MN, Düren, Germany) NucleoSpin Mini-Kit following the instructions of the manufacturer.

4.2.3.3. Cloning (BP/LR reaction)

Cloning was performed using the Invitrogen (Heidelberg, Germany) Gateway BP/LR clonase II-Enzyme Mix following the instructions of the manufacturer. All constructs cloned in this study are based on Gateway-system compatible vectors.

4.2.3.4 Isolation of RNA

Total RNA was extracted from 100 mg plant leaf material of 4-5 week old *Arabidopsis* plants. Frozen samples were homogenized with ~20 1 mm Zirconia Beads'(BioSpec) in a Mini-BeadBeater-6 (BioSpec) for 30 seconds or using a TissueLyser (Qiagen, Hilden, Germany) for 30 sec at 30 strokes/sec. RNA was isolated using the TRI Reagent (Ambion) following the manufacturer's protocol. Concentration and quality was determined using a NanoDrop

photometer (PeqLab). RNA with a 260/280 and 160/230 ratio of ~2.0 was used for cDNA synthesis.

4.2.3.5. cDNA synthesis

Five µg total RNA was used for cDNA synthesis with oligo-dT-primers employing the 'SuperScript II first-strand synthesis system for reverse-transcription PCR' (Invitrogen) following the manufacturer's protocol. cDNA was solved in 50 µl water and subsequently used for quantitative real-time PCR.

4.2.3.6. Polymerase chain reaction (PCR)

Standard PCR was performed using the Ampliqon (Odense, Denmark) *Taq* polymerase while amplification of DNA for cloning purpose was done with high accuracy *Ta**K**a**R*a *Taq* polymerase (Clontech, Mountain View, USA). The standard PCR reaction mix (Table 11) and thermal profile (Table 12) is depicted below.

Table 11: Standard PCR reaction mix

Reagent	Amount
DNA template	10-50 ng
PCR amplification buffer	1/10 of reaction volume
dNTP mix (dATP, dGTP, dCTP, dTTP)	0,2 mM each
forward primer	0,5 µM
reverse primer	0,5 µM
Taq DNA polymerase	2,5-5 units
sterile H_2O	Variable

Table 12: Standard PCR thermal profile

Step	Temp.	Time	
Initial denaturation	95°C	3 min.	
Denaturation	95°C	30 sec.	20-35x
Annealing	55°C	30 sec.	
Extension	72°C	20-300 sec	
Final extension	72°C	3 min.	

4.2.3.7. Quantitative Real-Time PCR (qPCR)

Expression kinetics in time course experiments were performed by qPCR analyses. All qPCR analyses were performed with cDNA corresponding to ~15 ng RNA before reverse transcription on an 'iQ5 multicolor real-time PCR detection system' (BioRad) using 'iQ5 SYBR Green Ready Mix' (BioRad). Gene-specific primers were designed using Primer3 software (Rozen and Skaletsky, 2000) and further analyzed using NetPrimer (Premierbiosoft). Primers were designed with a NetPrimer rating value of 85-100, as recommended by Czechowski and colleagues (Czechowski et al., 2005). Expressed protein (AT4G26410) was used as the reference gene, as it shows very constant expression throughout various biotic and abiotic stresses (Czechowski et al., 2005). qPCR results were analyzed using the ΔΔCt-method as described by Livak and colleagues (Livak and Schmittgen, 2001). Fold-changes were calculated relative to wildtype untreated (0 hpi) samples or to the same time point set to 1. Data were shown as the mean +/- standard deviation (SD) from two biological and two technical replicates (n=2). All expression kinetics were repeated in two individual experiments with similar results, except for verification of microarray data, which was based on only one replicate.

The standard qPCR-program is depicted in Table 13.

Table 13: Standard qPCR program

q-PCR program			
Initial denaturation	95°C	2 min.	
Denaturation	95°C	20 sec.	40x
Annealing	55°C	30 sec.	
Extension	72°C	25 sec.	
Final extension	72°C	1 min.	
Melting Curve	55-95°C	10 sec.; á 0.5°C; 81x	

4.2.3.8. Microarray experiment

For microarray analysis, total RNA was isolated from 100 mg of plant leaf tissue prior to and 6 and 14 h after infection with *G. orontii* using TRI reagent following basically the instructions of the manufacturer with some modifications. To allow isolation of low molecular weight RNA (small RNAs), the volume of isopropanol was increased to 1 ml/ml

