Members of the *Arabidopsis* WRKY Group III Transcription Factors Are Part of Different Plant Defense Signaling Pathways

Monika Kalde, Meike Barth, Imre E. Somssich, and Bernadette Lippok

Max-Planck-Institut für Züchtungsforschung, Abteilung Molekulare Phytopathologie, Carl-von-Linné Weg 10, D-50829 Köln, Germany

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WRKY proteins are a large group of transcription factors restricted to the plant kingdom. In *Arabidopsis thaliana*, the gene family consists of 74 members. Here, we analyzed the expression of all 13 members of one main WRKY subgroup and found that the majority are responsive both to pathogen infection and to salicylic acid. Temporal expression studies during compatible, incompatible, and nonhost interactions and employing plant defense-signaling mutants allowed us to define four distinct WRKY subsets responding to different signaling queues along defense pathways. These subsets did not reflect phylogenetic relationships. Promoter studies of one member, *AtWRKY54*, using a reporter gene construct in transgenic *Arabidopsis* plants, revealed that regulatory regions mediating pathogen and SA inducibility are clearly separable. In an *AtWRKY54* knockout line, resistance to *Peronospora parasitica* was not compromised, but the transient expression kinetics of several WRKY genes was affected, suggesting both the existence of functional redundancy and intense cross-talk between signaling networks.

Additional keywords: ascomycete *Blumeria graminis*, *nahG*, oomycete, *pad4*, *RPP2*, *RPP4*.

The mechanisms protecting the majority of plants against most potential microbial pathogens are highly regulated processes. An important step in their regulation is the transcriptional activation or repression of numerous defense-related genes by distinct transcription factors. Previous studies have identified several transcription factors that are involved in plant defense, including members of plant-specific families like NAC proteins (Ren et al. 2000), the group of ethylene-responsive element-binding proteins (Gu et al. 1992), and WRKY proteins (Eulgem et al. 2000).

The family of WRKY proteins is becoming more and more a subject of interest. Members of this family have been identified in numerous plant species, e. g., wild oat (Rushton et al. 1995), parsley (Rushton et al. 1996), sweet potato (Ishiguro and Nakamura 1994), potato (Dellagi et al. 2000), tobacco (Wang et al. 1998), rice (Kim et al. 2000), winter bittersweet nightshade (Huang and Duman 2002), orchardgrass (Alexandrova and Conger 2002), desert legume (Pnueli et al. 2002), and *Arabidopsis thaliana* (de Pater et al. 1996). WRKY

proteins have been shown to be strongly involved in the alteration of gene expression in response to microbial pathogens, pathogen elicitors, and viruses and upon treatment with signaling substances such as salicylic acid (SA) (Asai et al. 2002; Chen and Chen 2002; Dellagi et al. 2000; Eulgem et al. 1999; Yoda et al. 2002).

A common feature of all WRKY proteins is the WRKY domain, a highly conserved stretch of about 60 amino acids. Each WRKY domain contains a C-terminal located novel zinc finger and the strictly conserved amino acid sequence WRKYGQK at its N-terminus. This sequence is required for proper folding of the zinc finger and, therefore, is coresponsible for DNA binding (Maeo et al. 2001). The WRKY domain binds specifically to various W box elements, all containing a TGAC core sequence (de Pater et al. 1996; Rushton et al. 1995, 1996; Wang et al. 1998). W boxes have been identified in the promoters of many defense-related genes, including the well studied *PR* genes (Maleck et al. 2000; Rushton et al. 1996) and within WRKY gene promoters themselves, thereby suggesting that WRKY factors can regulate their own expression (Eulgem et al. 1999). Recently, putative target genes for WRKY factors, encoding receptor-like kinases (Asai et al. 2002; Du and Chen 2000; Ohtake et al. 2000; Robatzek and Somssich 2002) and an ankyrin-repeat protein (NPR1) (Yu et al. 2001), have been identified. However, the physiological function of most of the WRKY factors identified so far remains unclear.

