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e-Xtra*

Analyses of *wrky18 wrky40* Plants Reveal Critical Roles of SA/EDS1 Signaling and Indole-Glucosinolate Biosynthesis for *Golovinomyces orontii* Resistance and a Loss-of Resistance Towards *Pseudomonas syringae* pv. *tomato* AvrRPS4

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Simultaneous mutation of two WRKY-type transcription factors, WRKY18 and WRKY40, renders otherwise susceptible wild-type *Arabidopsis* plants resistant towards the biotrophic powdery mildew fungus *Golovinomyces orontii*. Resistance in *wrky18 wrky40* double mutant plants is accompanied by massive transcriptional reprogramming, imbalance in salicylic acid (SA) and jasmonic acid (JA) signaling, altered *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) expression, and accumulation of the phytoalexin camalexin. Genetic analyses identified SA biosynthesis and EDS1 signaling as well as biosynthesis of the indole-glucosinolate 4MI3G as essential components required for loss-of-*WRKY18 WRKY40*–mediated resistance towards *G. orontii*. The analysis of *wrky18 wrky40 pad3* mutant plants impaired in camalexin biosynthesis revealed an uncoupling of pre- from postinvasive resistance against *G. orontii*. Comprehensive infection studies demonstrated the specificity of *wrky18 wrky40*–mediated *G. orontii* resistance. Interestingly, WRKY18 and WRKY40 act as positive regulators in effector-triggered immunity, as the *wrky18 wrky40* double mutant was found to be strongly susceptible towards the bacterial pathogen *Pseudomonas syringae* DC3000 expressing the effector *AvrRPS4* but not against other tested *Pseudomonas* strains. We hypothesize that *G. orontii* depends on the function of WRKY18 and WRKY40 to successfully infect *Arabidopsis* wild-type plants while, in the interaction with *P. syringae* AvrRPS4, they are required to mediate effector-triggered immunity.

The plant immune system can be subdivided into two main branches, both depending on the perception of nonself or modified-self structures (Maekawa et al. 2011). The first active layer of plant defense is based on nonself recognition by detecting molecular components that are structurally conserved across a wide range of microbes but that are normally not present in the host (Chisholm et al. 2006; Dodds and Rathjen 2010). Extracellular perception of these slow-evolving microbe-associated molecular patterns (MAMPs) by pattern recognition receptors triggers downstream cell-autonomous responses leading to MAMP-triggered immunity (MTI). Although effective against most pathogenic threats, MAMP-induced defense responses are insufficient against virulent pathogens. Those pathogens have evolved the ability to evade MTI by secreting specific effector molecules into plant cells that suppress or interfere with MAMP-triggered immune responses. As a consequence, plants have acquired intracellular immune sensors that recognize isolate-specific pathogen effectors. Perception of an effector by an intracellular resistance (R) protein leads to the activation of downstream responses culminating in effector triggered immunity (ETI). Well studied are two signaling branches of ETI activated by Toll-interleukin-like receptor (TIR) or coiled-coil (CC) types of nucleotide binding-leucine-rich repeat (NB-LRR) proteins, which require the lipase-like ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) or NON–RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) proteins for effective defense activation, respectively. Whereas resistance induced upon infection with the avirulent bacterial strain *Pseudomonas syringae* pv. *tomato* expressing the effector *AvrRPS4* is EDS1-dependent, effective defense against *P. syringae* pv. *tomato* AvrRPM1 requires NDR1 (Aarts et al. 1998; Falk et al. 1999; Moreau et al. 2012).

Both, MTI and ETI involve similar downstream events comprising transcriptional reprogramming, the activation of defense hormone pathways, and the production of (antimicrobial) secondary metabolites. Two major plant defense hormones are jasmonic acid (JA) and salicylic acid (SA). In general, JA signaling is associated with defense against necrotrophic pathogens, whereas SA plays essential roles against biotrophic pathogens (Glazebrook 2005). Moreover, EDS1 was shown to be a core component of both MTI and ETI with *eds1* mutants revealing defects in basal defense but also in defense responses mediated via TIR-NB-LRR R proteins (Heidrich et al. 2011; Parker et al. 1996; Wiermer et al. 2005).

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In barley, the CC-NB-LRR R protein MILDEW LOCUS A10 (MLA10) mediates effector-triggered resistance towards the obligate biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. This resistance necessitates effector-dependent association of MLA10 with the transcriptional repressors WRKY1 and WRKY2 in the nucleus (Shen et al. 2007). In *Arabidopsis*, transcription of the barley homologs WRKY18 and WRKY40 is rapidly activated in response to the host-adapted powdery mildew fungus *Golovinomyces orontii* (Pandey et al. 2010). Whereas *wrky18 wrky40* double mutant plants are resistant against *G. orontii*, wild-type plants are susceptible, with strong fungal sporulation observed on infected leaf surfaces at 7 days postinfection (dpi) (Eichmann and Huckelhoven 2008; Shen et al. 2007). During the last decade, numerous powdery mildew–resistant mutants have been identified in forward genetic screens (Frye and Innes 1998; Vogel and Somerville 2000). Many of these exhibit broad-spectrum resistances or reveal constitutive defense activation (Ellis and Turner 2001; Ellis et al. 2002; Nishimura et al. 2003; Vogel and Somerville 2000; Vogel et al. 2002, 2004). Identified components of defense execution include the syntaxin PENETRATION1 (PEN1), the atypical myrosinase PEN2, and the ABC transporter PEN3 (Bednarek et al. 2009; Collins et al. 2003; Stein et al. 2006). The PEN2-dependent breakdown of the antimicrobial tryptophan (Trp)-derived indole-glucosinolate methoxyindol-3-ylmethyl-glucosinolate (4MI3G) was recently implicated in resistance towards a variety of fungal pathogens and its sequential action with camalexin, the major *Arabidopsis* phytoalexin, in restricting nonhost powdery mildew infection (Bednarek et al. 2009; Schlaeppi and Mauch 2010; Schlaeppi et al. 2010).

We previously demonstrated that lack of *WRKY18* and *WRKY40* results in a massive transcriptional reprogramming during early stages of *G. orontii* infection (Pandey et al. 2010). Expression of several positive and negative regulators of JA and SA signaling and crucial biosynthesis genes of Trp-derived antimicrobial compounds was significantly altered in resistant *wrky18 wrky40* compared with susceptible wild-type plants. In addition, camalexin was found to accumulate to high levels upon infection in *wrky18 wrky40* plants.

In this study, we provide genetic evidence that preinvasive fungal entry restriction in *wrky18 wrky40* depends on the production of the Trp-derived antimicrobial compounds camalexin and the indole-glucosinolate 4MI3G or its breakdown products. Moreover, we found late disease resistance independent of early host cell-entry restriction and *wrky18 wrky40* resistance towards *G. orontii* independent of the PEN1 secretion pathway. Furthermore, we demonstrate that loss-of-WRKY18 WRKY40–dependent pre- and postinvasive *G. orontii* resistance depends on the SA/EDS1 signaling pathway. Finally, we discovered that *wrky18 wrky40* mutant plants do not exhibit a broad-spectrum resistance towards powdery mildew fungi but reveal increased susceptibility specifically towards *P. syringae* pv. *tomato* AvrRPS4. Thus, WRKY18 and WRKY40 negatively regulate resistance against *G. orontii* but positively influence resistance against *P. syringae* pv. *tomato* AvrRPS4.