TRI reagent. After extraction, 25 µg total RNA was adjusted to 100 µl in DEPC water and used for an additional purification step using the Qiagen RNeasy-Plant Mini Kit with some modifications. 350 µl of RLT buffer (Qiagen) without β–mercaptoethanol was added and mixed well for 5 sec. Afterwards, 350 yl of 100% EtOH was added (to reach a final EtOH proportion of ~60%) and mixed by pipetting up and down. 750 µl of the mixture was transferred to RNeasy spin column and centrifuged 20 sec at 13.000 rpm. Flowthrough was discarded and washing step with RW1 buffer (Qiagen) was skipped, as it would remove low molecular RNAs. 500 µl of RPE buffer (Qiagen) containing EtOH was added to the column. After centrifugation for 20 sec at 13.000 rpm, flow-through was discarded and the washing step was repeated once. A final washing step with 500 µl 80% EtOH was performed before elution of RNA with DEPC water.

For microarray analysis, high quality total RNA (50 ng) were reverse-transcribed into double-stranded cDNA and subsequently in vitro transcribed in the presence of biotin-labeled nucleotides using the Affymetrix GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, USA) including polyA controls as recommended by the manufacturer. Quantity and quality of the biotinylated cRNA was determined by using NanoDrop ND 1000 (PeqLab, Erlangen, Germany) and Bioanalyzer 2100 (Agilent, Böblingen, Germany). 15 µg of biotin-labeled cRNA samples were fragmented randomly to 35-200 bp at 94°C in Fragmentation Buffer (Affymetrix, P/N 901229).

Array hybridization was carried out by mixing biotin-labeled cRNA samples with 300 µl Hybridization Mix (Affymetrix, P/N 900720) containing Hybridization Controls and Control Oligonucleotide B2 (Affymetrix, P/N 900454). Afterwards, samples were hybridized onto Affymetrix AGRONOMICS1 *Arabidopsis* tiling arrays for 16 h at 45°C. Hybridized arrays were washed using an Affymetrix Fluidics Station 450 running the FS450_0004 protocol. The Affymetrix GeneChip Scanner 3000 was used to measure fluorescence intensity emitted by the labeled target.

4.2.3.8. Microarray data analysis

Microarray data processing was performed by Emiel Ver Loren van Themaat at the MPIPZ in Cologne. Normalization and statistical analysis of the data was carried out as described by Irizarry and colleagues and Smyth (Irizarry et al., 2003; Smyth, 2004). Fold-changes were calculated by comparing the following genotypes upon *G. orontii* infection:

- Col-0 0 hpi vs. *wrky18* 0 hpi
- Col-0 6 hpi vs. *wrky18* 6 hpi

- Col-0 14 hpi vs. *wrky18* 14 hpi
- Col-0 0 hpi vs. *wrky40* 0 hpi
- Col-0 6 hpi vs. *wrky40* 6 hpi
- Col-0 14 hpi vs. *wrky40* 14 hpi

1.5-fold up- and 0.66-fold down-regulated genes with a p-value ≤ 0.05 were used for further analysis using BioVenn (http://www.cmbi.ru.nl/cdd/biovenn/) to visualize co-regulated genes. Moreover, Gene Ontology analysis was performed using the online tool of The Arabidopsis Information Resource (http://www.arabidopsis.org/tools/bulk/go/index.jsp).

4.2.3.9. Chromatin immunoprecipitation (ChIP)

For ChIP experiments, 2 g leaf material of 4-5 weeks old plants were harvested prior (0 hpi), 2 and 6 hpi *G. orontii* infection, kept on ice, and subsequently processed following basically the protocol by Gendrel et al. (2005) with some modifications. Cross-linking of harvested leaf material was carried out by vacuum infiltration of 1% formaldehyde solution for 15 min, followed by a second 5 min vacuum infiltration. Sonication of extracted nuclei was performed using a Diagenode BioRuptor UCD300 T0 (Liege, Belgium) sonicator. Sonication was performed at 4°C and samples were additionally cooled with ice-water during the sonication process. Sonication was performed at high efficiency, 30 sec sonication, 30 sec pause (repeated 5 times). After that, ice-water was exchanged and the setup was repeated. After pre-clearing with proteinA agarose beads, the sheared chromatin was incubated overnight with rabbit polyclonal HA-antibodies (Sigma) at 4°C on a nutator. Afterwards, immunocomplexes were collected by 2 h incubation with proteinA agarose beads and after washing, reverse cross-linking was performed overnight at 65°C. DNA extraction was performed by phenol-chloroform isolation and DNA precipitated with ethanol. Input sample DNA was resolved in 70 µl and immunoprecipitated DNA in 20 µl TE buffer pH 8.0. 10.5 µl of 1:20-dilutions were used for qPCR analysis.

