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LARGE-SCALE BIOLOGY ARTICLE

Induced Genome-Wide Binding of Three Arabidopsis WRKY Transcription Factors during Early MAMP-Triggered Immunity

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Short title: WRKY Factors in MAMP-triggered Immunity

One-sentence summary: Genome-wide analysis reveals the in vivo binding sites of Arabidopsis WRKY18, WRKY33 and WRKY40 transcription factors during early MTI and the consequences of WRKY18 and WRKY40 binding on transcriptional output.

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ABSTRACT

During microbial-associated molecular pattern (MAMP)-triggered immunity (MTI) molecules derived from microbes are perceived by cell surface receptors and upon signaling to the nucleus initiate a massive transcriptional reprogramming critical to mount an appropriate host defense response. WRKY transcription factors play an important role in regulating these transcriptional processes. Here, we determined on a genome-wide scale the flg22-induced *in vivo* DNA-binding dynamics of three of the most prominent WRKY factors, WRKY18, WRKY40 and WRKY33. The three WRKY factors each bound to more than 1000 gene loci predominantly at W-box elements, the known WRKY binding motif. Binding occurred mainly in the 500 bp promoter regions of these genes. Many of the targeted genes are involved in signal perception and transduction not only during MTI but also upon damage-associated molecular pattern (DAMP)-triggered immunity (DTI), providing a mechanistic link between these functionally interconnected basal defense pathways. Among the additional targets were genes involved in the production of indolic secondary metabolites and in modulating distinct plant hormone pathways. Importantly, among the targeted genes were numerous transcription factors, encoding predominantly ethylene response factors, active during early MTI, and WRKY factors, supporting the previously hypothesized existence of a WRKY sub-regulatory network. Transcriptional analysis revealed that WRKY18 and WRKY40 function redundantly as negative regulators of flg22-induced genes often to prevent exaggerated defense responses.

Introduction

Plants are constantly exposed to a wide range of pathogens in their environment but owing to their intricate and efficient basal defense system can often ward off such threats. Key to this successful defense is the ability of plants to recognize various conserved microbial structures, termed microbe-associated molecular patterns (MAMPs), through dedicated plasma membrane-localized pattern recognition receptors (PRRs), and to rapidly initiate intracellular signaling leading to MAMP-triggered immunity (MTI) (Monaghan and Zipfel, 2012; Schwessinger and Ronald, 2012; Newman et al., 2013; Vidhyasekaran, 2014; Li et al., 2016). FLS2 is currently the most intensively studied Arabidopsis PRR and is activated upon binding of bacterial flagellin or flg22, which is a conserved epitope present in the flagellin N-terminus (Zipfel et al., 2004; Sun et al., 2013; Kadota et al., 2014). Upon flg22 perception, several immediate host responses can be observed including the influx of H^+ and Ca^{2+}, the generation of reactive oxygen species (ROS), and the activation of calcium-dependent protein kinases and MAP kinase cascades (Vidhyasekaran, 2014). Subsequently, as with other ligand-PRR interactions, binding of flg22 to FLS2 results in rapid and massive transcriptional reprogramming within the host cell (Zipfel et al., 2004; Zipfel et al., 2006; Wan et al., 2008).

Transcriptional profiling has revealed that the expression of thousands of host genes is significantly altered during MTI. Wildtype Arabidopsis seedlings treated for 30-60 min with flg22 showed altered expression of more than 1000 genes and a rapid induction of gene sets classified to be involved in signal perception, signal transduction and transcriptional regulation (Navarro et al., 2004; Zipfel et al., 2004). Prominent among the transcription factor (TF) genes that were induced by flg22 early on during MTI are members of the WRKY TF family. Fifteen WRKY TF genes were already strongly (> 4-fold) induced 30 min post flg22 treatment in Arabidopsis seedlings including *WRKY18* (>10-fold), *WRKY33* (>15-fold), and *WRKY40* (>20-fold) (Zipfel et al., 2004). These latter three WRKYs were also identified to be important functional HUBs within a proposed WRKY regulatory network (Choura et al., 2015). The potential importance of WRKY factors in modulating early MTI responses was further supported by the analysis of promoter sequences of flg22-induced genes, which revealed an over-representation of the W-box *cis*-acting DNA element, the consensus binding site of WRKY TFs (Navarro et al., 2004). Similarly, the W-box was over-represented within promoters of the large group of early flg22-induced receptor-like kinase (RLK) genes (Zipfel et al., 2004).

WRKY factors have been demonstrated to fulfill essential regulatory functions to modulate pathogen-triggered cellular responses in numerous plant species (Rushton et al., 2010; Tsuda and Somssich, 2015). For instance, Arabidopsis WRKY33 is a key positive regulator of resistance against the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Zheng et al., 2006; Birkenbihl et al., 2012). WRKY33 indirectly interacts with MAP kinase 4 via the VQ motif-containing protein MKS1. Upon flg22 treatment WRKY33 is released from this complex and subsequently is associated with the promoter of the camalexin biosynthetic gene *PAD3* (Qiu et al., 2008). Arabidopsis WRKY18 and WRKY40 act redundantly in negatively regulating resistance towards the obligate hemi-biotrophic fungus *Golovinomyces orontii* (Pandey et al., 2010). Genetic studies on these two TFs have also clearly demonstrated that they have dual functions namely acting as negative regulators of MTI, and as positive regulators of effector-triggered immunity mediated by the major resistance gene *RPS4,* and in resistance towards the herbivore *Spodoptera littoralis* (Xu et al., 2006; Lozano-Durán et al., 2013; Schön et al., 2013; Schweizer et al., 2013).

Recently, we succeeded in using ChIP-seq to idenitfy genome-wide binding sites of WRKY33 following infection of mature Arabidopsis leaves with the fungus *B. cinerea* 2100 (Liu et al., 2015). In this study, we aimed to define the genome-wide binding patterns of WRKY 18, WRKY40, and WRKY33 during early MTI in Arabidopsis seedlings treated with the potent MAMP flg22, and to determine the consequence of the observed TF binding on the transcriptional output of the identified target genes.

RESULTS AND DISCUSSION

The function of transcription factors is mainly determined by the genomic sites they bind to and by the genes they regulate. Therefore, to investigate the function of WRKY18, WRKY40 and WRKY33 in early MTI, we determined genome-wide binding sites for those TFs upon flg22 treatment. For ChIP-seq we used transgenic complementation lines of WRKY18 (WRKY18-HA), WRKY40 (WRKY40-HA) and WRKY33 (WRKY33-HA), which expressed the HA-epitope tagged WRKY proteins under control of their native promoters in the respective knockout mutants (see Materials and Methods). In this way, we could avoid the problem that no appropriate antibodies exist against these WRKY factors, and were able to use the same anti-

HA antibody to determine the respective protein levels and to isolate proteo-DNA complexes containing the three WRKY proteins. Moreover, it was possible to use wildtype (WT) plants as negative controls, which are genotypically similar to the complementation lines and express the investigated *WRKY* genes without the HA-tag. To induce MTI and achieve a highly synchronous response to the elicitor, which is a precondition for the sensitive and clear detection of binding sites, we grew seedlings in liquid medium and treated them with the bacterial flagellin-derived peptide flg22 (Felix et al., 1999).

To investigate *WRKY18*, *WRKY40* and *WRKY33* expression and inducibility at the RNA level, WT seedlings were treated with 1 µM flg22 and samples taken for qRT-PCR analysis, either untreated or at 1 h, 2 h and 4 h post treatment. All three *WRKY* genes were induced after 1 h flg22 treatment (Figure 1A). While *WRKY18* was constitutively expressed with a moderate flg22-dependent increase at 1 h post treatment, *WRKY40* and *WRKY33* were strongly induced at 1 h with elevated RNA levels observed up to the 4 h time point.

Flg22-induced HA-tagged WRKY protein accumulation in the complementation lines followed the RNA expression patterns with a short delay (Figure 1B). For WRKY18, significant amounts of protein were visible in the non-induced state, the peak of protein abundance was at 1.5 h, and afterwards the protein level stayed high up to the 6 h time point. A second lower molecular weight protein band was consistently detected at all time points and was very likely a consequence of constant protein degradation in the plants. For WRKY40 and WRKY33, only very low protein levels were detected without elicitation. Upon flg22 treatment, their protein abundance strongly increased with peaks at 3 h and 2 h post elicitation, respectively. Based on the kinetics of protein accumulation, the 2 h time point after flg22 treatment was chosen to determine early MTI-induced WRKY binding sites by ChIP-seq.

Analysis of WRKY18, WRKY40 and WRKY33 binding sites

In the course of this study we established comprehensive lists of genome-wide binding sites for the three WRKY factors, WRKY18, WRKY40 and WRKY33, which provide a valuable source of information about general genome-wide in vivo W-box occupancy by WRKY factors, the inducibility of binding elicited by flg22, and genome-wide experimental evidence for the hypothesized WRKY regulatory network (Eulgem, 2006; Eulgem and Somssich, 2007; Chi et

al., 2013; Choura et al., 2015). For our ChIP-seq experiments we analyzed material from the three WRKY complementation lines before (0 h) and 2 h after flg22 treatment using the corresponding non-HA-antigen-containing WT plants as negative control.

DNA-binding sites in each WRKY-HA line were determined separately for two biological replicates by comparing their observed sequencing read distribution with that of the similarly treated WT sample. Binding sites were scored as reproducible if the corresponding peak region overlapped with a peak region in the other replicate by at least 50 % of the length of the smaller peak region. Furthermore, a binding site in the pooled samples of both replicates was counted as consistent if its peak region overlapped with a reproducible peak region in both of the original replicates by at least 50 % of the length of the smaller region.

After 2 h of flg22 treatment, 1403 consistent binding sites were detected for WRKY18, 1622 for WRKY40, and 1208 binding sites for WRKY33 (Supplemental Data Set 1). Since many gene loci contained more than one binding site, the number of predicted target genes was lower, namely 1290 for WRKY18, 1478 for WRKY40 and 1140 for WRKY33 (Table 1). For WRKY40 and WRKY33, the observed binding was almost exclusively dependent on flg22 treatment, while for WRKY18 binding to only 380 genes was defined as solely flg22-dependent. This was probably due to the detected constitutive protein levels in the WRKY18 complementation line, which appeared to also reflect the true situation, since elevated *WRKY18* RNA levels were also observed prior to induction by flg22 in WT plants under our tested conditions (Figure 1A). Nevertheless, manual inspection of binding sites using the Integrative Genomics Viewer (IGV; Thorvaldsdóttir et al., 2013) revealed that most of the WRKY18 binding peaks visible at 0 h were significantly higher upon flg22 treatment. We found that half of the 209 consistent WRKY18 binding sites defined for 0 h had a more than 1.5 times higher ChIP score or enrichment (ef) score at 2 h (Supplemental Data Set 2). Taking this fact into account, these sites can also be considered to display flg22-dependent enrichment of WRKY18 binding, which is in agreement with the higher WRKY18 protein and RNA levels detected upon flg22 stimulation.

In certain cases, our automated analysis pipeline failed to detect important target genes, for example due to the applied stringent peak calling, or mis-assignments during the peak annotation step, or for neighboring genes on opposite strands that shared common overlapping promoter regions. To address these issues, we manually inspected the loci of some prominent

defense genes not detected by the initial analysis in the IGV for obvious binding peaks. Genes that could be verified as binding targets in this way were added to the corresponding WRKY target lists. Using this strategy, we additionally classified the cysteine-rich receptor-like protein kinase gene *CRK2*, *ISOCHORISMATE SYNTHASE1* (*ICS1*), the glycosyl hydrolase gene *PEN2* and the cytochrome genes *CYP83B1* and *CYP71A13* as targets of WRKY40 (Supplemental Figure 1A-E). Moreover, we also rated the leucine-rich repeat serine/threonine protein kinase gene *FLS2*, which is the receptor for flg22, as a likely WRKY target gene, although the automated peak annotation assigned the corresponding binding region to a nearby tRNA gene (Supplemental Figure 1F).

As anticipated for transcription factors, more than 97 % of the the binding sites for each of the studied WRKY TFs were located in non-coding DNA regions (Figure 2A), with a clear enrichment in the 1000-bp promoter regions (55 %). About 5 % of the peaks were located in introns, suggesting that some of these introns might also have regulatory capacity. For all three WRKY factors, the binding region peaks were located preferentially within the 400-bp region upstream of the transcription start site (TSS), with the highest frequency of peaks at around position -200 bp (Figure 2B).