RESULTS

wrky18 wrky40 resistance against *G. orontii* is independent of preinvasive host cell-entry restriction.

In our previous study, the ERF/AP2-type redox-responsive transcription factor 1 (*RRTF1*; Khandelwal et al. 2008) displayed 12-fold higher basal transcript levels in *wrky18 wrky40* compared with wild-type plants, and binding of WRKY40 protein to the *RRTF1* promoter was demonstrated in planta (Pandey et al. 2010). To test its putative contribution to the *G. orontii* resistance phenotype, we generated homozygous *wrky18 wrky40 rrtf1* triple mutant plants. We first determined the ratio of spores showing secondary hyphae formation to all spores that formed an appressorium 48 h postinfection (hpi), indicative of successful host cell entry by the pathogen (Fig. 1A). While *rrtf1* single mutants showed wild-type-like fungal entry rates of about 70%, *wrky18 wrky40 rrtf1* triple mutants displayed a reduction in host cell entry to about 40%. However, this reduction was indistinguishable from resistant *wrky18 wrky40* double mutant plants. At later infection stages (9 dpi), loss of *RRTF1* did not alter the resistant phenotype of *wrky18 wrky40* plants characterized by the absence of macroscopically visible fungal sporulation on the leaf surface (Fig. 1B). Together these data suggest RRTF1 function to be redundant or dispensable for *wrky18 wrky40* resistance towards *G. orontii*.

We previously reported rapid and significant accumulation of the antifungal secondary metabolite camalexin and strong

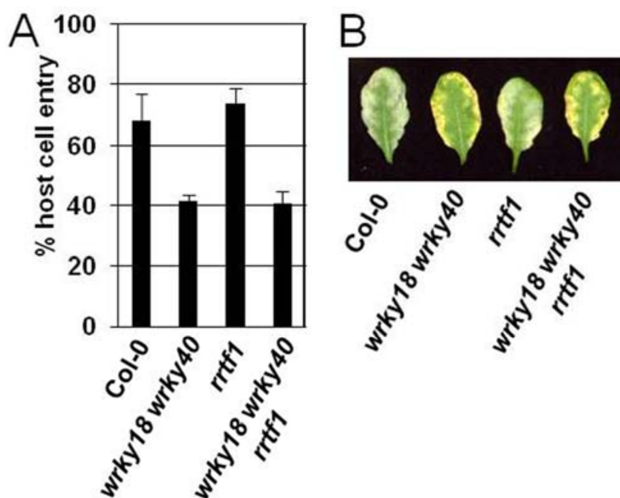

Fig. 1. *RRTF1* is dispensable for resistance of *wrky18 wrky40* plants towards *Golovinomyces orontii*. **A,** Host cell-entry rates of fungal spores determined 48 h postinoculation and **B,** phenotypic characterization of homozygous *rrtf1* single, *wrky18 wrky40* double, and *wrky18 wrky40 rrtf1* triple mutant plants at 9 days after infection with *G. orontii*. Values in A represent means ±standard deviation. *n* = 4.

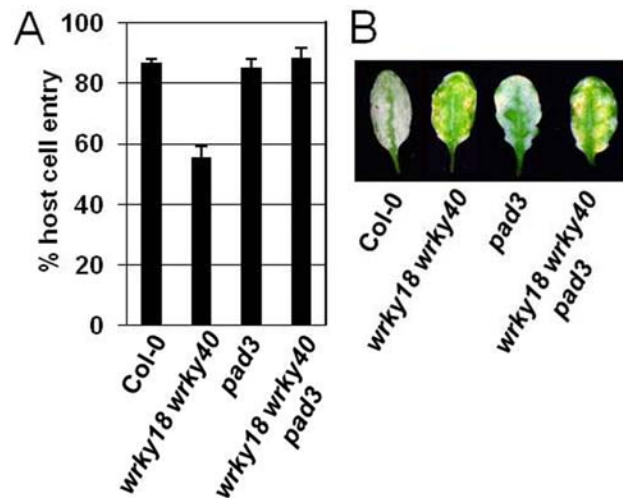

Fig. 2. *PAD3* is required for preinvasive but not for postinvasive resistance in *wrky18 wrky40* plants. **A,** Host cell-entry rates of fungal spores determined at 48 h postinoculation and **B,** phenotypic characterization of *pad3* single, *wrky18 wrky40* double, and *wrky18 wrky40 pad3* triple mutant plants at 9 days after infection with *Golovinomyces orontii*. Values in A represent means ±standard deviation. *n* = 4.

induction of the crucial biosynthesis genes *CYP71A13* and *PHYTOALEXIN-DEFICIENT3* (*PAD3*, *CYP71B15*) in resistant *wrky18 wrky40* double mutant plants within 24 h after *G. orontii* infection (Pandey et al. 2010). To test whether camalexin is an essential component of resistance in *wrky18 wrky40* plants, we generated a camalexin-deficient *wrky18 wrky40* mutant lacking the key camalexin biosynthesis gene *PAD3*. Homozygous *wrky18 wrky40 pad3* plants were infected with *G. orontii* and were examined for successful fungal host cell entry at 48 hpi. Camalexin-deficient *pad3* single mutants revealed wild-type-like fungal entry rates, whereas *wrky18 wrky40* plants showed reduced penetration rates, as expected. Indeed, knockdown of camalexin biosynthesis in the *wrky18 wrky40* double mutant background resulted in the reconstitution of wild-type-like fungal entry rates up to 90% (Fig. 2A), indicating that rapid elevation of camalexin levels in response to *G. orontii* infection in resistant *wrky18 wrky40* plants is required for early fungal host cell-entry restriction. Interestingly, however, at later infection stages, *wrky18 wrky40 pad3* triple mutants displayed a *wrky18 wrky40*-like resistant phenotype, characterized by macroscopically undetectable fungal sporulation and formation of necrotic lesions on infected leaf surfaces (Fig. 2B). In contrast, *pad3* single mutants exhibited susceptibility with wild-type-like *G. orontii* sporulation. These data demonstrate an essential role of PAD3 for preinvasive fungal host cell-entry restriction but not for later postinvasive defense against *G. orontii* in the *wrky18 wrky40* double mutant.

4MI3G biosynthesis is indispensable for pre- and postinvasive resistance of *wrky18 wrky40*.