4.2.3.10. Denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, 100 mg of plant tissue were collected and frozen in liquid nitrogen until use. Frozen samples were homogenized with ~20 1 mm 'Zirconia Beads' (BioSpec) in a Mini-BeadBeater-6 (BioSpec) for 30 seconds. 100 µl 2x SB-buffer were added to the frozen and homogenized samples and mixed rigorously. Samples were incubated for 3 min at 95°C and subsequently centrifuged for 2 min at 13.2 rpm. 5-30 µl of the supernatant and 10 µl of a prestained molecular-weight marker (Precision plus protein standard dual color; BioRad)

were loaded into a10-12% SDS resolving gel containing a 4% stacking gel. Gels were run in electrophoresis tanks (Mini-Protean 3 Cell; BioRad) in 1x running buffer for 60 min at 40 mA (constant) until the marker suggested a sufficient separation of the proteins.

4.2.3.11. Immuno-blot analysis (Western blot)

After proteins were separated in SDS-PAGE, gels were transferred to nitrocellulose membranes (Tans-Blot, BioRad). For this, the stacking gel was removed, discarded and the blotting apparatus (Mini-Protean 3 Cell; BioRad) was assembled according to the manufacturer instructions. Transfer was performed in 1x transfer buffer at 150 mA (constant) for 2 h. Afterwards, membranes were removed from blotting apparatus and washed for 2 min in H_20. To check for equal protein loading, membranes were stained in Ponceau S solution for 5 min before washed for 5 min in H_2O. Stained membranes were scanned and thereafter destained in PBS-T (PBS+0.1% Tween20) for 10 min, before they were blocked 2 h at RT or overnight at 4°C slowly shaking on a rotary shaker in 5% non-fat dry milk (Roth) diluted in PBS-T. After blocking, blocking solution was removed and membranes washed 3 times 5 min in PBS-T. Primary antibodies treatment was carried out 2 h at RT or overnight at 4°C slowly shaking on a rotary shaker in 5% non-fat dry milk. After primary antibody treatment, membranes were washed 3 times 5 min in PBS-T, before a secondary antibody in 5% non-fat dry milk was added to the membranes and incubated 1-2 h at RT slowly shaking on a rotary shaker. After washing the membranes 3 times with PBS-T, HRP-conjugated secondary antibodies were detected by chemiluminescence, using Pierce Western substrate purchased from Thermo Fisher Scientific (Rockford, USA) according to the manufacturer instructions. Luminescence was detected by exposing the membrane to photographic film.

4.2.4. Hormone and secondary metabolite measurements

4.2.4.1. Hormone measurements

Four week old plants were used for JA and free SA measurements. 200 mg plant leaf material of four individual plants was collected (n=4), frozen in liquid nitrogen and stored at -80°C until use. Frozen tissue was transferred to FastPrep tubes (Qbiogene) containing 900 mg FastPrep lysing matrix and 1 ml ethyl acetate spiked with 200 ng of D4-SA and D2-JA as internal standards. Samples were homogenized by reciprocal shaking (FastPrep speed 6.5) twice for 45 seconds each and centrifuged at 13.000 rpm for 20 minutes at 4°C. The supernatant was transferred to fresh 2 ml Eppendorf tubes and the extraction was once repeated by adding 0.5 ml ethyl acetate without internal standard to the same tissue, followed

by a centrifugation step at 13.000 rpm for 20 minutes at 4°C. Both supernatants were combined and evaporated using a vacuum concentrator until dryness at 30°C. The dried samples were dissolved in 500 µl 70% methanol, mixed for at least 5 minutes and subsequently centrifuged at 13.000 rpm for 10 minutes. 400 µl of the supernatant were transferred into fresh HPLC vials. SA and JA measurements were conducted on a liquid chromatography tandem mass spectrometry system (Varian 1200).

4.2.4.2. Secondary metabolite measurements by HPLC

200 mg of plant leaf tissue were collected and frozen in liquid nitrogen until use. 10 µl of DMSO per 4 mg fresh weight material and 10-30 'Zirconia Beads' (BioSpec) were added to the samples and homogenized 30 seconds in 'BeadBeater' (BioSpec). Homogenized samples were centrifuged at 15.000 rpm at 4°C for 20 minutes and supernatants were transferred to HPLC well plates and used for HPLC analysis on an Agilent 1100 HPLC system equipped with DAD and FLD detectors. The concentration of the metabolites of interest were quantified on the basis of the comparison of their peak areas with those obtained during HPLC analyses of known amounts of the respective compound.