The W-box is the predominant WRKY factor binding motif

WRKY factors are known to bind to the non-palindromic consensus nucleotide sequence TTGACT/C, termed the W-box. Within the 120 MB Arabidopsis Col0 genome (Tair10), 136,087 W-boxes can be identified, with equal numbers on the plus (68,029) and the minus (68,058) DNA strand, which averages roughly to one W-box per 880 bp. The W-box motifs are almost equally distributed among the regions classified as transcription start sites (TSS), transcription termination sites (TTS), exons (CDS), 5'UTRs, 3'UTRs, introns and intergenic regions, again with no preference for the plus or minus strand (Supplemental Figure 2). To detect new or previously known binding motifs in the identified WRKY binding regions, we used the DREME/MEME software suite (Bailey, 2011; Machanick and Bailey, 2011) to perform stringent motif searches within a 500-bp region encompassing the peak summit of each binding region. The corresponding DREME searches for short motifs identified the W-box as the most frequently occurring motif for all three WRKY factors, with one or more W-boxes found in 67 %,

72 % and 75 % of the analyzed peak summit regions for WRKY18, WRKY40, and WRKY33, respectively (Figure 3A-C). This indicated that the W-box was the likely binding motif in most of the cases. For all three WRKY factors, the identified W-box motifs were preferentially located close to the binding peak summits, however the probability curves did not display a single sharp central peak, as one would expect for a single binding site per binding region (Figure 3A-C). Consistent with this observation, the number of W-boxes per binding region (which can be larger than the 500 base pairs surrounding the peak summits analyzed in Figure 3A-C) was often higher than one for all tested WRKY factors (Figure 3D), with the number of observed W-boxes being independent of the sizes of the binding regions (Supplemental Figure 3). About 90 % of the binding regions contained at least one W-box, and more than 60 % contained two or more. Considering the average size of the binding regions of 730 bp, this clearly indicates an overrepresentation of W-boxes compared to the statistically expected 0.8 W-boxes per region. One prominent example for multiple WRKY factor binding sites within one promoter is the *FLS2* locus, where ten W-boxes within a one kb region with multiple binding peaks for all three WRKY factors were detected (Supplemental Figure 1F). For the identified binding regions a two-fold preference for the W-box sequence TTGACT over the TTGACC motif was observed, mirroring their genomic abundances.

Despite the predominance of the W-box motif, binding regions without W-boxes were also observed for all three WRKY factors. This may be indicative of additional W-box sequence-unrelated WRKY factor binding sites or alternatively sites of indirect WRKY binding via other DNA-associated proteins with different DNA binding specificities. When we performed comparable motif searches with the identified binding regions lacking W-boxes, no obvious consensus motifs could be detected.

WRKY18 and WRKY40 can bind independently from each other

WRKY18 and WRKY40 were shown previously to form both homo-dimers and hetero-dimers that are capable of binding DNA, and to be redundant in function (Xu et al., 2006; Pandey et al., 2010; Schön et al., 2013). When searching for overlaps between the identified target gene sets of the three WRKY factors, an almost complete overlap of target genes was detected between WRKY18 and WRKY40 after flg22 treatment (87 %), while significantly fewer common

targets were observed between WRKY33 and WRKY18 (53 %) or WRKY40 (57 %) (Figure 4, see Suppl. Data Set 3 for sector-specific gene lists).

This could suggest that upon flg22 treatment WRKY18 and WRKY40 might have bound as hetero-dimers to the majority of their target sites, while WRKY33 binding occurred more independently. However, when WRKY18-HA or WRKY40-HA were individually expressed in a *wrky18 wrky40* double mutant and ChIP-qPCR was performed on such samples, both factors were still able to bind to tested prominent target loci such as *FLS2*, *BZIP60* and *ERF-1* (Supplemental Figure 4). This observation indicates the ability of WRKY18 and WRKY40 to bind as either homo-dimers or monomers, which could also be the binding modes of WRKY18 in the non-induced state. The observed large overlap of WRKY factor target genes could be the result of simultaneous binding to closely spaced W-boxes within the same binding region. It is also possible that seemingly overlapping binding was the result of using whole seedlings for ChIP analyses, as such samples cannot resolve potential selective binding by different WRKY factors within specific tissues (e.g. roots and shoots) or cell types.

Binding of WRKY18, WRKY40 and WRKY33 to promoters of genes implicated in MAMP-triggered immunity

To investigate the relevance of the identified WRKY target genes for plant defense responses we performed a gene ontology (GO) term enrichment analysis using the GO tool on the TAIR webpage. We found that, compared to the corresponding prevalence in the complete genome, the target gene sets for WRKY18, WRKY40 and WRKY33 were most enriched for genes associated to the cellular component GO term *plasma membrane*, where perception of MAMPs and recognition of pathogens takes place. With respect to molecular functions, genes associated to the GO terms *transferase activity*, *kinase activity*, and *receptor binding or activity* were significantly overrepresented, and these functions are strongly linked to signal transduction to the nucleus upon recognition of pathogens or MAMPs. In agreement with this, the strongest enrichment among the WRKY target genes affected in biological processes was observed for genes connected to the GO terms *response to stress*, *response to abiotic or biotic stimulus* and *signal transduction* (Supplemental Figure 5). This result indicates that upon

248 elicitation all three WRKY factors showed a clear preference for binding gene loci involved in
249 the early processes of MTI perception and signaling.

250

251 **Receptor-like kinase (RLKs) genes as targets**

252 Up to 75 receptor (-like) and 64 receptor (-like) kinase genes were identified as targets of
253 WRKY18, WRKY40 and WRKY33 (Supplemental Data Set 4). Among these were genes
254 encoding the prominent plasma membrane-bound MAMP receptors for flagellin (FLS2), chitin
255 (CERK1 and LYM2), and lectin (CES101), as well as genes for the flg22-induced receptor
256 kinase FRK1, the abscisic acid receptor PYL4, the phytosulfokin receptor 1, and four glutamate
257 receptors (GLRs). Further identified WRKY targets were the gene encoding the receptor-
258 associated kinase BIK1 that phosphorylates the ROS-producing NADPH oxidase RBOHD
259 (Macho and Zipfel, 2014) and the *RBOHD* gene itself, as well as genes encoding BIR1, which
260 interacts with BAK1 to negatively regulate different plant resistance pathways (Gao et al.,
261 2009), the Gα protein XLG2, which regulates immunity by direct interaction with FLS2 and
262 BIK1 (Liang et al., 2016), AGB1, a heterotrimeric Gβ protein that acts together with XLG2 and
263 AGG1/AGG2 (Gγ proteins) to attenuate proteasome-mediated degradation of BIK1, and
264 PUB12, an E3 ubiquitin ligase involved in the negative regulation of FLS2 by degradation (Lu et
265 al., 2011; Table 2).

266 Interestingly, genes encoding components of damage-associated molecular pattern (DAMP)
267 signaling, including the main receptor PEPR1, the expressed DAMP ligands PROPEP2 and
268 PROPEP3, but not the non-expressed PROPEP1 (Figure 5; Bartels and Boller, 2015), and
269 RLK7, the receptor of PIP1 peptide signaling (Hou et al., 2014), were bound by all three WRKY
270 factors upon flg22 treatment. This suggests that besides WRKY33, which positively regulates
271 expression of *PROPEP2* and *PROPEP3* (Logemann et al., 2013), also the flg22-induced
272 WRKY18 and WRKY40 factors provide a mechanistic and functional link between MTI and
273 DAMP-triggered immunity (DTI). Additionally, several genes encoding receptors of the
274 membrane-bound TIR-NBS-LRR class resistance proteins, usually active during effector-
275 triggered immunity (ETI), were identified as WRKY targets during MTI (Supplemental Data Set
276 1). Taken together, these findings are in full agreement with earlier predictions that numerous

RLK genes would be targets of WRKY factors, based on the over-representation of W-box elements within their respective promoters (Zipfel et al., 2004).

The perception of MAMPs by dedicated receptors triggers various signaling pathways that ultimately give rise to appropriate transcriptional responses within the nucleus. In many cases, such signaling is executed by kinases, transferases and redox-active and reactive proteins through posttranslational modification of proteins within signaling cascades. Protein kinase genes were identified as putative targets of all three WRKY factors, being two-fold overrepresented among the target genes compared to their abundance within the genome. Besides the receptor kinase genes, we additionally identified about 110 kinase genes as WRKY targets, most of which belong to the large families of proteins classified as *kinase-like proteins* or *concanavalin A-like lectin kinase proteins*. Furthermore, there were also several kinases from *MAP kinase cascades* and *calcium-dependent protein kinases* identified as WRKY targets, both of which are important signaling components leading to multiple defense responses (Tena et al., 2011; Supplemental Data Set 4). In the case of WRKY40 and WRKY33, all detected binding was dependent on flg22 treatment.

WRKY targets connected to hormone pathways

Numerous studies have shown that treatment with pathogens or MAMPs induces hormone signaling and causes the rapid production of plant hormones as well as up-regulation of hormone-response genes (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). In particular, the ethylene, salicylic acid and jasmonic acid pathways constitute major defense pathways that are highly interconnected, thereby enabling the plant to quickly adapt to changing biotic environments (Tsuda et al., 2009).

Ethylene (ET) pathway

The induction of the ethylene (ET) pathway is one of the earliest events during MTI (Broekgaarden et al., 2015). Two hours after flg22 treatment, we observed induced binding of WRKY18, WRKY40 and WRKY33 to the promoters of key genes in this pathway (Table 3). Among the observed WRKY targets were genes encoding the enzymes of ET biosynthesis ACS2, ACS6 and ACS8, as well as MKK9, which was reported to function upstream of

MPK3/MPK6 in the activation of ACS2 and ACS6 by phosphorylation and binding of WRKY33 (Yoo et al., 2008; Li et al., 2012). Also, the ACC oxidase gene *ACO2*, which catalyzes the final step in the biosynthesis of ET, was a target of the WRKY proteins. Other prominent target genes were *EIN2*, encoding an essential transducer of ET signaling that stabilizes the key ET-dependent gene activator EIN3 (Alonso et al., 1999), and as targets of WRKY18 and WRKY40, the negative regulators of ET signaling *EBF1* and *EBF2* (Gagne et al., 2004). In addition, 34 ET-responsive transcription factor genes (*ERFs*) were found to be targets of the three WRKY factors. Among them were positive regulators like *ERF1*, *ERF-1*, *ERF2*, *ERF5* and *ERF6*, and the negative regulator *ERF4* (Huang et al., 2016). Furthermore, the ERF gene *ORA59* encoding a major integrator of ET and jasmonic acid (JA) signaling (Pré et al., 2008), was identified as an flg22-induced target of WRKY18 and WRKY40. *ORA59* was previously shown also to be a direct target of WRKY33 upon *Botrytis cinerea* infection (Birkenbihl et al., 2012; Liu et al., 2015). Additional targets of all three WRKYs were *MYB51*, which connects ET signaling with indole glucosinolate biosynthesis, and *ABA REPRESSOR1* (*ABR1*), encoding an ERF factor that negatively regulates abscisic acid (ABA)-activated signaling pathways, thereby possibly influencing ET–ABA signal antagonism (Pandey et al., 2005; Clay et al., 2009).

The observed binding of WRKY factors to the promoters of numerous genes encoding components of distinct parts of the pathway, including ET biosynthesis, ET signaling, and transcriptional regulation of the ET response, suggests that these WRKY TFs participate in the regulation of the entire pathway at various levels.

Salicylic acid (SA) pathway

Among the identified WRKY target genes associated with the SA pathway were *ICS1* encoding a key SA biosynthetic enzyme (Garcion et al., 2008), *EDS1* encoding a major component required for host resistance towards various pathogens and an upstream regulator of SA signaling, *SAG101* coding for a key EDS1 interacting protein, *EDS5* encoding a multidrug transporter required for SA accumulation upon pathogen challenge (Serrano et al., 2013), the SA receptor genes *NPR3* and *NPR4* (Fu et al., 2012), *NIMIN1* encoding a negative regulator of NPR1 function (Weigel et al., 2005), *PBS3* encoding an amino acid conjugating GH3 family member regulating SA levels (Okrent et al., 2009), and the resistance gene *SNC1* encoding an immune receptor acting in the EDS1 pathway and requiring SA signaling (Li et al., 2001).

Furthermore, several *MYB* and *WRKY* TF genes involved in the regulation of downstream SA responses were identified as targets (Table 4).

Jasmonic acid (JA) pathway

Several genes that encode functions related to JA signaling were also identified as direct targets of the three tested WRKY factors. The two JA biosynthetic genes *LIPOXYGENASE3* (*LOX3*) and *ALLENE OXIDE CYCLASE3* (*AOC3*) were targets, as was the amidohydrolase gene *ILL6* that contributes to the turnover of active JA-isoleucine upon wounding (Widemann et al., 2013). Additional targets included *JAZ6* coding for a repressor of JA signaling (Chini et al., 2007), *BOP2* encoding a paralog of *NPR1* essential for pathogen resistance induced by methyl jasmonate (Canet et al., 2012), and the transcription factor genes *ORA59*, *WRKY50* and *WRKY51*. The WRKY50 and WRKY51 TFs negatively regulate JA responses following SA treatment or under low 18:1 fatty acid conditions (Gao et al., 2011; Table 5).