Previous reports describe the sequential action of camalexin and the PEN2-dependent turnover of 4MI3G in *Arabidopsis* nonhost resistance (Bednarek et al. 2009; Schlaeppi et al. 2010). Another independent secretion pathway is represented by the plasma membrane (PM)-resident syntaxin PEN1, which together with soluble adaptor protein SNAP33 and vesicle-associated membrane protein VAMP721/VAMP722 forms PM-localized ternary SNARE complexes required for penetration resistance towards nonadapted powdery mildews (Collins et al. 2003; Kwon et al. 2008). To analyze putative contributions of these pathways to *wrky18 wrky40*–dependent *G. orontii* resistance, we attempted to generate the respective triple mutants. Since we failed to identify a homozygous *wrky18 wrky40 pen2* mutant, we generated triple mutants impaired in 4MI3G biosynthesis and the PEN1 secretion pathway by mutations in *CYP81F2* and *PEN1*, respectively. Fungal entry rates of *wrky18 wrky40 pen1* triple mutants did not differ from *wrky18 wrky40* double mutant plants (Fig. 3A). However, at later infection stages, resistance in *wrky18 wrky40 pen1* plants was associated with the appearance of large brownish necrotic leaf areas that were absent in resistant *wrky18 wrky40* plants (Fig. 3B). In contrast, wild-type-like penetration rates and a susceptible phenotype were reconstituted in *wrky18 wrky40 cyp81f2* triple mutants, indicating a crucial role of 4MI3G for both pre- and postinvasive *G. orontii* resistance in *wrky18 wrky40* plants (Fig. 3A and B). Detailed expression kinetic studies by quantitative

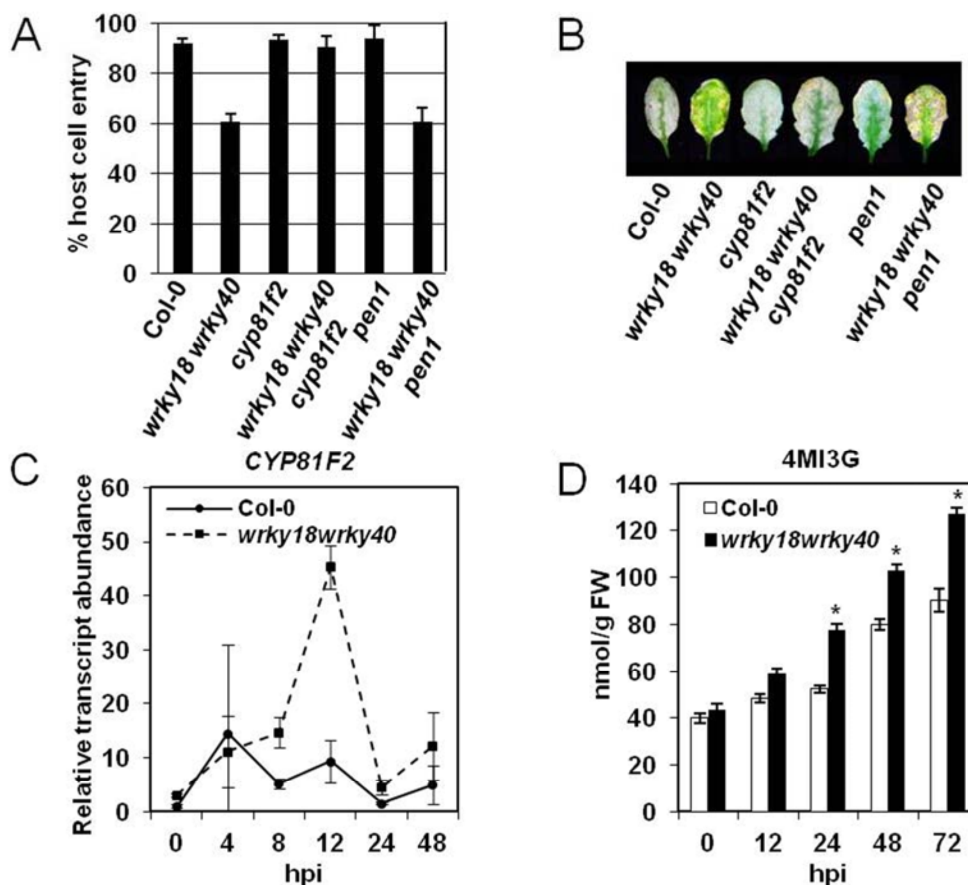

Fig. 3. 4MI3G accumulates more strongly in *wrky18 wrky40* plants and is required for the *wrky18 wrky40* resistance phenotype. **A,** Host cell-entry rates of fungal spores determined 48 h postinoculation and **B,** phenotypic characterization of *wrky18 wrky40 cyp81f2* and *wrky18 wrky40 pen1* triple mutant plants and additional indicated genotypes at 9 days postinoculation with *Golovinomyces orontii*. **C,** Temporal expression analysis of 4MI3G biosynthesis gene *CYP81F2* during *G. orontii* infection in susceptible wild-type Col-0 (solid line) and resistant *wrky18 wrky40* plants (dashed line) and **D,** 4MI3G accumulation in Col-0 (white bars) and *wrky18 wrky40* plants (black bars) during the first 72 h after *G. orontii* infection. Values represent means ±standard deviation. $n = 4$ (A and D), and 2 (C). Asterisks (*) indicate a P value < 0.01 compared with wild-type samples at the same time point based on Student's t-test.

reverse transcription polymerase chain reaction (qRT-PCR) confirmed and extended previous microarray studies showing that the crucial 4MI3G biosynthesis gene *CYP81F2* is strongly induced in the resistant double mutant upon *G. orontii* infection, with infected wild-type plants showing similar expression kinetics but with strongly reduced transcript levels (Fig. 3C). To test whether induced expression of the biosynthesis gene correlates with the production of the respective compound, 4MI3G measurements were performed in the course of *G. orontii* infection. Indeed, from 24 h onwards, resistant *wrky18 wrky40* plants accumulated significantly more 4MI3G upon *G. orontii* infection as compared with wild-type plants (Fig. 3D). At 72 hpi, *wrky18 wrky40* plants accumulate approximately 25% more 4MI3G than susceptible wild-type plants. Together, these data indicate that *wrky18 wrky40* resistance towards *G. orontii* is independent of PEN1 but requires CYP81F2 function for pre- and postinvasive fungal growth restriction.

EDS1 and SA are required for *G. orontii* resistance by *wrky18 wrky40* plants.