4.2.4.3. Root growth assay

Seeds were sterilized by two washing steps with 70 and 100% ethanol. Sterile seeds were put on MS Phytagel (Sigma) agar plates containing 0-12.5 µm MeJA and 0.5% sucrose. Plates were stored for one day at 4°C before transfer to closed growing cabinets (Percival chambers) under long-day conditions (14 hours light/10 hours darkness). Primary root length was determined after 10-12 days. MeJA-insensitive *jar1* was included as a positive control (Staswick et al., 1992).

4.2.5. Yeast 2-hybrid screening

Yeast 2-hybrid screening was performed using the Clontech (Mountain View, USA) yeast strains AH109 and Y187 and a cDNA library in pGADT7-vectors were kindly provided by Birgit Kemmerling. WRKY18 and WRKY40 cDNA was cloned into pGBKT7 vectors as baits and transformed into yeast strain AH109 using LiAc-transformation. For screening against the cDNA-library, 50 ml liquid culture of each yeast bait was inoculated in SD-Trp (containing 4% glucose) and incubated overnight at 30°C shaking at 200 rpm. The next day, 1 ml cDNA library was thawed in a water-bath at 42°C and incubated in YPDA liquid medium adjusted to 1 OD_{600}/ml at 30°C at 200 rpm shaking for 1-2 h. 10 OD_{600} bait and 10 OD_{600}

library were mixed in a 50 ml reaction tube and centrifuged for 5 min at 4000 rpm at RT. Supernatant was discarded and pellet was resuspended in 10 ml YPDA (containing 10% PEG6000), transferred to 100 ml flasks and incubated overnight at 30°C shaking at 80 rpm. The next day, the bait-library mix was centrifuged for 5 min at 4000 rpm at RT and supernatant was discarded. The pellet was resuspended in 15 ml SD-Trp-Leu-His containing 0.05% Gelrite (Gelzan, Sigma) and added to 500 ml SD-Trp-Leu-His containing 0.05% Gelrite and mixed by slowly shaking the flask. For titer calculation, 10 µl were removed and added to 20 ml SD-Trp-Leu containing 0.05% Gelrite in a Petri-dish and incubated for 2 days at 30°C. Subsequently, yeast colonies were counted. The rest of the 500 ml SD-Trp-Leu-His Gelrite medium containing bait and prey were divided into Petri-dishes and incubated 7 days at 30°C.10 µl of each grown colony was removed and transferred into 100 µl SD-Trp-Leu-His liquid medium in a microtiter-plate. Colonies were plated on SD-Trp-Leu-His agar plates with and without 3AT (5 mM). Plates were incubated for 2 days at 30°C. Colonies grown on 3AT-containing plates were used for PCR analysis and subsequent sequencing of candidate cDNA following the Clontech Matchmaker™ GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech, Mountain View, USA).

4.2.5.1. LiAc yeast (Co-) transformation

Yeast transformation was done with yeast strain Y187 or AH109. For this, 50 ml liquid YPDA medium was inoculated with a fresh yeast colony and incubated overnight at 30°C shaking at 200 rpm. The next day, yeast culture was diluted with pre-warmed YPDA to OD_{600} 0.25 in 50 ml liquid YPDA medium and incubated for 3 more hours at 30°C at 200 rpm. Yeast culture was centrifuged 1 min at 2500 rpm at RT and pellet was resuspended in 1 ml 100 mM LiAc (in 1xTE buffer) and transferred to a fresh 1.5 ml reaction tube to make yeast cells competent for transformation. After centrifugation for 30 sec at 16.000 rpm, pellet was resuspended in 250 µl 100 mM LiAc in 1xTE buffer.

DNA for (co-)transformation was prepared by mixing 100 ng of (each) plasmid DNA with 25 µg herring sperm carrier DNA (Clontech) in a maximum of 10 µl and was added to 50 µl of freshly prepared competent yeast cells. 300 µl of yeast transformation buffer were added and mixed rigorously. Transformation mix was incubated for 30 min at 30°C shaking at 500 rpm and incubated for 15 min in a 42°C water-bath, before it was centrifuged for 30 sec at 2500 rpm. The pellet was resuspended in 250 µl 1xTE buffer and 50 µl were plated on SD-Trp-Leu-His agar plates and incubated for 2-3 days at 30°C until colonies appear.

4.2.5.2. Yeast crude protein extraction

For protein extraction, yeast was grown in appropriate liquid SD selection media overnight at 28°C shaking at 200 rpm. 3 ml of each cell culture was centrifuged for 1 min at 13.000 rpm and supernatant was discarded. The pellet was frozen in liquid nitrogen and subsequently incubated 95°C for 5 min. This 'freezing/heating'- procedure was repeated 4 times. 200 µl of 2x protein loading buffer was added to each sample, mixed and incubated again for 5 min at 95°C. After mixing rigorously, samples were centrifuged 5 min at 13.000 rpm and used for SDS-PAGE.