Tryptophan-derived secondary metabolite genes as targets

As was the case with the aforementioned signaling- and hormone pathway-associated target genes, the tested WRKY factors bound to the promoters of genes encoding diverse functional activities related to the indole secondary metabolites glucosinolates and camalexin, possibly to achieve a more synchronized and robust control of the entire defense system. We detected binding to genes encoding transcriptional regulators as well as biosynthesis enzymes and genes related to the transport of the metabolites. The indolic glucosinolate pathway, emanating from tryptophan, branches at indole-3-acetaldoxime (I3AOx) towards either the production of the phytoalexin camalexin or the generation of indole glucosinolates (Sønderby et al., 2010). In the common part of the pathway, as well as in the downstream branches genes encoding biosynthetic enzymes were detected as flg22-dependent targets of WRKY18, WRKY40 and WRKY33 (Table 6), including previously reported targets of WRKY33 upon infection with *B. cinerea* (Mao et al., 2011; Birkenbihl et al., 2012; Liu et al., 2015), but also several new gene loci. Among the identified targets were *CYP79B2* encoding the enzyme that participates in converting tryptophan to I3AOx, and *CYP71A12*, *CYP71A13, GSTF6*, *GGP1* and *PAD3*, coding for enzymes subsequently converting I3AOx to indole-3-acetonitrile (IAN) and finally to camalexin (Figure 6).

Among the genes encoding the indole-glucosinolate core structure-forming enzymes, which convert I3AOx to indol-3-yl-methyl glucosinolate (I3MG), *CYP83B1*, *GSTF9* and *GGP1* were identified as WRKY18 and/or WRKY40 and/or WRKY33 target genes. In addition, the genes coding for *CYP81F2*, and the two indole-glucosinolate methyltransferase genes *IGMT1* and *IGMT2*, which establish secondary modifications leading to 4 (and 1)-methoxy-indol-3-yl-methyl glucosinolate (4MI3MG); Pfalz et al., 2011), were identified as WRKY18, WRKY40 and WRKY33 targets. The genes encoding CYP81F3 and CYP81F4, both also reported to be capable of modifying the indole-ring of I3MG (Pfalz et al., 2011), were not targeted by the WRKY factors. Among the targets of the three tested WRKY TFs were the atypical myrosinase gene *PEN2*, the syntaxin gene *PEN1* and the ABC transporter gene *PEN3*, involved in the production or transport of toxic metabolites derived from 1MI3MG or 4MI3MG (Assaad et al., 2004; Xu et al., 2016). Further WRKY target genes were the flg22-induced TFs *MYB51*, also named *HIGH GLUCOSINOLATE1* (*HIG1*), and *ERF6* that regulate the production of I3AOx or 4MI3MG via direct binding to the promoters of the biosynthetic genes *CYP79B2/CYP79B3* (Frerigmann and Gigolashvili, 2014), or *CYP81F2*, *IGMT1*, and *IGMT2* (Xu et al., 2016), respectively.

Transcription factor genes as targets

A further predominant functional class of WRKY18, WRKY40 and WRKY33 targets upon flg22 treatment were transcription factor genes. We found that nearly 10 % of all target genes of the tested WRKY TFs encode transcription factors, which is markedly higher than their relative abundance within the Arabidopsis genome (about 6 %), and an indication that these WRKYs are master regulators of plant immunity. Among the targeted genes, *AP2/ERF* TF genes were highly over-represented (Table 7), which again reflects the importance of ET signaling in early MTI (Broekgaarden et al., 2015).

Even more pronounced was the binding of all three WRKY factors to gene loci of their own gene family. WRKY33 binding to the *WRKY33* locus was previously reported (Mao et al., 2011). Here, we found that all three WRKY factors bound to about one third of all WRKY gene promoters, including their own promoters, indicating extensive cross- and auto-regulation within the WRKY TF family upon flg22 elicitation. Of the 32 WRKY genes bound by the three WRKY

factors in total, all but three showed significantly up-regulated gene expression (see below and Supplemental Table 1) after 2 h of flg22 treatment (Suppl. Table 1). This preferred binding to the regulatory regions of the WRKY TF gene family was observed earlier for WRKY33 after infection of Arabidopsis with the necrotrophic fungus *Botrytis cinerea* (Liu et al., 2015). In fact, despite distinct modes of elicitation, nearly the same set of WRKY genes reported by Liu et al. (2015) to be targeted by WRKY33 were also found in our study (Supplemental Table 1). These findings suggest that there is a core set of WRKY factors that is activated upon elicitation by different stimuli, and add support to the hypothesis that WRKY factors form a complex sub-regulatory network during the establishment of rapid host defense responses (Eulgem, 2006; Eulgem and Somssich, 2007; Chi et al., 2013; Choura et al., 2015).

Transcriptional changes upon flg22 treatment

WRKY18 and WRKY40 have been reported to act as negative regulators of MTI (Xu et al., 2006)**.** To investigate the genome-wide effect of these two TFs in transcriptional changes induced by flg22 treatment, RNA-seq experiments were performed in wildtype plants and *wrky18, wrky40*, and *wrky18 wrky40* mutant lines. As for the ChIP-seq experiments, seedlings of each genotype were independently grown for biological replicates in liquid medium and treated with flg22 for 2 h or left untreated. Consistent with previous publications, massive transcriptional changes were observed upon elicitation (Navarro et al., 2004; Zipfel et al., 2004; Zipfel et al., 2006). About 7000 genes in each genotype were altered in their expression upon 2 h flg22 treatment compared to untreated seedlings, by at least two-fold (absolute FC ≥ 2) at a false discovery rate (FDR) of less than 0.05 (Supplemental Data Set 5). In all lines about 4000 genes were up-regulated and 3000 down-regulated upon flg22 treatment (Table 8, Figure 7A).

GO term analysis of the genes with altered expression levels upon flg22 treatment revealed (Supplemental Figure 6): i) Rather small differences in GO term enrichment between the different genotypes, probably due to the high number of differentially expressed genes upon flg22 treatment and the relatively low number of genotype-specific differentially expressed genes (not shown). ii) The terms *unknown cellular components*, *unknown molecular functions* and *unknown biological processes* were clearly under-represented among the flg22-affected genes in WT, reflecting that genes involved in MAMP-triggered immunity have been

extensively investigated. iii) The most obvious differences in GO term enrichment could be observed between up-regulated and down-regulated genes upon flg22 treatment. Thus, the down-regulated genes were strongly enriched for genes associated to the cellular component terms *chloroplast* and *plastid*, which were under-represented among up-regulated genes. Moreover, the up-regulated genes showed a markedly stronger enrichment for genes associated to the molecular function *kinase activity* and the biological processes *response to stress* and *signal transduction*, while the down-regulated genes showed stronger enrichment of genes associated to *cell organization and biogenesis* and *developmental processes*. Together this discrimination in GO terms mirrors the well-described trade-off between plant defense and growth during MTI (Huot et al., 2014; Li et al., 2015).

Differentially expressed genes (DEGs) in *wrky18*, *wrky40,* and *wrky18 wrky40*

When we searched for genotype-specific differentially expressed genes (DEGs) in the WRKY mutant lines compared to WT (absolute FC ≥ 2, FDR < 0.05), we identified in untreated and flg22-treated *wrky18* seedlings only three and six differentially regulated genes, respectively, with one of them being *WRKY18*. In *wrky40* seedlings under the same conditions 14 and 108 DEGs were identified, while in the *wrky18 wrky40* double mutant 112 and 426 genes, respectively, were affected (Figure 7B, Supplemental Data Set 6). The low number of DEGs in the *wrky18* and *wrky40* single mutants compared to the much higher number detected in the double mutant demonstrates the functional redundancy of WRKY18 and WRKY40 at this stage of MTI, with WRKY40 seemingly acting more independent than WRKY18. This redundancy was further confirmed by the high overlap between *wrky40* and *wrky18 wrky40* DEGs, which was 85 % with respect to the *wrky40* gene set, and by the finding that four out of the six *wrky18* DEGs also appeared in the *wrky40* gene set (Figure 7C, see Supplemental Data Set 7 for sector-specific gene lists). Functional redundancy of WRKY18 and WRKY40 was previously demonstrated both in their role as negative regulators of resistance towards the powdery mildew fungus *Golovinomyces orontii* (Pandey et al., 2010), and in positively regulating AvrRPS4 effector-triggered immunity against *Pseudomonas syringae* (Schön et al., 2013). Two hours after flg22 treatment, markedly more DEGs were up-regulated than down-regulated in *wrky40* (88 %) and *wrky18 wrky40* (61 %) compared to WT (Table 9, Figure 7B), indicating that

455 WRKY40, and potentially WRKY18 acting redundantly with WRKY40, were mainly negatively
456 affecting gene expression.

457

458 **Differentially regulated direct target genes (DRTs)**

459 About 55 % of all identified WRKY18, WRKY40 and WRKY33 target genes belonged to the
460 gene set showing altered expression in WT seedlings after flg22 treatment, which is clearly
461 higher than the expected 23 % based on the proportion of genes altered in expression in WT
462 relative to all annotated Arabidopsis genes. About 90 % of these target genes with altered
463 expression levels were up-regulated upon flg22 treatment (Supplemental Figure 7,
464 Supplemental Data Set 8) suggesting that these WRKY factors play a positive regulatory role
465 in reprogramming the transcriptome at this stage of MTI. This apparent discrepancy, that
466 these WRKY factors are negative regulators of most of the differentially expressed genes
467 identified between WT and the respective mutant on the one hand, while their flg22-
468 dependent binding is mainly observed to up-regulated genes in WT plants on the other hand,
469 suggests that they may function to negatively counterbalance the positive effect of other TFs
470 acting at such loci. Candidates for such activators are SARD1, CBP60g and CHE. SARD1
471 and CBP60g belong to the family of calmodulin binding TFs, while CHE is a member of the
472 TCP family. All three TFs have been shown to positively influence defense gene expression
473 and to regulate MTI responses (Truman and Glazebrook, 2012; Zheng et al., 2015). Recent
474 ChIP-seq studies have determined genome-wide binding sites for SARD1 during MTI, and
475 demonstrated substantial redundancy with CBP60g binding (Sun et al., 2015). Comparison
476 between the SARD1 data and our WRKY target gene set revealed a substantial overlap
477 despite differences in the experimental set-ups employed (Supplemental Figure 8, Suppl.
478 Data Set 9). Of the total 392 common targets, 29 are DRTs of WRK18 and WRKY40 and all
479 of these DRTs are up-regulated upon flg22 treatment in the *wrky18 wrky40* double mutant
480 (Suppl. Table 2). One of the common directly regulated target genes*, ICS1*, has been shown
481 to be a direct and positively regulated target not only of SARD1, CBP60g, and CHE (TCP21),
482 but also of other TFs including TCP8 and the NAC TF NTL9 (Wang et al., 2015; Zheng et al.,
483 2015), while in our experiments it was significantly down-regulated by WRKY18 and
484 WRKY40. Similarly, we see a clear negative effect of the WRKY factors on the expression of
485 the receptor-like kinase gene *CRK5*. *CRK5* expression is strongly induced upon flg22-

treatment and it is a direct target of WRKY18 and WRKY40, but not WRKY33 (Supplemental Data Set 1; Supplemental Data Set 5). Previous co-transfection assays performed in tobacco leaves revealed that expression of a reporter gene construct driven by the *CRK5* promoter was suppressed by W-box-dependent promoter binding of WRKY18 or WRKY40 (Lu et al., 2016). *ALD1* and *CRK45* are two additional genes that are direct negatively regulated targets of WRKY18 or WRKY40 but are also targets of SARD1. The *ald1* mutant was shown to be compromised in basal resistance and negatively affected in early flg22 responses, while elevated expression of *ALD1* enhanced basal resistance (Song et al., 2004; Cecchini et l., 2015). *CRK45* mutants also showed enhanced basal susceptibility to *Pseudomonas* whereas overexpression lines thereof increased basal resistance (Zhang et al., 2013). Thus, the proper coordinate regulation of defense genes appears rather complex and very likely involves dynamic binding of both positive and negative transcriptional regulators to fine-tune expression and to ensure robust responsiveness to the stimulus.