In vivo binding of WRKY40 to the *EDS1* promoter and altered *EDS1* transcript levels in *wrky18 wrky40* mutant plants upon infection provided hints that this central component of the plant immune system may be a vital contributor to resistance towards *G. orontii* (Pandey et al. 2010). EDS1 contributes to postinvasive nonhost resistance as well as to SA-dependent and SA-independent defense signaling in incompatible host pathogen interactions (Bartsch et al. 2006; Lipka et al. 2008; Wiermer et al. 2005). Moreover, SA-dependent defense responses acting downstream of EDS1 have been demonstrated to be induced upon *G. orontii* infection (Dewdney et al. 2000; Reuber et al. 1998). To estimate one or more EDS1 and SA contributions to *wrky18 wrky40* resistance, we first measured SA levels in *wrky18 wrky40* and wild-type plants during *G. orontii* infection. SA levels increased 24 hpi in wild-type and *wrky18 wrky40* plants and peaked 48 hpi with fourfold higher SA levels detected as compared with basal amounts (Fig. 4A). However, no variation in timing and magnitude of basal and induced SA levels were detected between the two genotypes

Table 1. Powdery mildew resistance or susceptibility in *wrky18 wrky40* mutant plants[a]

Pathogen	Col-0	*wrky18 wrky40*
Golovinomyces orontii[b]	S	R
Golovinomyces cichoracearum[b]	S	S
Golovinomyces cruciferarum[b]	S	S
Botrytis cinerea[c]	R	R
Phytophthora infestans[d]	R	R
Hyaloperonospora arabidopsidis Emco5[e]	S	S
H. arabidopsidis Emwa[e]	R	R
H. arabidopsidis Cala2[e]	R	R
Pseudomonas syringae pv. *tomato* DC3000[f]	S	S
P. syringae pv. *tomato* DC3000 AvrRPM1[f]	R	R
P. syringae pv. *tomato* DC3000 HopA1[f]	R	R
P. syringae pv. *tomato* DC3000 AvrRPS4[f]	R	S

[a] Four-week-old plants were infected with the indicated pathogens. Macroscopic characterization of infection phenotypes was performed for all pathogens. All infections were repeated at least twice with similar results. S = susceptible, R = resistant.

[b] Microscopically determined by comparing host cell-entry rates to conidiophore counts.

[c] Plant leaves were inoculated by droplet-infection and leaf lesion sizes were macroscopically compared.

[d] Susceptibility was determined by trypan blue staining of droplet-infected leaves.

[e] 10-day-old seedlings were spray-infected and susceptibility was determined by trypan blue staining.

[f] Analyzed by bacterial colony counts after spray infections.

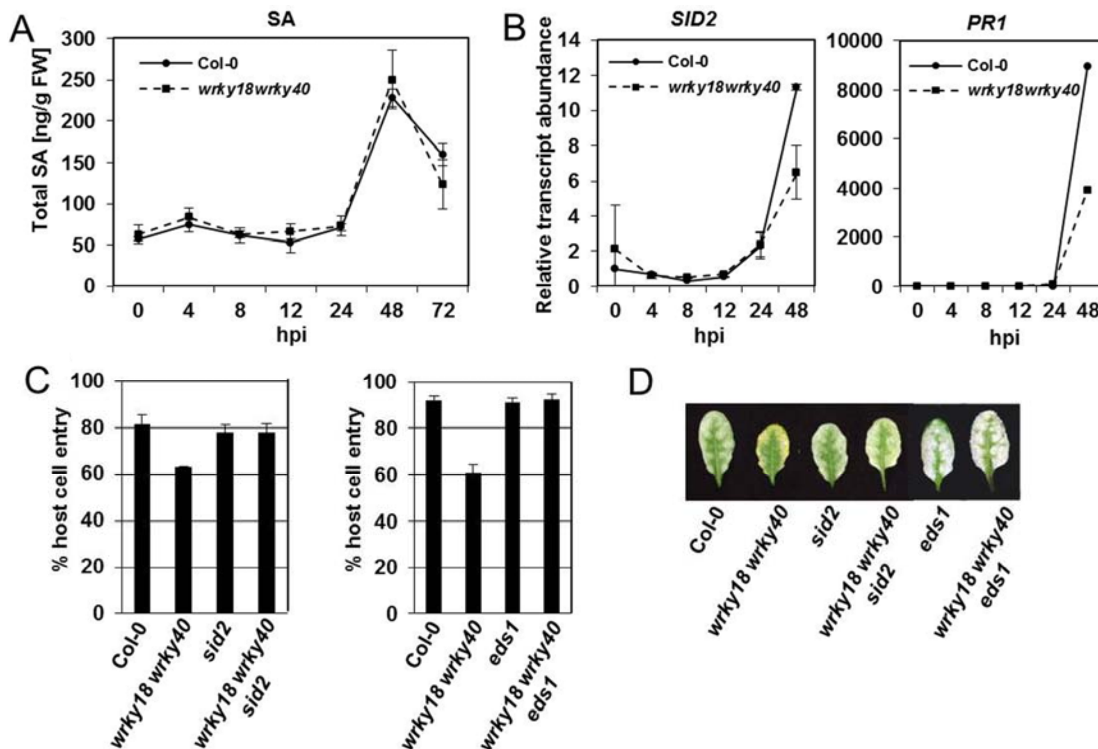

Fig. 4. Susceptible wild-type and resistant *wrky18 wrky40* plants accumulate similar levels of salicylic acid (SA) during *Golovinomyces orontii* infection but SA and EDS1 signaling is indispensable for resistance of *wrky18 wrky40* plants against *G. orontii*. **A,** SA levels during the first 72 h after *G. orontii* infection in wild-type Col-0 (solid line) and *wrky18 wrky40* plants (dashed line). **B,** Temporal expression of SA biosynthesis gene *SID2* and SA downstream marker gene *PR1* during the first 48 h after *G. orontii* infection in wild-type Col-0 (solid line) and *wrky18 wrky40* plants (dashed line). **C,** Host cell-entry rates of fungal spores at 48 h postinoculation and **D,** phenotypic characterization of *sid1*, *eds1*, *wrky18 wrky40*, *wrky18 wrky40 sid2*, and *wrky18 wrky40 eds1* mutant plants at 9 days after infection with *G. orontii*. Values represent means ±standard deviation. $n = 4$ (A), 2 (B), or 4 (C).

during the course of *G. orontii* infection. This data correlates with an invariant transcriptional activation of the SA biosynthesis gene *SID2/ICS1* in both genotypes in response to *G. orontii* infection (Fig. 4B). Next, we measured induction of the downstream marker gene of SA signaling *PATHOGENESIS RELATED1* (*PR1*) (Fig. 4B). Both genotypes showed a clear induction of *PR1* transcript at 48 hpi. Thus, altered *EDS1* transcription and loss of WRKY18 and WRKY40 function in *G. orontii* resistant had no direct detectable effect on SA levels and induction of SA-dependent downstream signaling. Despite these findings, additional SA- and EDS1-dependent processes may still be altered in *wrky18 wrky40* plants as compared with wild-type plants. Alternatively, SA and EDS1 may act upstream of WRKY18 and WRKY40 and, hence, contribute to *G. orontii* resistance in *wrky18 wrky40* plants. To test this, we generated *wrky18 wrky40 eds1* and *wrky18 wrky40 sid2* triple mutants, respectively. As described above, susceptible wild-type plants displayed fungal entry rates of around 90% and similar ratios were observed for *eds1* and *sid2* single mutants (Fig. 4C). In contrast, fungal entry rates were significantly reduced to 65% in the *wrky18 wrky40* double mutant. However, loss of *EDS1* or *SID2* in the *wrky18 wrky40* background restored fungal entry rates to wild-type-like levels (Fig. 4C), demonstrating that *wrky18 wrky40*–dependent enhanced preinvasive resistance towards *G. orontii* depends on functional EDS1 and SID2. At later infection stages, *wrky18 wrky40 sid2* and *wrky18 wrky40 eds1* triple mutant plants exhibited a wild-type-like susceptible phenotype (Fig. 4D). These findings demonstrate that, although *G. orontii*–induced SA levels do not differ between resistant *wrky18 wrky40* and susceptible wild-type plants, both SA and EDS1 are required for pre- and postinvasive defense against *G. orontii* in *wrky18 wrky40* plants.