In *Arabidopsis*, 74 members of the WRKY family are present (AGI 2000). These factors have been arranged in three distinct groups depending on the number and type of their WRKY domains (Eulgem et al. 2000). In this study, the transcriptional regulation of WRKY group III, consisting of 13 members, was analyzed and compared under different conditions for the first time. Group III differs from groups I and II in its altered C2-HC zinc finger motif C-X7-C-X23-H-X-C. The results presented here show that the transcriptional activation upon pathogen attack and treatment with SA is a common feature of most of the group III WRKY genes. Furthermore, most of these genes are expressed during both nonhost and *R* gene-dependent resistance. The analysis of mutants lacking key components of plant defense pathways showed that even closely related WRKY members display strong differences in their expression patterns and are most likely involved in distinct signaling pathways. A detailed analysis of one member, *AtWRKY54*, revealed that different promoter regions are responsible for its induction after treatment with SA or upon inoculation with pathogens, respectively.

RESULTS

Phylogenetic relationship of group III WRKY type factors.

Originally, 14 out of the 74 *Arabidopsis* WRKY factors were classified as group III members (Table 1). However, AtWRKY52 (also designated RRS1), proved to be rather exceptional. Not only does it have an altered zinc finger motif (C2-H2), but in addition, it contains other domains such as leucine-rich repeats and a motif with similarity to the signaling domain of the *Drosophila* Toll protein. Both types of motifs are known from *R* gene products but are usually not present in other WRKY factors, with the exception of AtWRKY16 (Deslandes et al. 2002). Because it does not fit neatly into group III but might represent a novel group within the *Arabidopsis* WRKY family, AtWRKY52 was not a subject in this study. A phylogenetic tree, based on amino acids of their highly conserved WRKY domains, shows that the remaining 13 proteins can be further subdivided into two subgroups (Fig. 1), group IIIa (AtWRKY38, AtWRKY62, AtWRKY63, AtWRKY64, AtWRKY66, and AtWRKY67) and IIIb (AtWRKY30, AtWRKY41, AtWRKY46, AtWRKY53, AtWRKY54, AtWRKY55, and AtWRKY70).

Although WRKY proteins do not share extended sequence homologies outside of the WRKY domain, some members contain other conserved amino acid patterns (Eulgem et al. 2000). The analysis for such conserved regions within the amino acid sequences of group III proteins was performed using the MEME and MAST program versions 3.0 (Bailey and Elkan 1994; Bailey and Gribskov 1998). Only members of group IIIa share a highly conserved region of about 50 amino acids located N-terminal to the WRKY domain. Furthermore, we found a second conserved sequence of 30 amino acids that is located C-terminal to the WRKY domain. This sequence is only present in AtWRKY63, AtWRKY64, AtWRKY66, and AtWRKY67, which group on a group IIIa sub-branch in the phylogenetic tree (Fig. 1). No putative functions can be assigned to these regions. Since both regions are strongly charged and acidic (pI of 4.35 and 3.65, respectively), they may represent transactivation domains. A BLASTP search using these sequences revealed that they are not present in any other known proteins. These results indicate that the relatedness of different WRKY proteins within group III is reflected by both their highly conserved WRKY domain and other conserved regions outside of this domain.

Table 1. Genomic loci and protein accession numbers of the original 14 group III WRKY type proteins from *Arabidopsis thaliana*

Protein name	Genomic locus	EMBL accession numbers
AtWRKY30	At5g24110	AAK96196
AtWRKY38	At5g22570	AAL35288
AtWRKY41	At4g11070	AAL35289
AtWRKY46	At2g46400	AAK96020
AtWRKY52 (RRS1)	At5g45270 ^a	
AtWRKY53	At4g23810	AAK28442
AtWRKY54	At2g40750	AAL29430
AtWRKY55	At2g40740	AAD32805
AtWRKY62	At5g01900 ^a	AAK28310
AtWRKY63	At1g66600	AAL50782
AtWRKY64	At1g66560	AAL61860
AtWRKY66	At1g80590	AAF27132
AtWRKY67	At1g66550 ^a	AAL13043
AtWRKY70	At3g56400	AAL13046

^a The predicted open reading frames of these accessions do not correspond to the experimentally determined sequences. The corrected sequence for AtWRKY52 has been deposited in the GenBank database (accession numbers AX103688, AX103684, AX103691, and AX103687).

In Figures 2 through 6, WRKY factors that show a similar position in the phylogenetic tree are organized together to allow a better comparison of the transcript accumulation among structurally related family members.

Expression of group III WRKY factors after treatment with SA.