To identify directly regulated target genes (DRTs) of WRKY18 and WRKY40, we searched for DEGs between WT and the mutant lines *wrky18*, *wrky40*, and *wrky18 wrky40* that overlapped with the target genes of the respective WRKY factor as identified by ChIP-seq. As the VENN diagram in Figure 8 demonstrates, four loci out of the six DEGs detected in *wrky18* seedlings were bound by WRKY18 after flg22 treatment, including *WRKY18* itself. Fifty-eight (54 %) of the *wrky40* DEG loci were bound by WRKY40, with binding detected only after induction by flg22. In the WRKY40 DRT set *WRKY40* was also found, hinting towards possible feed-back regulation via WRKY18 and WRKY40. Of the DEGs identified in *wrky18 wrky40* seedlings, 122 (29 %) and 131 (31 %) gene loci were identified as WRKY18 and WRKY40 direct targets, respectively (see sector specific gene lists in Supplemental Data Set 10 and Supplemental Data Set 11). The overlap between the WRKY18 and WRKY40 DRTs in *wrky18 wrky40* was 117 genes, including almost all WRKY18 DRTs (Supplemental Data Set 12), again supporting the notion of a strong functional redundancy of the two WRKY factors and possibly binding to DNA as heterodimers as was previously proposed (Xu et al., 2006). In the same study, WRKY60 was also shown to be capable of forming heterodimers with WRKY18 and WRKY40, and WRKY60 is also a DRT of both. However, its role in MTI remains unclear since expression of this gene is not influenced by flg22, based also on our own RNA-seq data, or by other

MAMPs including elf18, chitin, and OGs (oligogalacturonides), according to numerous perturbation studies available at Genvestigator (https://genevestigator.com/gv/index.jsp).

About 10 % of the WRKY18 or WRKY40 direct target genes showed altered expression upon flg22 treatment in a WRKY18- or WRKY40-dependent manner in the *wrky18 wrky40* double mutant. This relatively poor correlation between TF binding and transcriptional response of target genes has been consistently observed in genome-wide ChIP studies (for possible explanations see Heyendrickx et al. (2014). Conversely, about 30 % of the DEGs between *wrky18 wrky40* and WT identified in this study were bound by WRKY18 or WRKY40. The limited overlap could be due to the dynamics of TF binding, which is often transient and cannot be resolved by analyzing one time point by ChIP-seq as performed here, or to the requirement of additional factors besides TF binding to alter gene expression.

Gene ontology analysis of the WRKY18 and WRKY40 DRTs revealed further enrichment of GO terms related to MAMP perception and signaling compared to the overall set of target genes (Supplemental Figure 9). This again is indicative of the two WRKY factors preferentially regulating genes important for early MTI. This further enrichment was observed for the cellular component term *plasma membrane*, the molecular function terms *kinase activity* and *transcription factor activity*, and the biological functions *response to stress*, *response to abiotic and biotic stimulus* and *signal transduction*.

Among the 117 DRTs common for WRKY18 and WRKY40 were 25 genes encoding kinases, 20 of them receptor and receptor-like kinases (including *RK1, RK2*, *RLK1*, *CRK20*, *CES101* and *WAKL4*) and together with MKK9 potential perception and signaling components, 16 genes for TFs, mainly ERF-type factors important in early MTI and stress-responsive WRKY factors, five genes for VQ motif-containing proteins that can interact with specific WRKY proteins and positively influence their binding to DNA (Jing and Lin, 2015), and seven genes for cytochrome P450 proteins, all of them, together with IGMT1, catalyzing the biosynthesis of secondary metabolites (Bak et al., 2011). Additionally, the three SA pathway-related genes, *ICS1*, *PBS3,* and *NIMIN1*, are DRTs of WRKY18 and WRKY40.

As noted above, nearly all of these genes and most of the other DRTs, were flg22-dependently up-regulated in *wrky18 wrky40* compared to WT, indicating that WRKY18 and WRKY40 often fulfill negative regulatory functions in combination with positive regulators at target sites,

thereby very likely ensuring proper spatiotemporal transcriptional outputs (Table 10, Supplemental Data Set 7). This negative function in early MAMP-induced responses is in accordance with *wrky18 wrky40* double mutants being less susceptible to the virulent bacterium *Pst* DC3000 than WT (Xu et al., 2006), and with WRKY40 acting together with BZR1 in balancing the trade-off between defense and growth (Lozano-Duran et al., 2013). Moreover, it is also consistent with a recent large genome-wide study on ABA-treated Arabidopsis seedlings revealing that highly up-regulated genes are targeted by multiple TFs, and that they act in concert to dynamically modulate transcription and to rapidly restore basal expression levels post stimulation (Song et al., 2016).

. In summary, we have defined the genome-wide in vivo binding sites of three Arabidopsis WRKY TFs during early MTI. Our study revealed that the two related WRKY factors WRKY18 and WRKY40 targeted nearly the same set of genes during this response, resulting in the altered expression of an almost identical sub-group of genes consistent with these WRKY factors being in part functionally redundant. Moreover, although WRKY33 targets additional unique loci, in many cases it targets the same promoters, or even the same DNA sites as WRKY18 and WRKY40. An additional key finding was that these three WRKY factors target numerous genes encoding key components of both MAMP and DAMP perception and signaling, providing a mechanistic link between these two functionally interconnected basal defense pathways (Albert, 2013; Bartels and Boller, 2015). Finally, our data imply that WRKY18 and WRKY40 are negative-acting regulatory components of numerous flg22-induced early response genes that act together with TF activators to modulate expression in a robust manner. The comprehensive data provide valuable insights that should prove helpful in our endeavor to define the genome-wide transcriptional regulatory network affected by WRKY TFs in the course of plant immunity.

Materials and Methods

Plant material

For all experiments seedlings of the *Arabidopsis thaliana* ecotype Columbia (Col-0) or mutants in the Col-0 background were used. Besides wildtype plants, insertion mutants for *WRKY18* (GABI_328G03), *WRKY40* (SLAT collection of dSpm insertion lines, Shen et al., 2007), and

WRKY33 (GABI_324B11) were used. The double mutant *wrky18 wrky40* was obtained by crossing the corresponding single mutants (Shen et al., 2007). The *ProWRKY33:WRKY33-HA* complementation line in the *wrky33* mutant was described earlier (Birkenbihl et al., 2012). To generate the *ProWRKY18:WRKY18-HA* complementation line, the 5.9 kb genomic fragment (Chromosome 4: nt 15378866-15384809), consisting of the 4.4 kb upstream region up to the next gene and the 1.5 kb intron containing coding region without the stop codon was amplified by PCR, and cloned into the vector pAM-KAN-HA in front of the sequence encoding the HA-epitope tag. For *ProWRKY40:WRKY40-HA*, the 8.7-kb fragment (Chromosome 1: nt 30376646-30385353), consisting of a 7.2-kb upstream region and 1.5-kb coding region was used. In both cases, the 3´UTR was replaced by a *35S CaMV* terminator sequence. All oligonucleotides used in this study are described in Supplemental Table 3. The constructs were transformed via *Agrobacterium* into the respective single mutants.

Successfully transformed plants were identified by kanamycin selection. To express the same genomic constructs in the *wrky18 wrky40* double mutant, they were cloned into a pAMP-HYG-HA vector conferring hygromycin resistance to the transformed plants. To confirm functionality of the constructs, transformants were tested for their ability to render resistant *wrky18 wrky40* plants susceptible to the powdery mildew fungus *Golovinomyces orontii* (Pandey et al., 2010).

Plant growth and flg22 treatment

To establish seedling cultures, seeds were surface sterilized with ethanol and subsequently grown in 1x MS medium supplemented with 0.5 % sucrose and 0.1 % claforan. After 12 days in a light chamber at long day conditions (16 h light / 8 h dark) illuminated by daylight-white fluorescence lamps (FL40SS ENW/37; Osram) the seedlings were treated with flg22 by replacing the growth medium with medium containing 1 µM flg22.

ChIP-seq and ChIP-qPCR experiments

For the ChIP experiments, two independent biological replicates were created at different times with WT and the complementation lines WRKY18-HA, WRKY40-HA, and WRKY33-HA. For each line and treatment, 2 g of whole seedlings were collected from separate cultures 2 h after the medium exchange, either without flg22 (0 h) or with medium containing flg22 (2 h), and processed separately as described previously (Birkenbihl et al., 2012), following the modified protocol of Gendrel et al. (2005). The IPs were performed using a rabbit polyclonal anti-HA antibody (Sigma-

Aldrich, St. Louis, MO; catalog number H6908). The precipitated DNA was purified with the Qia quick PCR purification kit (Qiagen, Germany) and processed further for ChIP-seq. To prepare the ChIP-seq libraries, the DNA was first amplified by two rounds of linear DNA amplification (LinDA; Shankaranarayanan et al., 2011) and then libraries were constructed with the NEBNext ChIP-seq Library construction kit (New England Biolabs, Ipswich, MA). The libraries were sequenced at the Max Planck Genome Centre Cologne with an Illumina HiSeq2500, resulting in 7-20 million 100 bp single-end reads per sample.

ChIP-seq data analysis

ChIP-seq data processing and analysis was performed as described in Liu et al. (2015) using the TAIR10 *Arabidopsis thaliana* reference genome (*http://www.arabidopsis.org*). The ChIP-seq data created in this study have been deposited at the GEO repository (GSE85922).

To identify potential WRKY binding regions ('peak regions'), the QuEST peak calling program (version 2.4; (Valouev et al., 2008) was used as described in Liu et al. (2015) to search for genomic DNA regions enriched in sequencing reads in the WRKY complementation lines compared to the corresponding WT samples. The peak calling was performed separately for the two biological replicates and additionally the mapped reads of both replicates were pooled and peaks were also called for the pooled samples. Peaks were annotated with respect to nearby gene features in TAIR10 using the annotatePeaks.pl function from the Homer suite (Heinz et al., 2010) with default settings. Consistent peaks between the replicates were identified as described in Liu et al. (2015), i.e. peak regions were counted as consistent, if they were found to be overlapping between the both replicates as well as the pooled sample (by at least 50% of the smaller region).

To search for conserved binding motifs in the consistent WRKY binding regions, for each consistent peak the 500 bp sequence surrounding the peak maximum was extracted and submitted to the online version of MEME-ChIP (Machanick and Bailey, 2011). MEME-ChIP was run with default settings, but a custom background model derived from the Arabidopsis genome was provided and 'Any number of repetitions' of a motif was allowed. To extract the number/percentage of peak regions that contain a certain motif, the online version of FIMO (Grant et al., 2011) was run with the peak sequences and the motif of interest (either MEME/DREME output or known W-box motif) as input and a *p*-value threshold of 0.001. To

identify all locations of the W-box motif (TTGACT/C) in the complete Arabidopsis genome, the R function ‘matchPattern’ (Pagès, 2016: *BSgenome: Infrastructure for Biostrings-based genome data packages and support for efficient SNP representation*. R package version 1.40.1.) was used.

RNA Extraction and Quantitative RT-PCR

For RT-qPCR and the RNA-seq experiments (see below), three independent biological replicates were used: whole seedlings of the indicated genotypes for each treatment were separately grown in three parallel liquid culture sets and processed separately. Total RNA was extracted from 100 mg of untreated or flg22-treated seedlings using the TRI Reagent Solution (Applied Biosystems, AM9738) following the manufacturer´s instructions. The RNA was treated with DNAse I (Roche) and purified using the RNeasy MiniElute Cleanup Kit (Qiagen, Germany). For RT-qPCR, the RNA was reverse transcribed with an oligo(dT) primer to produce cDNA using the SuperScript First-Strand Synthesis System for Reverse-Transcription PCR following the manufacturer’s protocol (Invitrogen). cDNA corresponding to 2.5 ng of total RNA was subjected to qPCR with gene-specific primers (Supplemental Table 3) using the Brilliant SYBR Green qPCR Core Reagent Kit (Stratagene). The qPCRs were performed on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) with two technical replicates in the same run and the three biological replicates in different runs. The data were analyzed using the DDCt method (Livak and Schmittgen, 2001) and RNA levels are indicated as relative expression compared to the endogenous reference gene At4g26410 (Czechowski et al., 2005). Error bars represent the standard deviation of the three biological replicates.

Immunoblot analysis

To analyze the levels of HA-tagged WRKY proteins from seedlings, total proteins were extracted with Laemmli Buffer and equal amounts separated by 8 % SDS-PAGE. The blot was probed with a monoclonal rat antibody against the HA-tag (Roche, Cat. No. 1867423) and developed with a secondary, peroxidase conjugated goat anti rat antibody (Sigma-Aldrich, A9037) using the ECL system according to standard protocols.