***wrky18 wrky40* mutant plants do not exhibit broad-spectrum resistance.**

Accumulation of antimicrobial secondary metabolites and *G. orontii*–induced activation of major defense pathways in *wrky18 wrky40* plants may suggest a broad-spectrum resistance against powdery mildews, as was reported for other mutant plants (Consonni et al. 2006; Ellis et al. 2002; Nishimura et al. 2003; Vogel and Somerville 2000). Therefore, we investigated the virulence of two additional *Arabidopsis*-colonizing powdery mildew species, *G. cichoracearum* and *G. cruciferarum*, on the *wrky18 wrky40* double mutant. However, in macroscopic analyses, *wrky18 wrky40* plants appeared as susceptible as wild-type plants towards both powdery mildew fungi (Table 1). This was verified by wild-type-like conidia formation on *wrky18 wrky40* plants (Supplementary Fig. 1). Thus, *wrky18 wrky40* plants do not exhibit a broad-spectrum resistance against powdery mildews.

Generally, WRKY18 and WRKY40 are thought to act as repressors of basal defense (Shen et al. 2007). It remains unclear, however, whether both WRKY factors generally antagonize basal resistance against a broad spectrum of invading microbes

Table 2. List of pathogens used in this paper

Name	Provided by
Golovinomyces orontii	V. Lipka, Georg-August-University, Göttingen. Germany
G. cichoracearum	V. Lipka, Georg-August-University, Göttingen. Germany
G. cruciferarum	V. Lipka, Georg-August-University, Göttingen. Germany
Pseudomonas syringae pv. *tomato* DC3000	R. Innes, Indiana University, Bloomington, Indiana, U.S.A.
P. syringae pv. *tomato* DC3000 AvrRPS4	K. Heidrich, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
P. syringae pv. *tomato* DC3000 AvrRPM1	K. Heidrich, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
P. syringae pv. *tomato* DC3000 HopA1	K. Heidrich, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
Hyaloperonospora arabidopsidis Emco5	J. Parker, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
H. arabidopsidis Emwa1	J. Parker, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
H. arabidopsidis Cala2	J. Parker, Max-Planck Institute for Plant Breeding Research, Cologne, Germany
Phytophtora infestans	D. Scheel, Leibnitz Institute for Plant Biochemistry, Halle Germany
Botrytis cinerea (isolate 2100)	R.Birkenbihl, Max-Planck Institute for Plant Breeding Research, Cologne, Germany Spanish Type Culture, Collection Universidad de Valencia, Burjassot, Spain

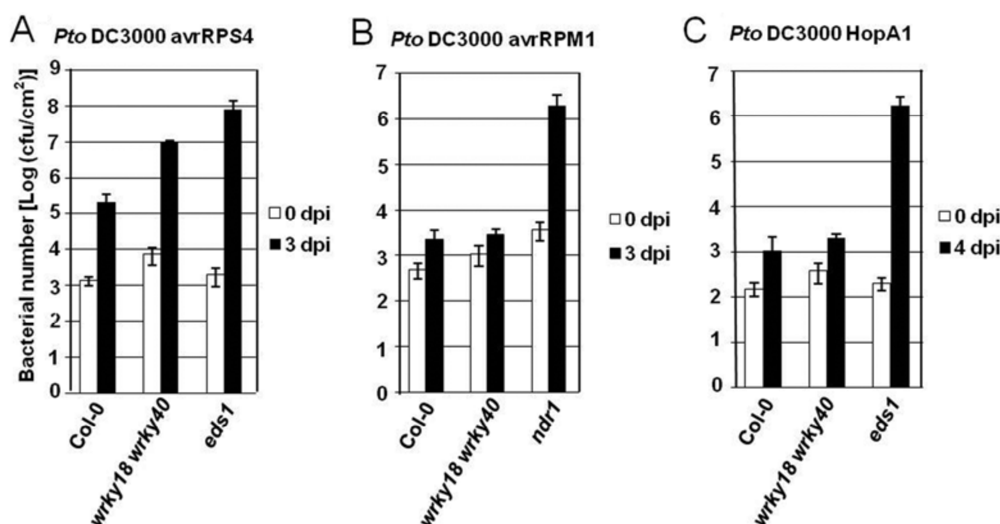

Fig. 5. Increased susceptibility of *wrky18 wrky40* plants towards *Pseudomonas syringae* pv. *tomato* AvrRPS4 infection. Four-week-old wild-type and *wrky18 wrky40* plants were spray-infected with the bacterial strains *P. syringae* pv. *tomato* AvrRPS4, *P. syringae* pv. *tomato* AvrRPM1, and *P. syringae* pv. *tomato* HopA1. Bacterial counts were performed **A,** at 3 days after *P. syringae* pv. *tomato* AvrRPS4 and **B,** *P. syringae* pv. *tomato* AvrRPM1 and **C,** at 4 days after *P. syringae* pv. *tomato* HopA1 infection. The *eds1* and *ndr1* single mutant plants were included as susceptible controls. Values represent means ±standard deviation (n = 4). The experiments were repeated three times with similar results.

or whether they modulate the complex interplay of rather specific processes favorable for colonization of a few but inappropriate for other pathogenic intruders. We therefore tested the *wrky18 wrky40* mutant for resistance or increased susceptibility against distinct pathogens and compared these infection phenotypes with those of wild-type plants after challenge (Table 2). First we infected wild-type and *wrky18 wrky40* plants with the compatible oomycete *Hyaloperonospora arabidopsidis* isolate Emco5 and the virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000. We did not observe any clear difference in the growth rate of *P. syringae* pv. *tomato* DC3000 or the macroscopical infection phenotype of *H. arabidopsidis* Emco5 between wild-type and double mutant plants (Supplementary Fig. 2). We further tested the incompatible oomycete *Phythophtora infestans* and the necrotrophic fungus *Botrytis cinerea* isolate 2100. Again, both genotypes did not differ in the outcome of the infection, showing a resistant phenotype (Table 1). Thus, simultaneous knockout of *WRKY18* and *WRKY40* does not alter resistance or susceptibility towards these pathogens.