4.2.5.3. Fluorescence recovery after photobleaching (FRAP)

Two days after co-transformation of *A. tumefaciens*, expressing the constructs of interest, into *N. benthamiana* leaves, freshly cut pieces of leaves were employed for FRAP analyses. FRAP experiments were performed by Dr. E. Schmelzer at the CeMic unit at the MPIPZ in Cologne.

4.2.5.4. Confocal microscopy

Freshly cut pieces of leaves of *N. benthamiana* transiently expressing YFP and GFP fusion proteins were imaged by a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss MicroImaging, Jena, Germany). The CFP and YFP fluorescence was excited with the Argon laser lines at 458 and 514 nm, respectively. The emission of CFP was detected between 465 and 520 nm and of YFP between 530 and 600 nm. Images were taken and processed using the Zeiss LSM software (version 3.2) and the Zeiss image examiner software (version 3.1).

5. Abbreviations

%	percent
°C	degree Celsius
3’	downstream region (of a gene or sequence)
4MI3G	4-Methoxy-indol-3-ylmethylglucosinolate
5’	upstream region (of a gene or sequence)
A	Ampere
ABA	abscisic acid
Avr	avirulence
bp	basepair(s)
Bgh	*Blumeria graminis forma specialis hordei*
C	carboxy-terminal
CC	coiled-coil
cDNA	copied DNA
CFP	cyan fluorescent protein
ChIP	chromatin immunoprecipitation
COI1	CORONATINE INSENSITIVE 1
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
CYP81F2	CYTOCHROME P450, FAMILY 81
dpi	days post induction
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	deoxynucleosidetriphosphate
dSpm	defective Suppressor-mutator
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDS1	ENHANCED DISEASE SUSCEPTIBILITY 1
EDTA	ethylenediaminetetraacetic acid
EIL1	ETHYLENE-INSENSITIVE3-LIKE 1
EIN3	ETHYLENE-INSENSITIVE3
ET	ethylene
ETI	effector-triggered immunity
EtOH	ethanol
Fig.	figure
FL	full length
flg	flagellin
FLS2	FLAGELLIN SENSING RECEPTOR 2
g	gram
g	gravity constant (9.81 ms-1)
G. cichoracerarum	*Golovinomyces cichoracearum*
G. cruciferarum	*Golovinomyces cruciferarum*
GFP	green fluorescent protein
G. orontii	*Golovinomyces orontii*
h	hours
Hpa	*Hyaloperonsopora arabidopsidis*
hpi	hours post induction
HPLC	high performance liquid chromatography
HR	hypersensitive response
HRP	horseradish peroxidase
Hv	*Hordeum vulgare*
ICS1	ISOCHORISMATE SYNTHASE 1

Ile	isoleucine
JA/JAs	jasmonic acid/jasmonates
JA-Ile	JA-isoleucine
JAR1	JASMONATE RESISTANT 1
JAZ1-12	JASMONATE-ZIM-DOMAIN PROTEIN 1-12
kb	kilobase(s)
kDa	kilo Dalton
l	liter
LOX2	LIPOXYGENASE 2
LRR	leucine rich repeats
m	milli
M	molar (mol/l)
µ	micro
MAPK	mitogen activated protein kinase
MeJA	methyl-jasmonate
MeOH	methanol
min	minutes
MLA	Mildew Locus A
MLO	MILDEW RESISTANCE LOCUS O
mRNA	messenger ribonucleic acid
MW	molecular weight
n	nano
N	amino-terminal
NaCl	sodium chloride
NPR1	NONEXPRESSER OF PR GENES 1
OD	optical density
OEC	*Orontii* effector candidate
OPDA	12-oxo-phytodienoic acid
OPR3	OXOPHYTODIENOATE-REDUCTASE 3
ox	overexpressor
p35S	35S promoter of CaMV
PAD3	PHYTOALEXIN DEFICIENT 3
PAD4	PHYTOALEXIN DEFICIENT 4
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDF1.2	PLANT DEFENSIN 1.2
PEG	polyethylene glycol
PEN1-3	PENETRATION 1-3
pH	negative decimal logarithm of H+ concentration
PMR4	POWDERY MILDEW RESISTANCE 4
PR1	PATHOGENESIS-RELATED 1
PRR	pattern recognition receptor
Pto	*Pseudomonas syringae* pv. *tomato*
pv.	pathovar
P value	probability value
qRT-PCR	quantitative real-time PCR
R	resistance
RLK	receptor-like kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RPM1	RESISTANCE TO P. SYRINGAE PV