RNA-seq experiment

For RNA-seq DNAse I-treated and purified RNAs from three biological replicates (as described for RT-qPCR) were used. Libraries for mRNA sequencing were constructed using the TrueSeq RNA Sample preparation Kit (Illumina) and the three biological replicates for each condition were sequenced at the Max Planck Genome Centre Cologne with an Illumina HiSeq2500, resulting in 23–44 million 100 bp single end reads per sample. The obtained reads were mapped to the Arabidopsis genome as described in Liu et al. (2015). The RNA-seq data created in this study have been deposited at the GEO repository (GSE85923).

RNA-seq data analysis

The mapped RNA-seq reads were transformed into a read count per gene per sample using the htseq-count script (s=reverse, t=exon) in the package HTSeq (Anders et al., 2015). Genes with less than 100 reads in all samples together were discarded, and subsequently the count data of the remaining genes was TMM-normalized and log2-transformed using functions 'calcNormFactors' (R package EdgeR; Robinson et al., 2010) and 'voom' (R package limma; Law et al., 2014). To be able to analyze differential gene expression both between treated and untreated samples within each genotype and between the different mutants (*wrky18*, *wrky40*, *wrky18 wrky40*) and the WT genotype (Col-0), a linear model with the explanatory variable 'genotype_treatment' (i.e., encoding both genotype and treatment information) was fitted for each gene using the function lmFit (R package limma). Subsequently, moderated t-tests were performed over the different contrasts of interest, comparing flg22-treated with untreated samples within each genotype (four contrasts) and comparing each of the mutants with the wildtype within each treatment (six contrasts). In all cases, the resulting p values were adjusted for false discoveries due to multiple hypothesis testing via the Benjamini–Hochberg procedure. For each contrast, we extracted a set of significantly differentially expressed genes between the tested conditions (adj. p value $\leqslant$ 0.05, |log2FCI $\geqslant$ 1).

Gene ontology analysis

For gene ontology analysis, the corresponding tool on the TAIR webpage (http://www.arabidopsis.org/tools/bulk/go/index.jsp) was used. The numbers of genes associated to each GO-term in the identified sets of ChIP-seq targets, transcriptionally regulated genes (DEG) and directly regulated target genes (DRT) for each WRKY factor were

transformed to relative abundances within each data set and compared to the relative abundance of the respective GO-term in the whole genome. To assess statistical significance of the observed enrichment or under-representation of each GO-term in the different analyzed gene sets, the *p*-values were calculated using a hypergeometric test.

Accession Numbers

The ChIP-seq data and the RNA-seq data created in this study have been deposited at the GEO repository with the accession numbers GSE85922 and GSE85923, respectively. The related GEO Super Series Number is GSE85924.

Supplemental Data

Supplemental Figures

Supplemental Figure 1. IGV images of WRKY18, WRKY40 and WRKY33 binding to the *CRK2*, *ICS1*, *PEN2*, *CYP83B1*, *CYP71A13* and *FLS2* loci.

Supplemental Figure 2. Distribution of W-box locations in the Arabidopsis genome.

Supplemental Figure 3. The number of identified W-boxes per binding region is independent of the region size for WRKY18, WRKY40 and WRKY33.

Supplemental Figure 4. Binding of WRKY18 or WRKY40 to selected target genes in the *wrky18, wrky40*, or *wrky18 wrky40* mutant.

Supplemental Figure 5. GO analysis of WRKY18, WRKY40 and WRKY33 target genes.

Supplemental Figure 6. GO analysis of genes that exhibit flg22-induced changes of gene expression in WT seedlings.

Supplemental Figure 7. Up- and down-regulated WRKY target genes upon 2 h flg22 treatment.

Supplemental Figure 8: Target genes common to SARD1 and WRKY18, WRKY40 and WRKY33.

Supplemental Figure 9. GO analysis of WRKY18 and WRKY40 target genes and their directly regulated targets (DRTs) in *wrky18 wrky40*.

Supplemental Table 1. WRKY18, WRKY40 and WRKY33 binding to WRKY genes and fold changes of expression of these targets in WT plants upon flg22 treatment.

Supplemental Table 2: WRKY18 and WRKY40 DRTs in *wrky18 wrky40* that are also bound by SARD1.

Supplemental Table 3. Primers used for cloning, RT-qPCR and ChIP-qPCR.

Supplemental Data Set 1. Genome-wide binding sites and target genes of WRKY18, WRKY40 and WRKY33 detected after 2 h flg22 treatment.

Supplemental Data Set 2. Increased enrichment of WRKY18 at target genes defined by clearly higher binding scores at 2 h than at 0 h flg22.

Supplemental Data Set 3. Overlap between WRKY18, WRKY40 and WRKY33 target gene sets after 2 h flg22 treatment (corresponds to VENN diagram Figure 4).

Supplemental Data Set 4. WRKY18, WRKY40 and WRKY33 target genes related to the GO-term "kinase activity".

Supplemental Data Set 5. Flg22-responsive genes in WT plants and *wrky18*, *wrky40,* and *wrky18 wrky40* mutants.

Supplemental Data Set 6. Differentially expressed genes (DEGs) upon flg22 treatment in *wrky18*, *wrky40*, or *wrky18 wrky40* mutants compared to WT plants.

Supplemental Data Set 7. Overlap of DEGs in *wrky18*, *wrky40*, or the *wrky18 wrky40* mutant compared to WT plants upon flg22 treatment (corresponds to VENN diagram Figure 7C).

Supplemental Data Set 8. Up- and down-regulated target and non-target genes in response to flg22 treatment (corresponds to VENN diagram Supplemental Figure 7).

Supplemental Data Set 9: Target genes common to SARD1 and WRKY18, W40 and W33 (corresponds to VENN diagram in Supplemental Figure 8).

748 **Supplemental Data Set 10.** WRKY18 and WRKY40 directly regulated target genes (DRTs;
749 corresponding to VENN diagrams in Figure 8).

750 **Supplemental Data Set 11.** Expression and binding data of WRKY18 and WRKY40 DRTs.

751 **Supplemental Data Set 12.** Directly regulated targets (DRTs) common to WRKY18 and
752 WRKY40.

753

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757

758 **Author Contributions**

759 RPB, conception and design, conducted experiments, acquisition of data, analysis and
760 interpretation of data, drafting and revising the manuscript; BK, analysis and interpretation of
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762 interpretation of data, drafting and revising the manuscript.

763

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Figure Legends

Figure 1. Induction of *WRKY18*, *WRKY40* and *WRKY33* by flg22 treatment. A. RT-qPCR analysis of flg22-induced RNA levels. Total RNA was isolated from seedlings treated for 0, 1, 2 and 4 h with flg22 and analyzed by qPCR using gene-specific primers. Shown are the mean and standard deviation (error bars) calculated from three biological replicates. B. Immunoblot analysis of flg22-induced protein levels. Protein extracts from seedlings of the HA-tagged complementation lines were treated for indicated times with flg22 and subsequently analyzed by immunoblot using an anti-HA antibody. Ponceau S staining served as loading control.

Figure 2. Distribution of flg22-induced WRKY18, WRKY40 and WRKY33 binding regions in the Arabidopsis genome. A. Prevalence of WRKY binding regions in different genomic categories. Promoters are defined as the 1000-bp region upstream of the transcription start site (TSS). Transcription termination site (TTS) refers to the 1000-bp region downstream of the 3'UTR, and genome regions located in between a TTS and the promoter of the next gene are regarded as intergenic. B. Distance of WRKY binding region peaks to the transcription start site. The number of binding region peaks for each 50-bp region relative to the TSS is indicated.

Figure 3. The W-box is the predominant motif within WRKY binding regions. A, B, C. Motif position probability graphs for WRKY18 (A), WRKY40 (B) and WRKY33 (C) established by CentriMo motif search (Bailey and Machanick, 2012). Indicated is the most frequent motif, its rate of occurrence, and the probability of this motif occurring at a given position relative to the binding peak summit (0) in the 500-bp binding regions. The included p-value describes the significance for central enrichment. D. Distribution of W-box abundances in WRKY18, WRKY40 and WRKY33 binding regions.

Figure 4. Overlap of WRKY18, WRKY40 and WRKY33 target gene sets after 2 h flg22 treatment. Indicated are the number of target genes in each section, and the fraction of overlapping genes between each pair of WRKY target gene sets with respect to the smaller set.

Figure 5. WRKY18, WRKY40 and WRKY33 binding to the *PEPR1*, *PROPEP2* and *PROPEP3* loci. A. Integrative Genome Viewer (IGV) images of the *PEPR1* (A) and *PROPEP1-3* loci (B). Binding of WRKYs is visualized by read coverage histograms indicating sequencing read accumulation before (0 h) or after flg22 treatment (2 h). WT samples served as negative control. The three lower tracks show the corresponding gene structures, position of W-boxes and the direction of transcription (arrows).

Figure 6. WRKY18, WRKY40 and WRKY33 bind to genes involved in biosynthesis and transport of secondary metabolites camalexin and indole-glucosinolates (biosynthetic pathways adapted from Sonderby et al., 2010; Pfalz et al., 2011; Morldrup et al., 2013). Identified WRKY18, WRKY40 or WRKY33 target genes are indicated in yellow. TFs are underlined.

Figure 7. Differentially expressed genes in *wrky18*, *wrky40*, and *wrky18 wrky40* plants. A. Number of significantly (FC ≥ 2, FDR < 0.05) up- or down-regulated genes at 2 h flg22 treatment compared to 0 h in the indicated genotype. B. Number of up- and down-regulated, differentially expressed genes (DEGs) (FDR < 0.05, FC ≥ 2) in the respective mutant lines compared to WT at 0, 1, or 2 h post flg22 treatment. C. Overlap of the identified sets of DEGs in the respective mutant lines relative to WT at 2 h post flg22 treatment. Indicated are the number of DEGs in each section.

Figure 8. Directly regulated target genes (DRTs) of WRKY18 and WRKY40. DRTs were identified by the overlaps of the WRKY18 and WRKY40 target gene sets with the sets of differentially expressed genes (DEGs) compared to WT in *wrky18*, *wrky40* and *wrky18 wrky40* mutants at 2 h post flg22 treatment. Indicated are the number of target genes, DEGs and DRTs in the respective sections, and the fraction DEGs identified as DRTs in each comparison.

Tables

Table 1: Flg22-induced WRKY18, WRKY40 and WRKY33 binding sites and target genes

TFs	Binding Sites	Total Target Genes	Induced Target Genes
WRKY18	1403	1290	380
WRKY40	1622	1478	1477
WRKY33	1208	1140	1104

		Score ChIP			
Gene ID	Gene name	W18	W40	W33	Gene Product Description
AT3G21630	*CERK1*	22.5	29.5	30.4	chitin elicitor receptor kinase 1
AT3G16030	*CES101*	56.2	56.2	23.8	lectin receptor kinase CES101
AT4G23130	*CRK5*	16.2	17.8	-	cysteine-rich receptor-like protein kinase 5
AT4G23220	*CRK14*	63.1	66.1	34.6	cysteine-rich receptor-like protein kinase 14
AT4G23250	*CRK17*	23.6	34.3	16.6	cysteine-rich receptor-like protein kinase 17
AT4G23260	*CRK18*	21.1	23.2	11.3	cysteine-rich receptor-like protein kinase 18
AT4G23280	*CRK20*	31.2	44.6	-	putative cysteine-rich receptor-like protein kinase 20
AT4G21400	*CRK28*	22.9	21.5	23.7	cysteine-rich receptor-like protein kinase 28
AT5G46330	*FLS2*	36.0	36.0	22.6	LRR receptor like kinase
AT2G39660	*BIK1*	23.8	30.7	-	serine/threonine-protein kinase BIK1
AT5G48380	*BIR1*	21.5	25.5	20.4	leucine-rich repeat receptor-like protein kinase
AT4G34390	*XLG2*	19.3	18.6	17.5	extra-large GTP-binding protein 2
AT4G34460	*AGB1*	10.8	-	16.3	guanine nucleotide-binding protein subunit beta
AT2G28830	*PUB12*	29.1	38.1	11.3	plant U-box 24 protein
AT4G18710	*BIN2*	26.3	26.0	16.1	Shaggy-related protein kinase eta
AT5G47910	*RBOHD*	-	12.3	-	respiratory burst oxidase-D
AT2G19190	*FRK1*	12.7	17.5	14.5	flag22 induced receptor kinase 1
AT5G48410	*GLR1.3*	-	-	20.8	glutamate receptor 1.3
AT5G11210	*GLR2.5*	12.1	-	14.2	glutamate receptor 2.5
AT2G29120	*GLR2.7*	16.3	20.3	-	glutamate receptor 2.7
AT2G29100	*GLR2.9*	-	18.8	20.7	glutamate receptor 2.9
AT5G01560	*LECRKA4.3*	20.8	25.6	-	Lectin-domain containing receptor kinase A4.3
AT2G17120	*LYM2*	32.3	44.2	-	LysM domain-containing GPI-anchored protein 2
AT1G73080	*PEPR1*	32.7	33.9	18.0	LRR receptor-like protein kinase PEPR1
AT5G64890	*PROPEP2*	18.9	20.3	21.2	elicitor peptide 2
AT5G64905	*PROPEP3*	26.1	35.0	40.3	elicitor peptide 3
AT1G09970	*RLK7*	13.0	15.2	16.3	leucine-rich receptor-like protein kinase LRR XI-23
AT2G02220	*PSKR1*	-	-	24.0	phytosulfokin receptor 1
AT2G38310	*PYL4*	13.4	16.6	-	abscisic acid receptor PYL4
AT1G65790	*RK1*	-	19.1	11.6	receptor kinase 1
AT1G65800	*RK2*	30.5	34.1	-	receptor kinase 2
AT4G21380	*RK3*	20.0	25.3	14.5	receptor kinase 3
AT5G60900	*RLK1*	37.8	43.8	15.5	receptor-like protein kinase 1
AT1G16150	*WAKL4*	30.6	35.9	20.2	wall associated kinase-like 4
AT1G16160	*WAKL5*	20.2	21.1	16.3	wall-associated receptor kinase-like 5

Table 2: Selected receptor and receptor-related target genes of WRKY18, WRKY40 and WRKY33. Indicated are ChIP scores for the respective WRKY factor and target gene at 2 h flg22 treatment. - , indicates ChIP scores below the threshold level.