In barley, the effector-dependent interaction of MLA10 with *WRKY* factors 1 and 2 provides a direct link between basal defense and R protein–mediated resistance (Shen et al. 2007). To test whether *wrky18 wrky40* plants are impaired in R protein–mediated defense, wild-type and *wrky18 wrky40* plants were infected with two *P. syringae* pv. *tomato* strains expressing either the bacterial effector *AvrRPM1* or *AvrRPS4*. Interestingly, infection with the avirulent bacterial strain *P. syringae* pv. *tomato* AvrRPS4 revealed an increased susceptibility of *wrky18 wrky40* plants, allowing about tenfold more bacterial growth as compared with resistant wild-type plants at 3 dpi (Fig. 5A). In contrast, resistance towards *P. syringae* pv. *tomato* AvrRPM1 was not altered in *wrky18 wrky40* plants (Fig. 5B).

As WRKY40 protein binds to the promoter region of *EDS1*, it is conceivable that mutations in one or both *WRKY18* or *WRKY40* affect EDS1 signaling. Therefore, *wrky18 wrky40* plants were challenged with additional pathogens against which resistance is known to depend on TIR-NB-LRR-type R proteins and functional EDS1. Resistance against *H. arabidopsidis* isolates Emwa1 and Cala2 is mediated by the TIR-NB-LRR R proteins RPP2 and RPP4, respectively, and both are EDS1-dependent (Slusarenko and Schlaich 2003). Infection with these two isolates revealed no phenotypic differences between wild-type and *wrky18 wrky40* plants (Table 1). Together, this data suggests that increased susceptibility of *wrky18 wrky40* plants towards *P. syringae* pv. *tomato* AvrRPS4 is 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–*P. syringae* pv. *tomato* interactions, a second avirulent strain, *P. syringae* pv. *tomato* HopA1, was used for infection of *wrky18 wrky40* mutant and wild-type plants. Resistance of wild-type Col-0 plants towards *P. syringae* pv. *tomato* HopA1 is also mediated by a TIR-NB-LRR-type R protein and requires functional EDS1 (Kim et al. 2009). However, infections of both genotypes revealed no differences in the macroscopic phenotype and bacterial colonization (Fig. 5C). Thus, *wrky18 wrky40* plants reveal a rather specific susceptibility phenotype against *P. syringae* pv. *tomato* AvrRPS4 infection that was not observed with other tested *P. syringae* pv. *tomato* strains and, on the other hand, a resistant phenotype against *G. orontii* without exhibiting a broad-spectrum resistance against other powdery mildews.

DISCUSSION

Previous work on *wrky18 wrky40* focused on the transcriptional changes occurring during *G. orontii* infection and in identifying gene expression differences between susceptible wild-type and resistant *wrky18 wrky40* plants (Pandey et al. 2010). In the current work, we performed genetic studies to determine crucial components required for loss-of-*WRKY18 WRKY40*–mediated *G. orontii* resistance. Several key findings were obtained.

Firstly, we uncovered an important role of *PAD3* for preinvasive but not for postinvasive defense. The *wrky18 wrky40 pad3* triple mutant exhibited wild-type-like penetration rates but showed a late *wrky18 wrky40*-like resistance phenotype. These data point to an active suppression of camalexin biosynthesis by *G. orontii* in a WRKY18- and WRKY40-dependent manner and an inability of *G. orontii* to degrade or tolerate camalexin as described for other fungi (Glawischnig 2007; Pandey et al. 2010). Notably, uninfected *wrky18 wrky40* plants already accumulate higher camalexin levels than wild-type plants (Pandey et al. 2010). Therefore, deregulation in timing and magnitude of camalexin accumulation may, at least in part, be the molecular basis for the preinvasive resistant phenotype observed in *wrky18 wrky40*. Additionally, our data suggest that camalexin-dependent preinvasive fungal restriction can be uncoupled from postinvasive resistance and, thereby, relegating effectiveness of camalexin accumulation to an early antifungal defense. However, host cell-entry restriction during the interaction of *G. orontii* with *wrky18 wrky40* plants appears to be rather weak. In *wrky18 wrky40* plants, 60% of the spores still enter the host tissue, whereas, for example, *mlo2* or *mlo2 mlo6 mlo12* mutant plants show 30% and less than 5% fungal entry success, respectively (Consonni et al. 2006). Thus, this preinvasive host-defense mechanism is insufficient to explain the explicit postinvasive resistance phenotype of *wrky18 wrky40* plants observed at later stages of fungal infection.

Secondly, induced expression of the 4MI3G biosynthesis gene *CYP81F2* correlated with elevated 4MI3G levels in *wrky18 wrky40* plants upon *G. orontii* infection and a requirement of 4MI3G for both pre- and postinvasive defense. Bednarek and colleagues (2009) described the consecutive requirement of various Trp-derived indole-glucosinolates for pre- and postinvasive defense against fungal nonhost pathogens. However, a direct role of 4MI3G in antifungal defense remains to be demonstrated. 4MI3G itself may have a yet-unknown signaling function required for resistance of the *wrky18 wrky40* mutant, as it was hypothesized to act as a signaling molecule for MAMP-induced callose formation (Clay et al. 2009). Alternatively, altered 4MI3G levels may influence the level of additional activated compounds, thereby affecting the outcome of the infection. The atypical myrosinase PEN2 was shown to be crucial for 4MI3G breakdown to an unknown compound and to be essential for pathogen-dependent callose deposition and resistance (Bednarek et al. 2009; Clay et al. 2009). PEN2 and CYP81F2 activities were also shown to be required for effective defense against nonadapted powdery mildews and other fungal pathogens (Bednarek et al. 2009; Maeda et al. 2009). Sanchez-Vallet and colleagues (2010) demonstrated a crucial role of CYP81F2 and PEN2 for nonhost resistance against the adapted necrotrophic fungal isolate *Plectospharella cucumerina* BMM, whereas resistance responses to nonadapted isolates were unaffected in *cyp81f2* and *pen2* mutants. On the other hand, mutations in *CYP79B1* and *CYP79B2* acting upstream of CYP81F2 and PEN2 in the Trp-derived indole-glucosinolate biosynthesis pathway revealed increased susceptibility also towards nonadapted isolates, strongly suggesting additional PEN2-independent indolic products with antifungal activities (Sanchez-Vallet et al. 2010). As homozygous *wrky18 wrky40 pen2* plants could not be identified, we currently cannot distinguish between the requirement of 4MI3G, its PEN2 breakdown product, or other derivatives for *G. orontii* resistance of *wrky18 wrky40* mutant plants.