	MACULICOLA 1
RPS4	RESISTANT TO P. SYRINGAE 4
RPS6	RESISTANT TO P. SYRINGAE 6
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SA	salicylic acid
SAG101	SENESCENCE-ASSOCIATED GENE 101
SB	sample buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
sec	seconds
SID2	SALICYLIC ACID INDUCTION DEFICIENT 2
Taq	*Thermophilus aquaticus*
T-DNA	transfer DNA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TF	transcription factor
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TRIS	tris-(hydroxymethyl)-aminomethan
Trp	tryptophan
U	unit
UV	ultraviolet
V	volt
vir	virulence
VSP2	VEGETATIVE STORAGE PROTEIN 2
WRKY18	WRKY transcription factor 18
WRKY40	WRKY transcription factor 40
wt	wildtype
YFP	yellow fluorescent protein

6. Literature

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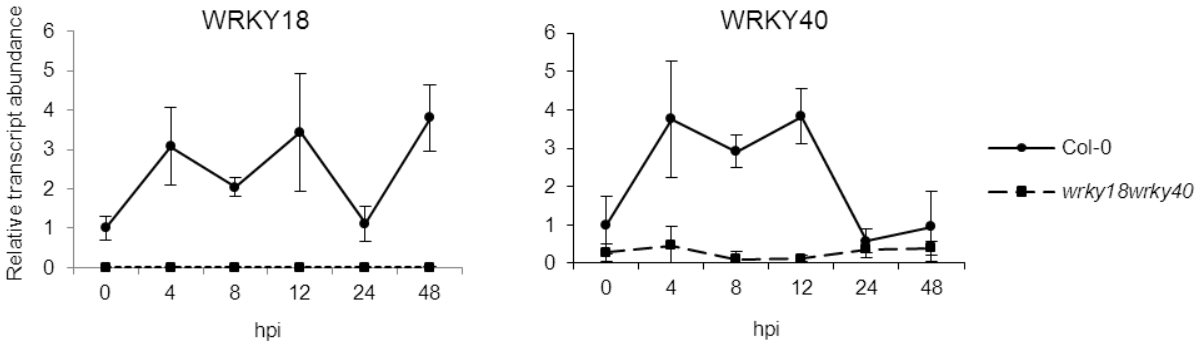
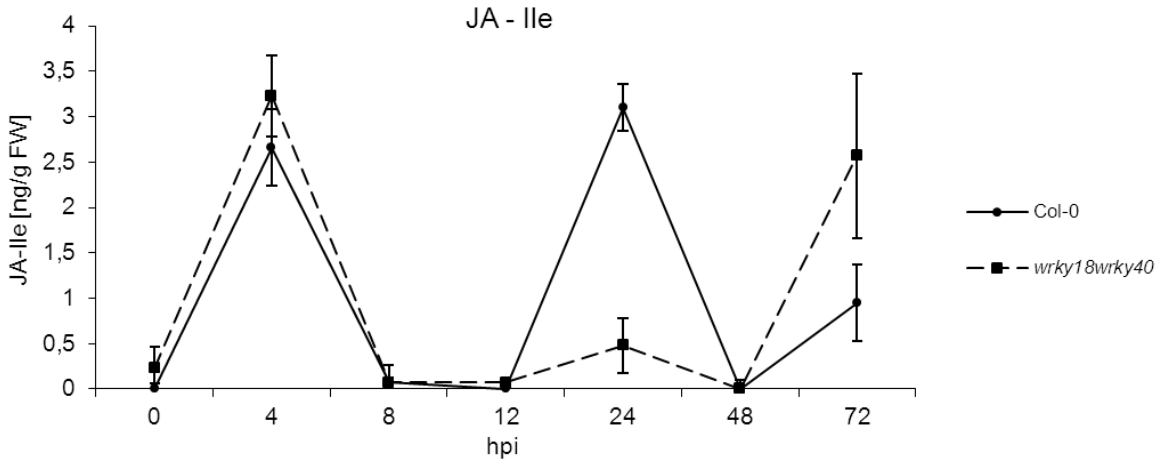
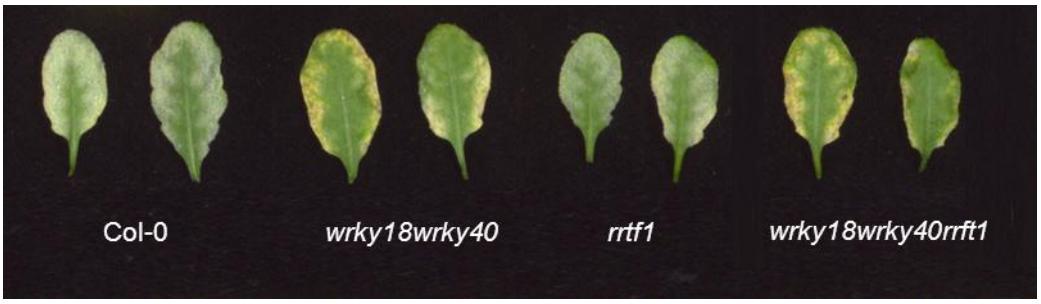
7. Supplementary Material