		Score ChIP			
Gene ID	Gene name	W18	W40	W33	Gene Product Description
AT1G73500	*MKK9*	28.9	26.3	-	MAP kinase kinase 9
AT1G01480	*ACS2*	27.3	36.4	-	1-aminocyclopropane-1-carboxylate synthase 2
AT4G11280	*ACS6*	60.9	90.0	29.9	1-aminocyclopropane-1-carboxylate synthase 6
AT4G37770	*ACS8*	23.4	22.3	30.5	1-aminocyclopropane-1-carboxylate synthase 8
AT1G62380	*ACO2*	18.6	12.5	-	aminocyclopropanecarboxylate oxidase
AT2G25490	*EBF1*	33.8	35.0	-	EIN3-binding F-box protein 1
AT5G25350	*EBF2*	73.4	67.7	22.8	EIN3-binding F-box protein 2
AT5G03280	*EIN2*	39.6	40.6	-	ethylene-insensitive protein 2
AT4G17500	*ERF-1*	54.0	65.7	41.1	ethylene-responsive transcription factor 1A
AT3G23240	*ERF1*	21.8	20.9	-	ethylene-responsive transcription factor 1B
AT5G47220	*ERF2*	15.4	17.4	-	ethylene-responsive transcription factor 2
AT3G15210	*ERF4*	16.2	22.3	-	ethylene-responsive transcription factor 4
AT5G47230	*ERF5*	-	15.3	24.2	ethylene-responsive transcription factor 5
AT4G17490	*ERF6*	21.5	23.2	-	ethylene-responsive transcription factor 6
AT1G06160	*ORA59*	21.8	22.5	-	ethylene-responsive transcr. factor ERF094
AT5G64750	*ABR1*	65.7	82.4	17.2	ethylene-responsive transcription factor ABR1
AT1G18570	*MYB51*	19.2	40.6	26.8	myb domain protein 51

Table 3: Selected ET pathway-related target genes of WRKY18, WRKY40 and WRKY33. Indicated are ChIP scores for the respective WRKY factor and target gene at 2h flg22 treatment. -, indicates ChIP scores below the threshold level.

		Score ChIP			
Gene ID	Gene name	W18	W40	W33	Gene Product Description
AT1G74710	*ICS1*	51.0	75.0	-	isochorismate sythase 1
AT3G48090	*EDS1*	26.1	24.9	-	enhanced disease susceptibility 1 protein
AT4G39030	*EDS5*	27.5	32.5	11.5	enhanced disease susceptibility 5, drug transport.
AT1G02450	*NIMIN1*	18.9	20.5	-	protein NIM1-interacting 1
AT4G16890	*SNC1*	17.4	19.3	15.9	protein SUPPRESS. OF npr1-1, CONSTITUT. 1
AT5G45110	*NPR3*	-	14.2	-	NPR1-like protein 3
AT4G19660	*NPR4*	-	14.9	-	NPR1-like protein 4
AT5G13320	*PBS3*	47.4	44.2	-	auxin-responsive GH3 family protein
AT5G14930	*SAG101*	-	-	21.2	senescence-associated protein 101
AT2G47190	*MYB2*	-	15.9	-	myb domain protein 2
AT3G61250	*MYB17*	15.1	-	-	myb domain protein 17
AT1G74650	*MYB31*	41.5	51.8	-	myb domain protein 31
AT1G18570	*MYB51*	19.2	40.6	26.8	myb domain protein 51
AT1G68320	*MYB62*	21.3	-	-	myb domain protein 62
AT5G62470	*MYB96*	40.7	48.2	-	myb domain protein 96
AT5G65790	*MYB68*	26.8	30.1	-	myb domain protein 68
AT4G31800	*WRKY18*	229.4	97.6	21.1	WRKY transcription factor 18
AT5G24110	*WRKY30*	24.0	26.0	25.2	WRKY DNA-binding protein 30
AT5G22570	*WRKY38*	21.9	22.7	12.5	putative WRKY transcription factor 38
AT4G23810	*WRKY53*	14.9	18.2	29.5	putative WRKY transcription factor 53
AT2G25000	*WRKY60*	78.2	62.5	-	putative WRKY transcription factor 60
AT5G01900	*WRKY62*	-	12.5	-	putative WRKY transcription factor 62

Table 4: Selected SA pathway-related target genes of WRKY18, WRKY40 and WRKY33. Indicated are ChIP scores for the respective WRKY factor and target gene at 2h flg22 treatment. -, indicates ChIP scores below the threshold level.

		Score ChIP			
Gene ID	Gene name	W18	W40	W33	Gene Product Description
AT1G72450	*JAZ6*	30.4	30.9	38.6	jasmonate-zim-domaine protein 6, or AT1g72460
AT1G17420	*LOX3*	28.5	30.4	-	lipoxygenase 3
AT3G25780	*AOC3*	-	10.3	10.1	allene oxide cyclase 3
AT1G15520	*PDR12*	23.1	32.3	21.5	ABC transporter G family member 40
AT2G41370	*BOP2*	17.6	19.9	10.4	ankyrin repeat and BTB/POZ domain-cont. protein
AT1G06160	*ORA59*	14.2	12.2	-	ethylene-responsive transcription factor ERF094
AT5G26170	*WRKY50*	-	14.6	-	putative WRKY transcription factor 50
AT5G64810	*WRKY51*	19.2	22.1	15.8	putative WRKY transcription factor 51

Table 5: Selected JA pathway-related target genes of WRKY18, WRKY40 and WRKY33. Indicated are ChIP scores for the respective WRKY factor and target gene at 2 h flg22 treatment. - , indicates ChIP scores below the threshold level.

		Score ChIP			
Gene ID	Gene name	W18	W40	W33	Gene Product Description
AT4G30530	*GGP1*	yes	yes	yes	gamma-glutamyl-peptidase 1
AT1G02930	*GSTF6*	16.9	18.1	19.2	glutathione S-transferase 6
AT2G30860	*GSTF9*	-	-	11.9	glutathione S-transferase 9
AT4G31500	*CYP83B1*	yes	yes	16.1	Cytochrome P450 83B1
AT4G39950	*CYP79B2*	19.5	25.1	23.1	tryptophan N-hydroxylase 1
AT5G57220	*CYP81F2*	31.0	43.4	15.0	cytochrome P450 81F2
AT2G30770	*CYP71A13*	yes	yes	yes	cytochrome P450 71A13
AT2G30750	*CYP71A12*	yes	14.1	20.7	cytochrome P450 71A12
AT3G26830	*PAD3*	28.4	36.2	27.8	cytochrome P450 71B15
AT3G11820	*PEN1*	19.1	18.5	14.7	syntaxin-121, vesicle traffic
AT2G44490	*PEN2*	38.9	31.7	36.4	beta-glucosidase 26
AT1G59870	*PEN3*	107.2	137.8	64.7	ABC transporter G family member 36
AT1G18570	*MYB51*	19.2	40.6	26.8	myb domain protein 51
AT4G17490	*ERF6*	21.5	23.2	-	ethylene-responsive transcript. factor 6
AT1G21100	*IGMT1*	25.0	24.1	15.1	O-methyltransferase-like protein
AT1G21110	*IGMT3*	18.7	17.0	10.4	O-methyltransferase family protein
AT1G21120	*IGMT2*	22.5	25.4	17.2	O-methyltransferase family protein
AT1G21130	*IGMT4*	31.4	37.3	11.8	O-methyltransferase-like protein

Table 6: Selected target genes of WRKY18, WRKY40 and WRKY33 associated with indole glucosinolate and camalexin. Indicated are ChIP scores for the respective WRKY factor and target gene at 2 h flg22 treatment. Manual inspection of the binding sites in the IGV browser identified additional targets that were included (and marked with yes, compare Supplemental Fig.1). - , indicates ChIP scores below the threshold level.

TF families	WRKY18	WRKY40	WRKY33	Genome
total TFs	129 / 10.0% **	156 / 10.5% **	102 / 9,0% **	1700 / 5.6%
AP2/ERF	27 / 19.6% **	34 / 24.6% **	13 / 9.4% *	138
NAC	6 / 6.3%	8 / 8.3%	5 / 5.2%	96
MYB	12 / 9.2% *	14 / 10.7% *	12 / 9.2% *	131
bHLH	10 / 6.2%	12 / 7.5%	7 / 4.3%	161
WRKY	22/ 30.6% **	27 / 37.5% **	20 / 27.8% **	72

Table 7: WRKY18, WRKY40 and WRKY33 binding to genes from selected TF families related to stress response at 2 h flg22 treatment. Indicated are the numbers of gene loci of a TF gene family bound by the respective WRKY factor and their fraction in percent of the entire TF gene family. Single and double asterisks indicate $p < 0.05$ and $p < 0.0001$, respectively, in a hypergeometric test for enrichment. The column “Genome” lists the total numbers of genes within the Arabidopsis genome (*agris,* http://arabidopsis.med.ohio-state.edu/AtTFDB/).

Genotype	Total	Up	Down
WT	6892	4099	2793
wrky18	7208	4244	2964
wrky40	7222	4230	2992
wrky18 wrky40	7297	4179	3118

Table 8: Numbers of genes altered in their expression in the respective genotype upon flg22 treatment. Indicated are the total numbers of genes with altered expression, and numbers of up- and down-regulated genes (absolute FC ≥ 2, FDR < 0.05) at 2 h flg22.

	0 h			2 h		
Genotype	Total	Up	Down	Total	Up	Down
wrky18	3	0	3	6	3	3
wrky40	15	12	3	108	96	12
wrky18 wrky40	112	92	20	426	259	167

Table 9: Numbers of differentially expressed genes (DEGs) in the respective genotype compared to WT. Indicated are the total numbers of DEGs, and numbers of up- and down-regulated genes (absolute FC ≥ 2, FDR < 0.05) at 0 h and 2 h flg22.

		DRTs		
Binding TF	DEGs In	Total	Up	Down
WRKY18	*wrky18*	4	2	2
WRKY40	*wrky40*	58	55	3
WRKY18	*wrky18 wrky40*	122	112	10
WRKY40	*wrky18 wrky40*	131	119	12
WRKY18 and WRKY40	*wrky18 wrky40*	119	109	10

Table 10: Directly regulated target genes (DRTs) at 2h flg22 of WRKY18 or/and WRKY40 in *wrky18*, *wrky40*, and *wrky18 wrky40*. DRTs are defined as differentially expressed genes (DEGs) in the respective genotype that are directly bound by the indicated WRKY TF. Shown are the total numbers and the numbers of up- and down-regulated DRT genes.