Thirdly, the PEN1 secretory pathway does not play a vital role in *wrky18 wrky40*–dependent *G. orontii* resistance. The membrane-associated syntaxin PEN1 focally accumulates in papillae formed underneath fungal entry sites of adapted and nonadapted powdery mildews but not in papillae triggered by the entry attempts of other ascomycetes (Assaad et al. 2004; Bhat et al. 2005; Meyer et al. 2009; Shimada et al. 2006). The mechanism underlying PEN1-mediated preinvasive resistance appears to be rather specific for nonhost powdery mildews such as *B. graminis* f. sp. *hordei* and *Erysiphe pisi* (Collins et al. 2003; Lipka et al. 2005), which is consistent with our findings that penetration resistance was completely unaffected in *wrky18 wrky40 pen1* plants. However, at later infection stages, resistance in *wrky18 wrky40 pen1* plants was associated with the appearance of large necrotic leaf areas that were absent in resistant *wrky18 wrky40* plants. Postpenetration resistance is often associated with the appearance of a hypersensitive response at the site of infection. Posthaustorial powdery mildew resistance was shown to depend on a set of genes including *EDS1*, *PAD4*, and *SAG101* (Lipka et al. 2005; Stein et al. 2006). EDS1 is required for the promotion of leaf cell death, contributes to basal defense and systemic resistance, and was differentially expressed in naive and *G. orontii*–infected *wrky18 wrky40* plants (Attaran et al. 2009; Pandey et al. 2010; Rusterucci et al. 2001; Vlot et al. 2008). Thus, the appearance of large necrotic areas in *wrky18 wrky40 pen1* plants might be the consequence of an altered basal or systemic defense programs or both.

Fourthly, both EDS1 and the SA signaling pathway, which have been associated with defense against powdery mildew fungi, impact resistance of *wrky18 wrky40* against *G. orontii* (Chandran et al. 2009; Dewdney et al. 2000; Lipka et al. 2005; Pandey et al. 2010). Yet, despite a deregulation of *EDS1* expression in *wrky18 wrky40* plants, expression of the crucial SA biosynthesis gene *SID2*, SA accumulation, and induction of the downstream SA marker gene *PR1* did not differ in resistant *wrky18 wrky40* and susceptible wild-type plants upon *G. orontii* infection. It is noteworthy that *G. orontii* only infects epidermal cells and our expression studies and hormone measurements were performed in whole leaf samples. Thus, it is conceivable that local differences in gene expression or hormone levels between infected and uninfected cells are masked. Nevertheless, activation of SA signaling upon *G. orontii* is in agreement with earlier reports demonstrating the induction of 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). In our analyses, the SA signaling pathway is activated after fungal penetration (approximately 48 hpi) in both genotypes, consistent with previous studies showing that the SA signaling pathway of the host plays a more important role at later infection stages (Chandran et al. 2009; Dewdney et al. 2000; Reuber et al. 1998). However, reconstitution of wild-type-like penetration rates in *wrky18 wrky40 sid2* plants points to a role of SA also for early penetration resistance in *wrky18 wrky40* mutant plants. Possibly, SA may influence processes required for penetration resistance in a loss-of-WRKY18 WRKY40-dependent manner. Clearly, the role of SA for preinvasive defense requires further investigation. However, at later infection stages, *wrky18 wrky40 sid2* plants revealed wild-type-like fungal proliferation, demonstrating a crucial role of SA for postinvasive resistance of *wrky18 wrky40* mutant plants. This is supported by recent studies reporting that SID2 and PAD4 are required for postinvasion resistance in *Arabidopsis* (Wen et al. 2011). Moreover, as *WRKY18* and *WRKY40* expression is strongly induced upon exogenous SA treatment (Dong et al. 2003), we hypothesize that processes influenced by loss-of-*WRKY18* and *WRKY40* act downstream of EDS1 and SA in defense against *G. orontii*.

In contrast to *G. orontii*, *wrky18 wrky40* plants did not show increased resistance against two other tested biotrophic powdery mildews. The *wrky18 wrky40* mutant does not constitutively express defense-associated genes at high levels, and the absence of broad-spectrum resistance clearly separates *wrky18 wrky40*–dependent *G. orontii* resistance from other powdery mildew resistant mutants (Pandey et al. 2010). Many of these mutants, including, *mlo2*, *mlo2 mlo6 mlo12*, *pmr4*, *edr1*, or *cev1*, reveal broad-spectrum resistances against powdery mildews caused by loss of susceptibility factors (Consonni et al. 2006), constitutive defense gene expression (Ellis and Turner 2001; Flors et al. 2008; Nishimura et al. 2003), or altered cell-wall compositions (Ellis et al. 2002; Nishimura et al. 2003; Vogel et al. 2002, 2004), which in some cases also leads to increased resistance against diverse pathogens. The *edr1* and *edr2* mutants exhibit broad-spectrum resistance against powdery mildews without constitutive defense activation but also confer resistance against other pathogens, including *P. syringae* pv. *tomato* DC3000 (Flors et al. 2008), which was not observed for *wrky18 wrky40* plants. Strikingly, however, infection of *wrky18 wrky40* plants with *P. syringae* pv. *tomato* AvrRPS4 showed clearly increased susceptibility towards this bacterial pathogen. This susceptibility phenotype appears specific to *P. syringae* pv. *tomato* AvrRPS4, since it was not observed with any other *P. syringae* pv. *tomato* strain tested. As the *wrky18 wrky40* mutant did not exhibit broad-spectrum resistance against powdery mildews and was not generally impaired in R protein–mediated resistance, we hypothesize rather specific defense perturbations during G. *orontii* and *P. syringae* pv. *tomato* AvrRPS4 infections. It is conceivable that these pathogens differentially affect distinct responses in *wrky18 wrky40* plants, as both pathogens secrete a substantial repertoire of unique effectors into the plant cell during infection (Lindeberg et al. 2009, 2012; Spanu et al. 2010). Recently direct physical association of the secreted effector AvrRPS4 and the R protein RPS4 with EDS1 in the plant cell nucleus was demonstrated (Heidrich et al. 2011). Moreover, interaction between an R protein and WRKY transcription factors is the basis for MLA-mediated resistance in barley upon *B. graminis* f. sp. *hordei* infection (Shen et al. 2007). We tested but failed to observe interaction of WRKY18 and WRKY40 with full-length RPS4 and truncated versions containing only the N-terminal TIR domain in yeast and in coinfiltration experiments in *Nicotiana benthamiana* epidermal cells, irrespective of the presence or absence of the cognate effector protein (data not shown). Thus, it remains unclear with which host components WRKY18 and WRKY40 are mechanistically linked for effective defense against *P. syringae* pv. *tomato* AvrRPS4 or which key genes required for resistance are regulated by these transcription factors.

Future studies will need to address several important aspects related to the negative role of WRKY18 and WRKY40 in *G. orontii* resistance and its positive function in mediating resistance towards *P. syringae* pv. *tomato* AvrRPS4. Recently, several *G. orontii*–specific secreted effector candidates were identified that interfere with the plant immune response (Spanu et al. 2010; Wessling et al. 2012). Whether WRKY18 or WRKY40 are targets of such effectors needs to be tested. Moreover, a more detailed analysis of altered host secondary metabolites, in particular of glucosinolates, in *wrky18 wrky40* plants is required. Additionally, by focusing on identifying proteins interacting with WRKY18 and WRKY40 and by pinpointing the in vivo promoter targets of these two transcription factors during the infection with *G. orontii* and *P. syringae* pv. *tomato* AvrRPS4, we hope to uncover some of the missing components that are essential in determining the distinct outcomes observed in the host. Finally, it is obvious that we must position these transcription factors in the increasingly complex regu-

latory network, in order to understand how their functions influence, both positively and negatively, host cellular responses.