To initiate studies concerning the physiological functions of the different members of group III, we characterized the SA-mediated accumulation of their mRNA transcripts. SA functions as signaling substance, and its accumulation plays a central role in the activation of defense reactions leading to plant disease resistance (Delaney et al. 1994). Two-week-old plants were sprayed with 5 mM sodium salicylate, and the transcript abundance of the chosen WRKY factors was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 2A). Most of the transcripts analyzed undergo a strong accumulation within the first 4 h of treatment. Particularly, AtWRKY38, AtWRKY46, AtWRKY53, AtWRKY54, AtWRKY62, AtWRKY64, AtWRKY67, and AtWRKY70 show a strong increase in expression within the first 2 h. Only AtWRKY30 and AtWRKY55 are not clearly induced after spraying with SA. Both are members of subgroup IIIb, albeit only distantly related, according to our phylogenetic analysis. This result indicates that most members of WRKY group III may have a related, SA-dependent function in signal transduction. The members differing in this feature do not share extended sequence similarity.

Our results were basically confirmed by Northern analysis (Fig. 2B), with two exceptions, namely, AtWRKY66 and AtWRKY41. Most likely the lower detection sensitivity of RNA blot analysis compared to RT-PCR is the cause of this discrepancy. Nevertheless, the similarity between the results derived from both methods and the higher sensitivity of RT-PCR favored the use of this method for subsequent analyses.

Expression of group III WRKY factors after inoculation with different pathogens.

We next analyzed the accumulation of group III WRKY transcripts upon inoculation with different microbes. We tested both compatible and incompatible interactions with the oomy-

Fig. 1. Phylogenetic analysis of the *Arabidopsis* WRKY group III. Amino acid sequences from the WRKY domains were aligned using CLUSTALW (Thompson et al. 1994). The diagram shows an unrooted tree constructed with the TREEVIEW program (Page 1996). Schematic representations of typical members of each subgroup are shown above and underneath the tree. Black boxes indicate the WRKY domain; boxes with stripes represent conserved acidic domains identified using MEME and MAST (Back and Chappell 1996; Bailey and Elkan 1994; Bailey and Gribskov 1998).

cete *Peronospora parasitica*, as well as nonhost interaction with the ascomycete *Blumeria graminis* f. sp. *hordei*, a natural pathogen of grasses (Fig. 3). The ecotype Columbia (Col-0) is resistant to *P. parasitica* isolates Cala2 and Emoy2 because of the race-specific resistance (*R*) genes *RPP2* or *RPP4*, respectively. Recently, it has been demonstrated that these two *R* genes transduce resistance responses via different signaling pathways (McDowell et al. 2000). After inoculation of plants with these isolates, neither hyphal growth nor host cell death is visible 72 h after inoculation (Fig. 3A and C). The ecotype Landsberg erecta (Ler-1) lacks the *RPP2* gene and is thus susceptible to *P. parasitica* Cala2, which forms an extended intercellular mycelium in rosette leaves (Fig. 3B). By using the nonhost pathogen *B. graminis* f. sp. *hordei*, a different type of interaction was analyzed. Nonhost resistance is thought to function independently of the occurrence of major *R* genes but shares with *R* gene pathways certain signaling components (Heath 2000). *B. graminis* f. sp. *hordei* fails consistently to infect cells of *Arabidopsis*. Although extracellular infection structures, including appressoria, are formed within 24 h post-spore inoculation on leaves, germinated spores almost quantitatively fail to grow invasively, even after 72 h (Fig. 3D).

Gene expression analyses revealed that most of the investigated genes are induced after inoculation with the described microbes (Fig. 4). Data presented here and for following figures were derived from two independent experiments. During *RPP2*-mediated resistance, the analyzed transcripts accumulate early, in general not later than 4 to 6 h postinoculation (Fig. 4A). At this time, no signs of pathogenic growth, even in the compatible interactions, are visible (data not shown). In susceptible Ler-1 plants however, the majority of analyzed *WRKY* genes are activated later than 6 h postinoculation (Fig. 4B). Expression analysis of *WRKY* group III genes during *RPP4*-mediated resistance also showed delayed induction kinetics for most of the analyzed genes compared with *RPP2*-mediated resistance (Fig. 4C). These findings indicate that the early expression of the majority of *WRKY* group III genes is specific

for *RPP2*-mediated resistance. The delayed induction in the compatible interaction and after inoculation with *P. parasitica* Emoy2 may be due to other defense signals that do not depend on the recognition of an avirulence gene product. During non-host resistance of Col-0 against *B. graminis* f. sp. *hordei*, most of the tested *WRKY* genes are induced within 24 h postinoculation (Fig. 4D) and thus expressed simultaneously with the differentiation of extracellular infection structures on the leaf surface.