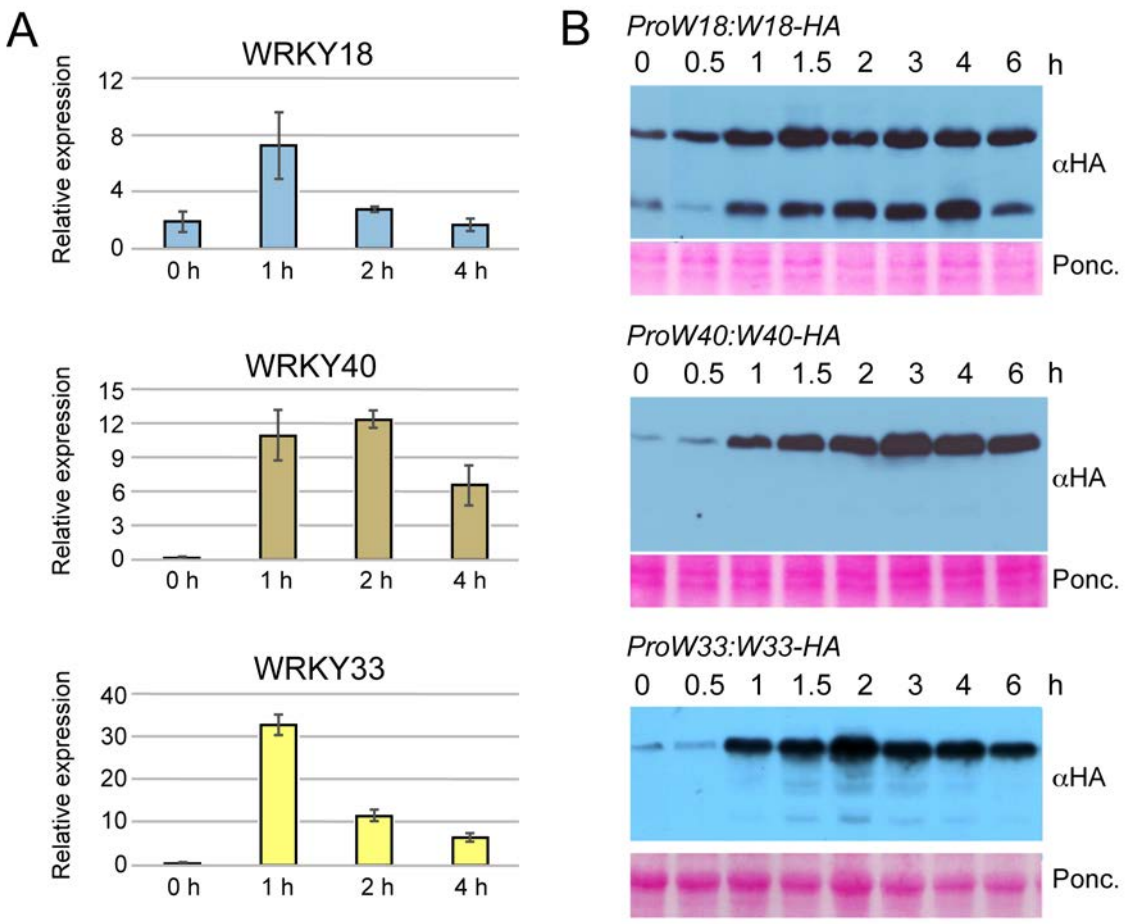

Figure 1. Induction of *WRKY18*, *WRKY40* and *WRKY33* by flg22 treatment. A. RT-qPCR analysis of flg22-induced RNA levels. Total RNA was isolated from seedlings treated for 0, 1, 2 and 4 h with flg22 and analyzed by qPCR using gene-specific primers. Shown are the mean and standard deviation (error bars) calculated from three biological replicates. B. Immunoblot analysis of flg22-induced protein levels. Protein extracts from seedlings of the HA-tagged complementation lines were treated for indicated times with flg22 and subsequently analyzed by immunoblot using an anti-HA antibody. Ponceau S staining served as loading control.

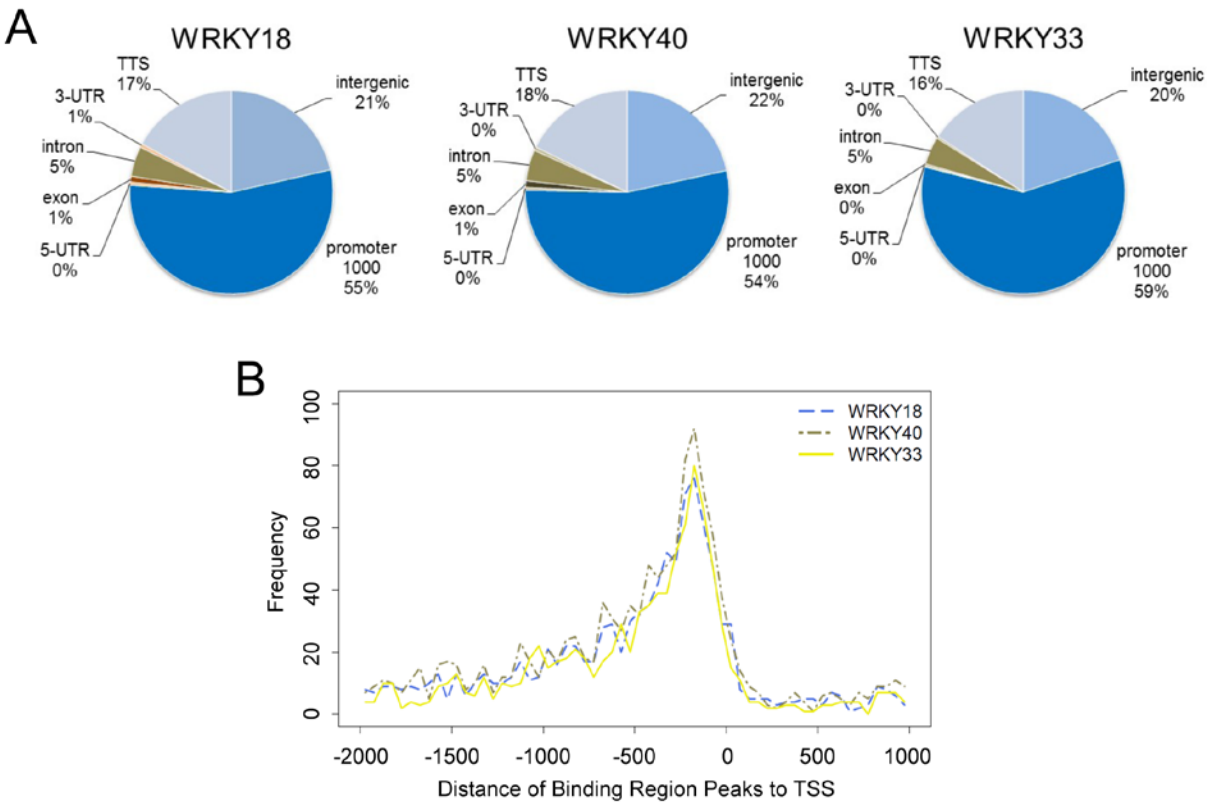

Figure 2. Distribution of flg22-induced WRKY18, WRKY40 and WRKY33 binding regions in the Arabidopsis genome. A. Prevalence of WRKY binding regions in different genomic categories. Promoters are defined as the 1000-bp region upstream of the transcription start site (TSS). Transcription termination site (TTS) refers to the 1000-bp region downstream of the 3'UTR, and genome regions located in between a TTS and the promoter of the next gene are regarded as intergenic. B. Distance of WRKY binding region peaks to the transcription start site. The number of binding region peaks for each 50-bp region relative to the TSS is indicated.

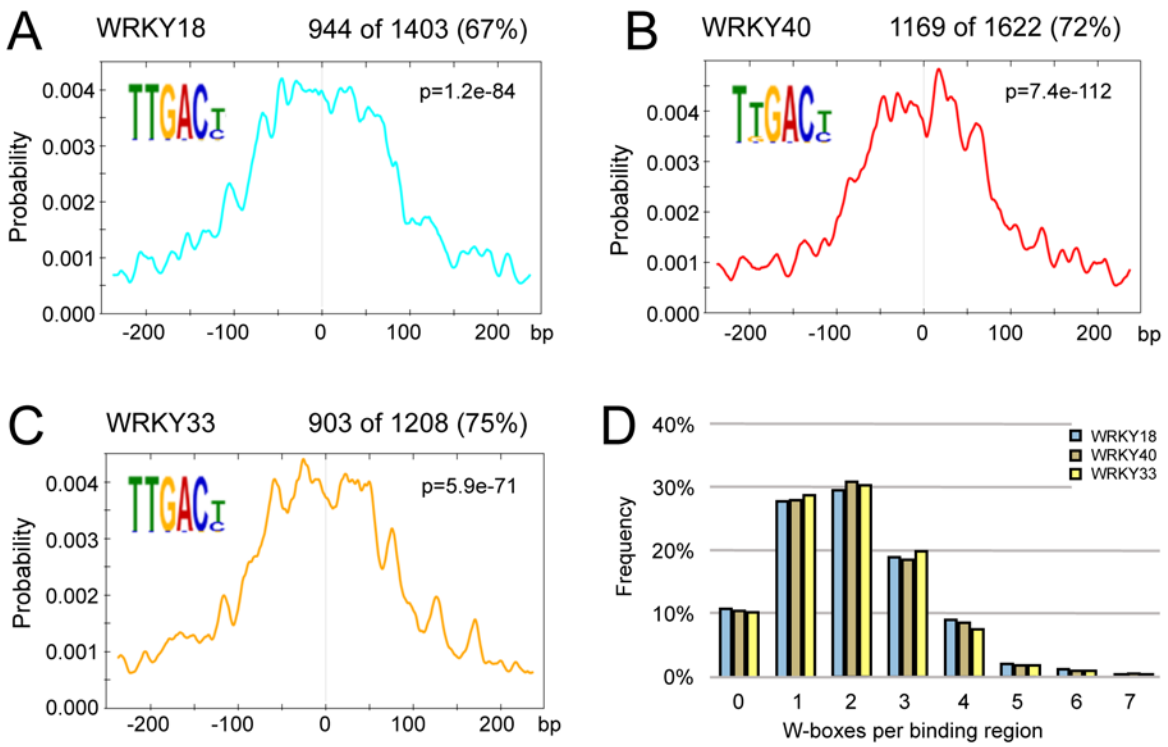

Figure 3. The W-box is the predominant motif within WRKY binding regions. A, B, C. Motif position probability graphs for WRKY18 (A), WRKY40 (B) and WRKY33 (C) established by CentriMo motif search (Bailey and Machanick, 2012). Indicated is the most frequent motif, its rate of occurrence, and the probability of this motif occurring at a given position relative to the binding peak summit (0) in the 500-bp binding regions. The included p-value describes the significance for central enrichment. D. Distribution of W-box abundances in WRKY18, WRKY40 and WRKY33 binding regions.

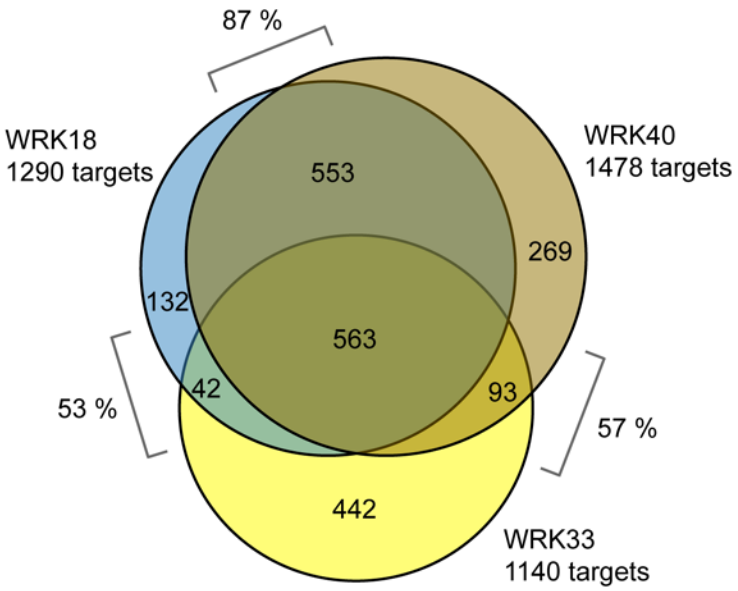

Figure 4. Overlap of WRKY18, WRKY40 and WRKY33 target gene sets after 2 h flg22 treatment. Indicated are the number of target genes in each section, and the fraction of overlapping genes between each pair of WRKY target gene sets with respect to the smaller set.