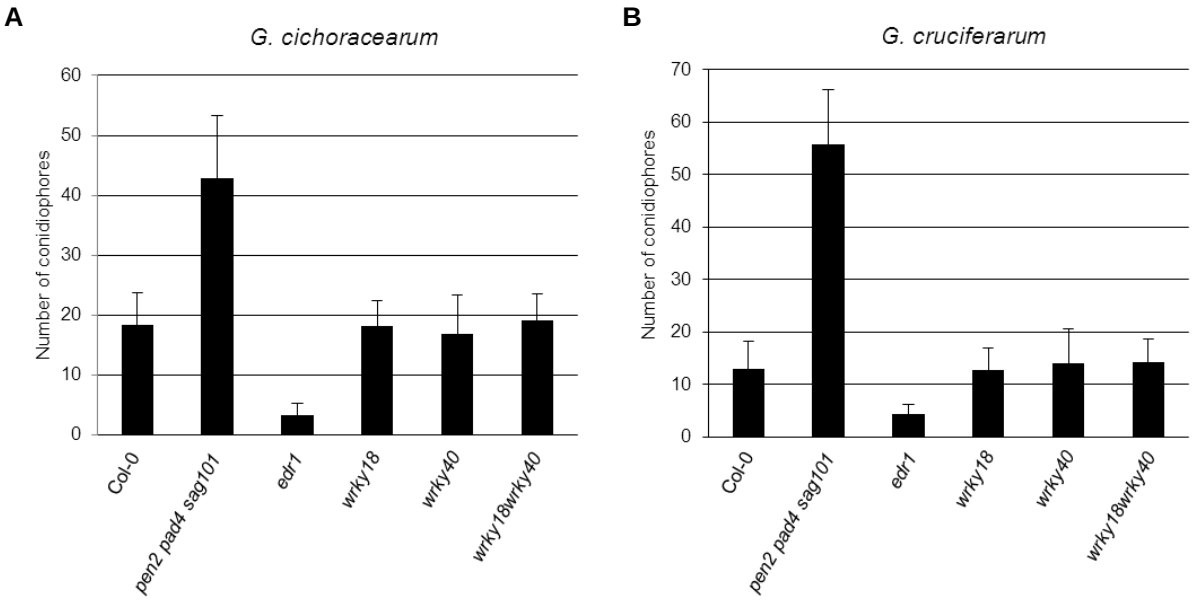
Supplementary Figure 1: Expression of *WRKY18* and *WRKY40* during early *G. orontii* infection. QPCR analysis of *WRKY18* and *WRKY40* transcript abundance during the first 48 h after *G. orontii* infection. Total leaf material of susceptible Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants was harvested at indicated time points. Four weeks old plants were used for the analysis and infection with *G. orontii* was carried out by spreading spores with a brush evenly over the plant. Infections were performed without touching the plant to avoid wound responses. Transcript abundance is relative to wildtype 0 hpi samples set to 1. Error-bars represent SD (n=3).



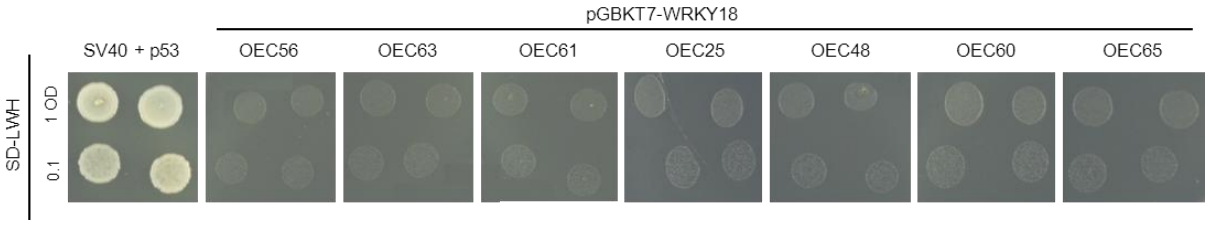
Supplementary Figure 2: JA-Ile accumulation during early *G. orontii* infection. JA-Ile levels in susceptible wildtype Col-0 (solid line) and resistant *wrky18wrky40* (dashed line) plants upon infection with *G. orontii*. Samples of 4 weeks old plants were collected during the first 72 hpi at indicated time points. Error-bars represent SD (n=4).