A closer look at the expression pattern of single *WRKY* genes in the different interactions reveals unexpected differences. Several expression patterns can be discriminated, suggesting distinct expression fingerprints for each interaction. Only one gene, *AtWRKY54*, is induced with similar kinetics during both *RPP2*- and *RPP4*-dependent interactions, suggesting that the encoded protein is shared by both *R* gene-triggered signaling cascades. The *AtWRKY54* protein exhibits 43% amino acid identity to its closest putative relative *AtWRKY70*. Their *WRKY* domains show over 80% identity. Nevertheless, the induction of *AtWRKY70* is delayed during *RPP4*-mediated resistance. Another gene, *AtWRKY62*, is induced after inoculation with *P. parasitica*, but not during nonhost resistance. However, the *WRKY* factor with highest similarity to *AtWRKY62* is *AtWRKY38*, which is clearly induced within 24 h postinoculation with *B. graminis* f. sp. *hordei*. Only one of the analyzed genes, *AtWRKY64*, is not expressed after inoculation with any of the pathogens tested but shows a clear induction after treatment with SA. Closely related to *AtWRKY64* are *AtWRKY63* and *AtWRKY67*, whose genes are strongly induced during interaction with *P. parasitica*. The only genes that are expressed consistently at 2 h postinoculation are *AtWRKY53* and *AtWRKY30*, which are both expressed in control plants, too, and thus may be responding to other factors (e.g., diurnal rhythms) than the inoculation with pathogens.

Besides the *R* genes themselves, additional components of pathways that lead to *R*-gene dependent resistance have been previously identified (Glazebrook 2001). For *RPP2*- and *RPP4*-mediated resistance, the *PAD4* protein is required; *pad4-1*

Fig. 2. Time course analysis of *WRKY* group III gene expression upon treatment with salicylic acid (SA). **A**, Reverse transcription-polymerase chain reaction (RT-PCR). Primers for actin were used as control. **B**, RNA blot analysis. Asterisks indicate an exposure time of more than 24 h. Ethidium bromide staining of rRNA is shown as loading control.

mutant plants show clearly diminished resistance against *P. parasitica* Cala2 (Glazebrook et al. 1997). Accumulation of SA is required for *RPP4*-mediated but is negligible for the *RPP2*-mediated resistance against *P. parasitica* Cala2 (McDowell et al. 2000). Transgenic plants, expressing a sequence coding for a SA-degrading bacterial salicylate hydroxylase (*nahG*) from *Pseudomonas putida*, show very weak effects on the growth of *P. parasitica* Cala2 (McDowell et al. 2000). To ascertain if the lack of PAD4 or SA effects the expression of *WRKY* genes, we inoculated *nahG* transgenic plants as well as *pad4-1* mutant plants with *P. parasitica* Cala2 (Fig. 5). In *pad4-1* plants, transcript accumulation of *WRKY* genes is generally delayed (exceptions being *AtWRKY30*, *AtWRKY53*, and *AtWRKY55*) (Fig. 5A). In *nahG* transgenic plants some of the analyzed genes are not affected in their timely induction (Fig. 5B). These include the genes *AtWRKY70*, *30*, *41*, *53*, and *55*. Other genes show a strongly delayed or even no detectable induction during the first 3 days postinoculation (*AtWRKY38*, *AtWRKY54*, *AtWRKY62*, *AtWRKY63*, and *AtWRKY66*).

PAD4, a lipase-like protein, is not only responsible for the synthesis of the phytoalexin camalexin in response to infection with *Pseudomonas syringae* pv. *maculicola* ES4326 (Glazebrook and Ausubel 1994) but, perhaps more importantly, is also a general component of the SA signal transduction cascade leading to systemic acquired resistance in plants. PAD4 is required for the accumulation of SA and thus acts upstream of SA accumulation in regulating defense response expression (Zhou et al. 1998). We tested whether the SA responsiveness of *WRKY* group III genes in wild-type plants (Fig. 2A) depends on a functional PAD4 protein by treatment of *pad4-1* plants with SA. As shown in Figure 5C, there is no difference between wild-type and mutant plants in the expression pattern of most of the tested *WRKY* genes, with the notable exception of *AtWRKY64*, whose expression is not induced by SA in *pad4-1* mutants. Thus, although the SA responsiveness generally occurs independently of PAD4, consistent with the proposed upstream position of PAD4 relative to SA accumulation, high SA levels must act together with a PAD4-derived signal to stimulate *AtWRKY64* expression. Similar to the wild-type situation, most analyzed

genes are clearly expressed transiently with a decreased transcript level after 24 h. However, expression of *AtWRKY66* is not transient in the *pad4-1* mutant, and expression of *AtWRKY41* is hardly detectable.