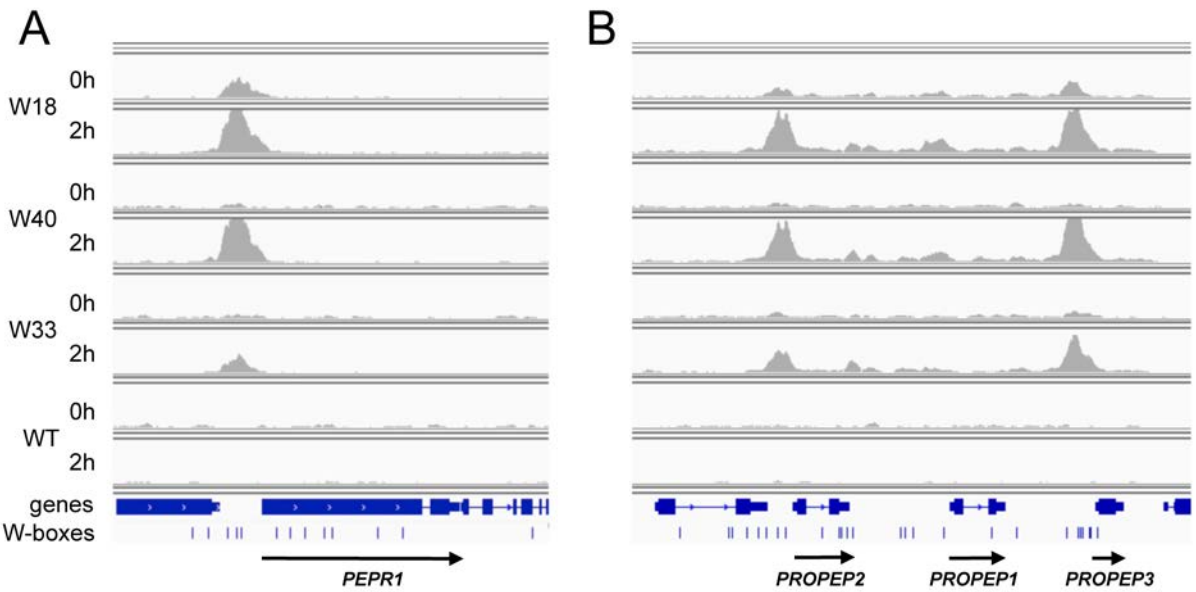

Figure 5. WRKY18, WRKY40 and WRKY33 binding to the *PEPR1*, *PROPEP2* and *PROPEP3* loci. A. Integrative Genome Viewer (IGV) images of the *PEPR1* (A) and *PROPEP1-3* loci (B). Binding of WRKYs is visualized by read coverage histograms indicating sequencing read accumulation before (0 h) or after flg22 treatment (2 h). WT samples served as negative control. The three lower tracks show the corresponding gene structures, position of W-boxes and the direction of transcription (arrows).

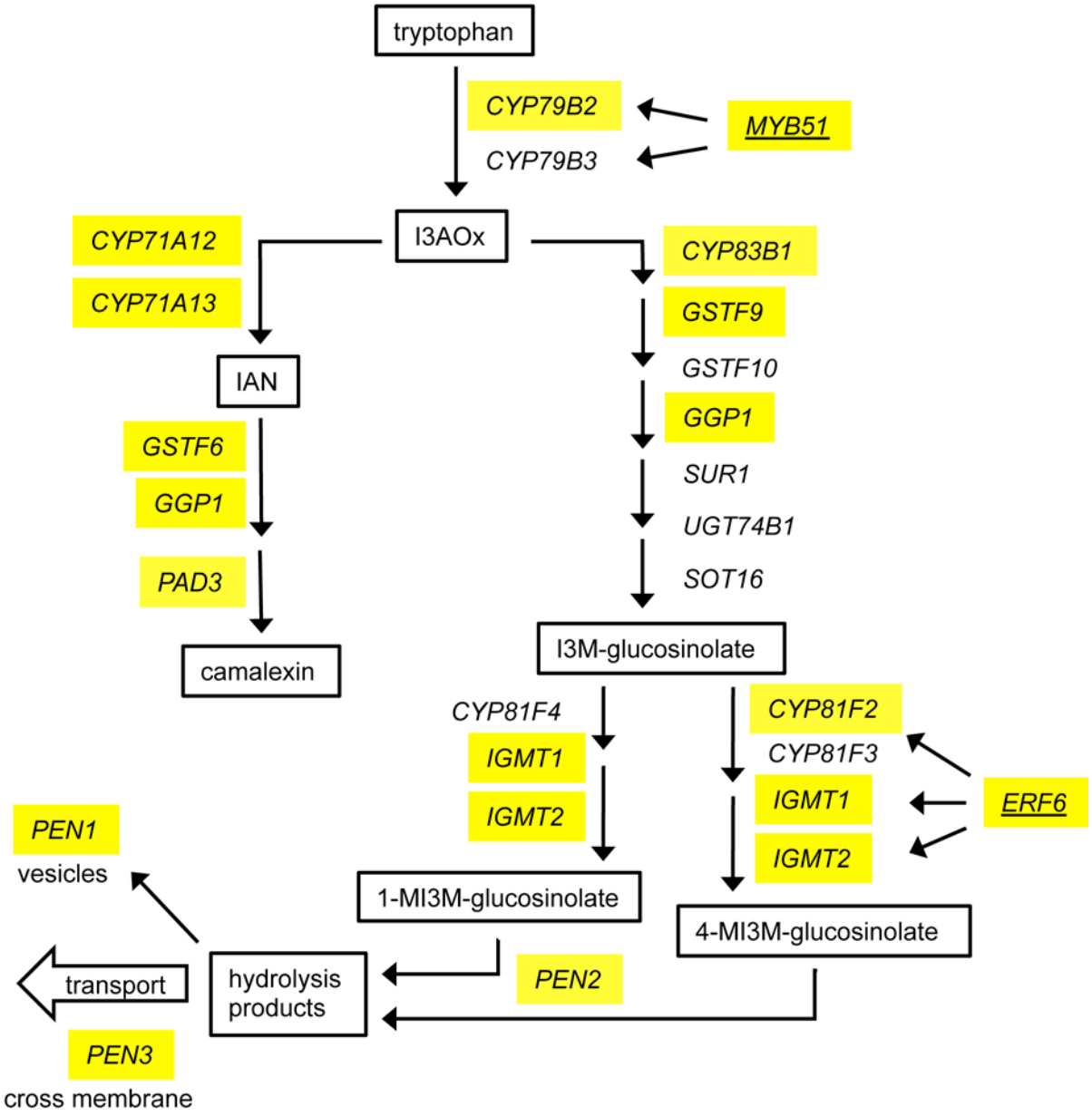

Figure 6. WRKY18, WRKY40 and WRKY33 bind to genes involved in biosynthesis and transport of secondary metabolites camalexin and indole-glucosinolates (biosynthetic pathways adapted from Sonderby et al., 2010; Pfalz et al., 2011; Morldrup et al., 2013). Identified WRKY18, WRKY40 or WRKY33 target genes are indicated in yellow. TFs are underlined.

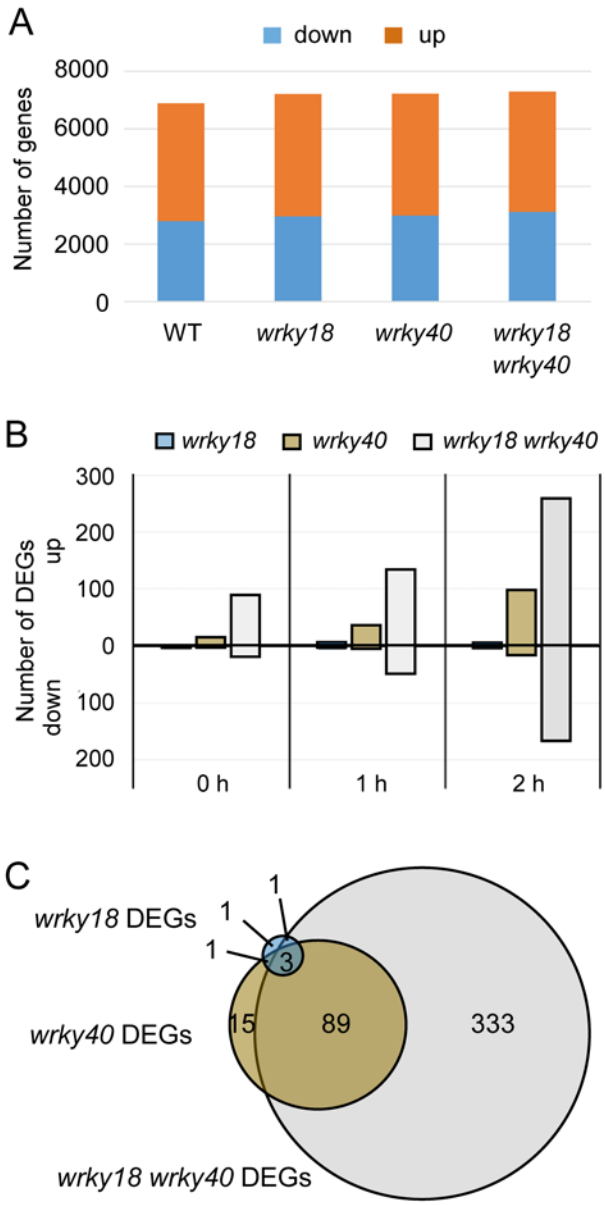

Figure 7. Differentially expressed genes in *wrky18*, *wrky40*, and *wrky18 wrky40* plants. A. Number of significantly (FC ≥ 2, FDR < 0.05) up- or down-regulated genes at 2 h flg22 treatment compared to 0 h in the indicated genotype. B. Number of up- and down-regulated, differentially expressed genes (DEGs) (FDR < 0.05, FC ≥ 2) in the respective mutant lines compared to WT at 0, 1, or 2 h post flg22 treatment. C. Overlap of the identified sets of DEGs in the respective mutant lines relative to WT at 2 h post flg22 treatment. Indicated are the number of DEGs in each section.

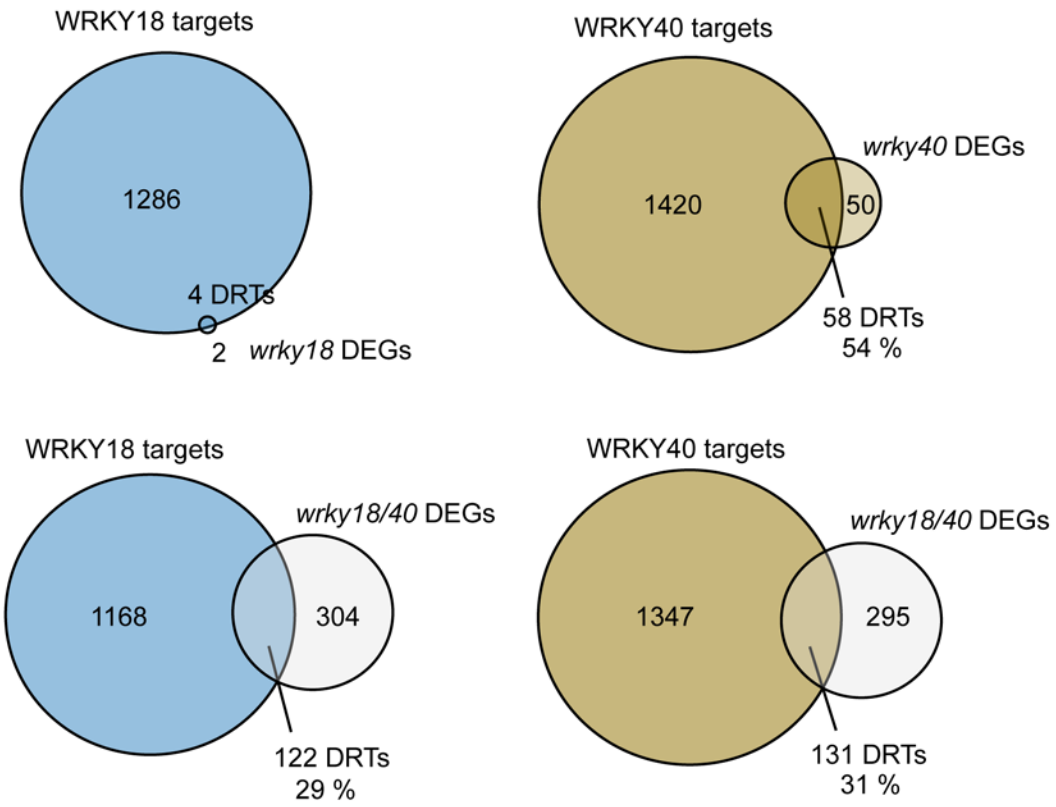

Figure 8. Directly regulated target genes (DRTs) of WRKY18 and WRKY40. DRTs were identified by the overlaps of the WRKY18 and WRKY40 target gene sets with the sets of differentially expressed genes (DEGs) compared to WT in *wrky18*, *wrky40* and *wrky18 wrky40* mutants at 2 h post flg22 treatment. Indicated are the number of target genes, DEGs and DRTs in the respective sections, and the fraction DEGs identified as DRTs in each comparison.

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