MATERIALS AND METHODS

Plant material.

All experiments were performed using *Arabidopsis thaliana* Columbia-0 (Col-0) wild-type plants or mutants in the Col-0 background. The *wrky18 wrky40* mutant published by Pandey and associates (2010) was used for all experiments. Triple mutants were generated by crossing *wrky18 wrky40* homozygous plants with *sid2-1*, *eds1-2*, *rrtf1*, *pad3-1*, *pen1-1*, *cyp81f2-1* homozygous plants. Homozygous triple mutants were identified by PCR or via cleaved amplified polymorphic sequences (CAPS) or both and were used for further experiments.

Pathogens.

Pathogens and their sources are listed in Table 2.

Powdery mildew infections.

Golovinomyces orontii was propagated on *pad4 sag101* double mutant *Arabidopsis* plants in closed cabinets under short-day conditions (10 h light and 14 h darkness) at 22°C with 70% humidity. Four-week-old plants were used for infections. All infections were performed by dusting without touching the plants, to avoid wound responses. A brush was used to spread spores over the plant. Infected plants were further kept under short-day conditions. Control samples (0 hpi) were taken before inoculation with the fungus. *G. cichoracearum* and *G. cruciferarum* were propagated on their natural host plants, zucchini (Gold rush) and *Brassica juncea*, respectively, under short-day conditions (8 h light and 16 h darkness) at 22°C with 80% humidity.

Powdery mildew penetration counts.

Infected leaf samples were taken 48 hpi, and chlorophyll was destained in an EtOH/acetic acid solution (3:1) for at least 24 h. To visualize epiphytic fungal growth, four leaves of one biological replicate (individual plant) of a representative experiment were stained in Coomassie Brilliant Blue solution (45% MeOH, 10% acetic acid, 0.05% Coomassie R 250) and were 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 ±standard deviation (SD) of one representative experiment with four individual plants ($n = 4$).

Powdery mildew conidiophore counts.

For conidiophore counts, at least nine leaves of three individual plants ($n = 3$) were harvested at 7 dpi and were 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 were prepared for microscopy. Microscopy was performed using a Leica epiflourescence microscope (Leica GmbH, Wetzlar, Germany). Transmitted light and filter A (BP 340 to 380) was used for conidiophore counting. This was done by determining the number of conidiophores of single sporulated spores at 7 dpi.

***Pseudomonas syringae* infections.**

For *Pseudomonas syringae* infections, bacteria were plated from glycerol stocks on fresh NYGA (per liter: 5 g of bactopeptone, 3 g of yeast extract, 20 ml of glycerol, with 15 g of agar) agar plates containing the appropriate antibiotics. Bacteria were grown for 2 days at 28°C, were plated again on fresh NYGA agar plates, and were incubated overnight at 28°C. Plants were kept under a hood sprayed with water, to increase humidity and induce stomata opening, for 3 h before infection. Infections were performed as described earlier (Heidrich et al. 2011), repeated at least three times. Values represent means ±SD of a representative experiment with four individual plants ($n = 4$).

***H. arabidopsidis* infections.**

A concentration of 4×10^4 spores/ml in H_2O was used for infection of 10-day-old plants. Infected plants were incubated in sealed trays under short-day conditions (10 h light and 14 h darkness, 22°C, 70% humidity) for 7 days. Afterwards, 20 to 30 leaves were harvested into 10-ml reaction tubes and were transferred to a 1:1 trypan blue/EtOH solution for staining of oomycete structures. Leaf samples were boiled for 5 min and trypan blue/EtOH solution was removed and exchanged by chloralhydrate for destaining plant leaves. Afterwards, chloralhydrate was removed and leaves were prepared for transmitted light microscopy. Infections were repeated at least twice with similar results.

***Phytophthora infestans* infections.**

Phytophthora infections were carried out with two different concentrations. Four-week-old plants were infected with a solution of 500 or 50 spores/µl in H_20, and 10 µl were used for drop-infection of plant leaves. Leaf samples were harvested 3 days after inoculation and were stained with trypan blue as described by Lipka and associates (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. Leaves infected with low spore concentrations were investigated by transmitted light microscopy. Infections were repeated twice with similar results.

***Botrytis cinerea* infections.**

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

Quantitative real-time PCR (qPCR).

qPCR analyses were performed with cDNA corresponding to approximately 15 ng of RNA before reverse transcription on an iQ5 multicolor real-time PCR detection system using iQ5 SYBR Green Ready Mix (BioRad, Munich). Gene-specific primers were designed with Primer3 software (Rozen and Skaletsky 2000) and were analyzed using NetPrimer (PREMIER Biosoft International, Palo Alto, CA, U.S.A.). Primers were used with a NetPrimer rating of 85 to 100, as recommended by Czechowski and associates (2005). Expressed gene AT4G26410 was used as reference (Czechowski et al. 2005). qPCR results were analyzed by the $\Delta\Delta C_t$ method as described earlier (Livak and Schmittgen 2001). Fold changes were calculated relative to wild-type untreated (0 hpi) samples set to 1. Data are shown as the mean ±SD from two biological and two technical replicates ($n = 2$). All expression kinetic experiments were repeated at least twice with similar results.

Hormone measurements.

Four-week-old plants were used for free SA measurements. Plant leaf material from four individual plants (200 mg) was collected ($n = 4$), was frozen in liquid nitrogen, and was stored at –80°C until use. Frozen tissue was transferred to FastPrep tubes (Qbiogene, Carlsbad, CA, U.S.A.) containing 900 mg of FastPrep lysing matrix and 1 ml of ethyl acetate spiked with 200 ng of D4-SA as internal standards. Samples were homogenized by reciprocal shaking (FastPrep speed 6.5) twice for 45 s each time and were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was transferred to fresh 2-ml reaction tubes, and the extraction was repeated once by adding 0.5 ml of ethyl acetate without internal standard to the same tissue, followed by a centrifugation step at 13,000 rpm for 20 min at 4°C. Both supernatants were combined and were evaporated, using a vacuum concentrator until dryness at 30°C. Dried samples were dissolved in 500 µl of 70% MeOH, were mixed for at least 5 min, and were subsequently centrifuged at 13,000 rpm for 10 min. supernatant (400 µl) was transferred into fresh high-pressure liquid chromatography (HPLC) vials. Measurements were conducted on a liquid chromatography tandem mass spectrometry system (Varian 1200; Agilent Technologies, Santa Clara, CA, U.S.A.).

Secondary metabolite measurements by HPLC.

Plant leaf tissue (200 mg) was collected and frozen in liquid nitrogen until use. Dimethyl sulfoxide (10 µl per 4 mg of fresh weight material) and 10 to 30 Zirconia Beads (BioSpec, Bartlesville, OK, U.S.A.) were added to the samples and were homogenized for 30 s in a BeadBeater (BioSpec). Homogenized samples were centrifuged at 15,000 rpm at 4°C for 20 min, 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.

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