Role of *AtWRKY54* during plant defense.

The only gene that is expressed with nearly the same temporal kinetics in the two tested *R* gene-specified interactions is *AtWRKY54*. Therefore, *AtWRKY54* may be a component of a signaling cascade, integrating both *RPP2*- and *RPP4*-derived signals. The finding that the expression of *AtWRKY54* is undetectable in the *nahG* transgenic line after inoculation with *P. parasitica* Cala2 and therefore is not specific for the SA-independent *RPP2*-mediated signaling pathway supports this idea. Thus, this member was selected for further analysis. *AtWRKY54* consists of three exons and two introns, the last intron being located within the region coding for the WRKY domain. To find out if *AtWRKY54* is required for the tested resistance responses, affects the expression of other group III members, or both, an *AtWRKY54* insertion mutant was identified in the ZIGIA lines (Wisman et al. 1998). The mutant contains a 2.6-kb fragment of the originally 8.1-kb *En-1* transposon from *Zea mays* inserted in the first exon of *AtWRKY54*. The first three base pairs of the insertion lead to a premature stop codon at position 78 in the predicted amino acid sequence of *AtWRKY54*, thus generating a truncated protein (Fig. 6). The truncated protein does not contain the DNA-binding WRKY domain. Therefore, the homozygous insertion line was assumed to be a loss of function mutant. Under short and long day conditions, this line did not show any obviously altered phenotype when compared with wild-type plants. After inoculation with *P. parasitica* Emoy2 and Cala2 or *B. graminis* f. sp. *hordei*, the resistance in the insertion mutant was also not affected (data not shown), suggesting that *AtWRKY54* is not alone crucial for resistance against these pathogens. However, lack of *AtWRKY54* expression clearly affected the expression kinetics of several other *WRKY* group III genes, including *AtWRKY38*, *AtWRKY62*, *AtWRKY53*, and *AtWRKY70*, upon treatment with SA (Fig. 7B). Unlike the transient expression pattern seen in wild-type plants, these genes showed a sustained expression 24 h after stimulus application, indicating that *WRKY* gene expression is directly or indirectly interconnected.

Analysis of the 5' noncoding region of *AtWRKY54*.

Induced *AtWRKY54* expression was more rapid upon treatment with SA than was observed following inoculation with *P. parasitica* Cala2 (Figs. 2 and 4A). We performed an analysis of the promoter sequence to identify potential *cis*-acting elements responsible for its induction upon these two stimuli. Using the PLACE database (Higo et al. 1999), 1.5 kb of the non-coding 5' flanking sequence were searched for previously identified *cis* regulatory elements known to mediate expression of defense-related genes. Two W boxes, the binding sites for WRKY factors, were identified at a distance of -481 and -537 bp upstream of the transcription start site. One of the identified W boxes is part of an elicitor responsive element known from the *CHN50* promoter (Fukuda 1997). With the exception of additional W box TGAC core sequences, no other previously identified elements, such as GCC boxes (Ohme-Tagaki et al. 2000) or SA-responsive *as-1* elements (Qin et al. 1994), were identified. To test whether regions containing the identified W boxes are important for the response to pathogens or SA, transgenic plants containing different length *AtWRKY54* promoter::GUS reporter gene constructs were generated (Fig. 6A). The T2 generation of stable transformants were tested for the induction of the reporter gene after spraying with 5 mM SA or upon inoculation with *P. parasitica* Cala2, respectively. Repre-

Fig. 3. Plant-microbe interactions chosen for expression analysis of the *Arabidopsis* group III *WRKY* family. **A**, Incompatible interaction of Columbia (Col-0) and *Peronospora parasitica* Cala2; **B**, Compatible interaction of Landsberg erecta (Ler-1) and *P. parasitica* Cala2. Arrows show hyphal growth of *Peronospora parasitica*. **C**, Incompatible interaction of Col-0 and *P. parasitica* Emoy2; **D**, Incompatible interaction of Col-0 and the nonhost pathogen *Blumeria graminis* f. sp. *hordei*. Cell wall of *B. graminis* f. sp. *hordei* is stained with Coomassie three days after inoculation. **A**, **B**, and **C**, show cotyledons stained with Trypan blue to detect hyphal growth three days after inoculation.

sentative results from the analysis of at least five independent transgenic lines for each construct are shown in Figure 7B.