Supplementary Figure 3: Phenotypic characterization of *wrky18wrky40rrtf1* triple mutants upon *G. orontii* infection. Four weeks old plants were infected with powdery mildew *G. orontii* and pictures were taken 10 dpi. As controls, susceptible Col-0 and resistant *wrky18wrky40* plants were included.



Supplementary Figure 4: Conidiophore counts after *G. cichoracearum* and *G. cruciferarum* infection. Indicated genotypes were evenly infected without touching the plants with **(A)** *G. cichoracearum* and **(B)** *G. cruciferarum* and the number of condidiophores was determined as a measure of susceptibility or resistance towards these obligate biotrophic fungi. Four weeks old plants were used for the analysis and conidiophore numbers were determined 7 dpi. *Edr1* and *pen2 pad4 sag101* mutants served as resistant and hypersusceptible controls. Experiments were performed by C. Roth, University of Göttingen. Error-bars represent SD (n=3). The experiment was repeated twice with similar results.

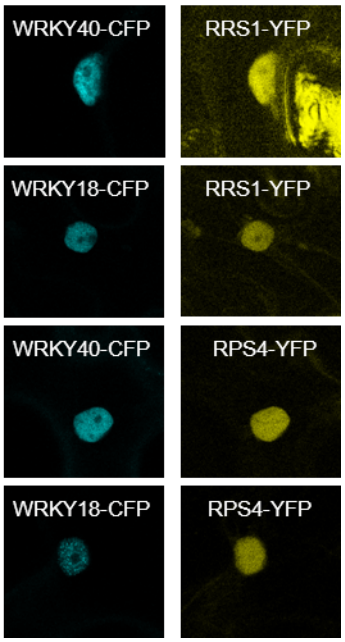


Supplementary Figure 5: Interaction studies of WRKY18 and *G. orontii* effector candidates (OECs). WRKY18 and selected *G. orontii* OECs were co-transformed into yeast and tested for interaction on triple selection media. Human SV40 T-antigen and murine p53 proteins were included as positive controls. Pictures were taken 3 days after transformation and incubation at 30°C. Similar results were obtained for co-transformation with WRKY40.

A

Bait	Prey	Growth on SD-LWH
SV40	p53	+++
RPS4	WRKY18	no
RPS4	WRKY40	no
WRKY18	RRS1	no
WRKY40	RRS1	no
WRKY18	RPS4 TIR	+
WRKY40	RPS4 TIR	no
RPS4 TIR	RRS1 TIR	++
Lam	RPS4 TIR	
Lam	RRS1 TIR	+
Lam	RRS1 FL	no
RPS4 FL	SPL3	no

B



Supplementary Figure 6: Interaction and localization studies of WRKY18 and WRKY40 with RPS4 and RRS1. (A) Interaction was tested by yeast co-transformation of either WRKY18 or WRKY40 with RPS4 (1218 aa), RRS1 (1288 aa) full-length or truncated RPS4 (183 aa) or RRS1 (155 aa) cDNA clones containing only the TIR domain. Yeast growth on triple selection media was determined 3 days after transformation and rated relative to positive control of human SV40 T-antigen and murine p53 proteins known to interact strongly in yeast. **(B)** Co-localization of WRKY18 and WRKY40 with RPS4 and RRS1 in the plant cell nucleus. Either CFP-tagged WRKY18 or WRKY40 was co-infiltrated with YFP-tagged RPS4 or RRS1 into *N.benthamiana* epidermal cells for Acceptor Photo Bleaching analyses (APB). Apart from nucelar co-localization, no direct interaction could be detected. Pictures were taken 2 days after co-infiltration.

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Wollte ich nur mal gesagt haben!
Fertig!

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegt worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.
Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Dr. Imre Somssich und Prof. Dr. Paul Schulze-Lefert betreut worden.

Ich versichere, dass ich alle Angaben wahrheitsgemäß nach bestem Wissen und Gewissen gemacht habe und verpflichte mich, jedmögliche, die obigen Angaben betreffenden Veränderungen, dem Dekanat unverzüglich mitzuteilen.

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Köln, 23. April 2012 _____

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