All three promoter versions allow expression of the reporter gene after treatment with SA. In contrast, only the -694 promoter construct is capable of inducing the reporter gene upon inoculation with *P. parasitica* Cala2. These results demonstrate that different regions within the 5' regulatory sequences of *AtWRKY54* are responsible for SA and pathogen responsive gene expression.

DISCUSSION

All 13 WRKY genes from *Arabidopsis thaliana* coding for transcription factors with the group III specific C2-HC zinc finger motif were chosen for expression analysis during biotic and abiotic stresses. WRKY proteins containing this special motif have previously been cloned from tobacco (Chen and Chen 2000) and parsley (Cormack et al. 2002) and were shown

Fig. 4. Timecourse expression analysis of WRKY group III genes in Columbia (Col-0) and Landsberg erecta (Ler-1) ecotype *Arabidopsis* plants upon pathogen challenge. **A**, Col-0 plants inoculated with *Peronospora parasitica* (*P.p.*) Cala2; **B**, Col-0 plants inoculated with *P. parasitica* Emoy2; **C**, Ler-1 plants inoculated with *P. parasitica* Cala2; **D**, Col-0 plants inoculated with *Blumeria graminis* (*B.g.*) f. sp. *hordei*. Control plants were mock-inoculated with water.

Fig. 5. Timecourse expression analysis of WRKY group III genes in *pad4-1* mutant and *NahG* transgenic plants after inoculation with *Peronospora parasitica* Cala2 or treatment with salicylic acid (SA). **A**, and **B**, Expression in *pad4-1* mutants and *nahG* transgenic plants after inoculation with *Peronospora parasitica* (*P.p.*) Cala2. The trypan blue-stained cotyledons illustrate the type of interaction by comparing the mycelial growth of the pathogen in both plant lines. **C**, Expression in *pad4-1* mutants after spraying with 5 mM SA.

to be able to bind specifically to W box elements and to activate gene expression. Data concerning WRKY group III genes remains very limited, despite several recent genome-wide expression profile studies dealing with plant defense-related responses (Chen et al. 2002b; Cheong et al. 2002). This is due to the fact that group III WRKY genes are nearly absent from currently used microarrays. Only for *AtWRKY53*, *AtWRKY70*, and *AtWRKY62* was altered expression upon pathogen attack, wounding, during leaf senescence, and upon treatment with SA demonstrated (Brodersen et al. 2002; Cheong et al. 2002; Hinderhofer and Zentgraf 2001; Yu et al. 2001).

Figure 8A summarizes the temporal expression patterns we obtained for the WRKY group III genes. Most of the analyzed genes are induced by SA, including *AtWRKY53*, *AtWRKY62* and *AtWRKY70*, which is consistent with previous reports (Brodersen et al. 2002; Yu et al. 2001). Some of the analyzed genes are induced within 2 h after treatment with SA and might be regarded as immediate early type WRKY genes (Cormack et al. 2002). This is partly supported by macroarray analyses demonstrating activation of *AtWRKY38*, *AtWRKY46*, *AtWRKY53*, *AtWRKY67*, and *AtWRKY70* transcription in the presence of the protein synthesis blocker cycloheximide (R. Stracke, I. Somssich, and B. Weißhaar, unpublished data). WRKY gene induction in pathogen-infected plants occurred generally later than after SA treatment. Possibly, this response requires the release of elicitors from germinating spores, a time-consuming process. Alternatively, germinating pathogens may release substances that temporarily suppress certain mechanisms involved in plant defense activation. However, based on our investigation of one member, namely *AtWRKY54*, it would appear that the signaling events leading to SA- and pathogen-induced expression are not identical. Analysis of *AtWRKY54* promoter::GUS reporter lines revealed that different regions within the 5' regulatory sequences of *AtWRKY54* are responsible for SA- and pathogen-responsive gene expression. Apart from WRKY-factor binding sites (W boxes), no other elements known to be involved in mediating plant defense gene activation were identified, with the exception of an EIRE sequence, which also contains a functional W box. Thus, expression of *AtWRKY54* upon infection or by SA treatment may require the binding of distinct WRKY factors to their respective W box elements. Similarly, therefore, association of WRKY factors to different, stimulus-specific regions in the promoters of other WRKY genes might influence their timely variable induction upon diverse biotic and abiotic signals.

The temporal expression of most WRKY group III genes in the compatible interaction *P. parasitica* Cala2/Ler-1 is delayed, compared to the incompatible interaction *P. parasitica* Cala2/Col-0 (Fig. 8A). This is consistent with previous findings demonstrating that timely expression of defense-associated genes is a crucial step during plant defense (Cao et al. 1994). Interestingly, most of the analyzed genes in the incompatible interaction *P. parasitica* Emoy2/Col-0 showed delayed expression, indicating that the early induction of such genes might be specific for *P. parasitica* Cala2 and, therefore, to be *RPP2* dependent. In contrast, during nonhost resistance, several genes are not expressed at all. This could be explained by the lack of tight coevolution between *Arabidopsis* and this non-host pathogen. Neither did the fungus develop specific mechanisms to colonize *Arabidopsis* plant tissue nor did adapted mechanisms emerge in the plant to recognize *B. graminis* f. sp. *hordei*-specific elicitors.

By analyzing the expression patterns in different interactions and mutants, it became obvious that distinct subsets of WRKY genes are activated by different queues along defined signal transduction pathways (Fig. 8B). At least four different induc-

tion groups can be distinguished. *AtWRKY55* and *AtWRKY46* are both induced *RPP2* dependently but do not require a functional PAD4 for expression upon infection with *P. parasitica* Cala2 and are thus induced prior to PAD4 function. The expression of other genes is dependent on a functional PAD4, such as *AtWRKY70* and *AtWRKY41*. Although *RPP2*-mediated resistance is independent of the accumulation of SA, expression of WRKY genes *AtWRKY62*, *AtWRKY66*, *AtWRKY63*, *AtWRKY38*, *AtWRKY67*, and *AtWRKY54* is delayed in *nahG* mutants after inoculation with *P. parasitica* Cala2. These genes require accumulation of SA for early induction and are, therefore, not direct targets of *RPP2* signaling but are, rather, secondarily induced by subsequent SA accumulation. The fourth group, including *AtWRKY30* and *AtWRKY53*, is induced independently of *RPP2*- and *RPP4*-specific signals. *AtWRKY53* has previously been shown to be rapidly but only transiently induced upon wounding (Chen et al. 2002b). This is consistent with our data showing elevated transcript levels in water-

Fig. 6. *AtWRKY54* transposon insertion mutant line. **A**, Schematic representation of the *AtWRKY54* gene. The gene is comprised of three exons (boxes) and two introns. Grey boxes indicate the region encoding the WRKY domain. The position of the *En-1* in the insertion line is indicated by a triangle. The *AtWRKY54* gene sequence at the *En-1* integration site is shown above. The *En-1* insertion leads to a premature stop codon, marked by an asterisk above the putative amino acid sequence after amino acid position 78. **B**, Expression of WRKY group III genes in the *AtWRKY54* mutant line after spraying with 5 mM salicylic acid.

Fig. 7. Analysis of *AtWRKY54* promoter::GUS transgenic *Arabidopsis* lines. **A**, Schematic representation of *AtWRKY54* promoter::reporter gene constructs. The *uidA* gene is inserted downstream of the first 30 bp of *AtWRKY54* exon 1 (black box). Colored boxes indicate positions of previously described *cis* acting elements in the 5' regulatory region. Blue boxes represent the invariant TGAC W box core sequence (Eulgem et al. 2000), yellow boxes represent known functional W box motifs (Eulgem et al. 1999; Yu et al. 2001). The red box represents the elicitor responsive *cis* element GGTCANNNAGTC, previously identified in the *CHN50* promoter of tobacco (Fukuda 1997). Numbers on the left indicate the length of the 5' regulatory sequence of each construct. +1 denotes determined transcriptional start site. **B**, GUS reporter gene activity observed in the indicated transgenic lines after treatment with salicylic acid (SA) and *Peronospora parasitica* (*P.p.*) Cala2. Mock control leaves were sprayed with water.

sprayed control plants. Nevertheless, this transient wound response is generally diminished after 24 h and, thus, only partly overlaps with the induction kinetics observed upon pathogen or SA treatments. The differential expression of *WRKY* genes at several distinct levels in the complex signal transduction network suggests that different *WRKY* factors may have discernible functions at various steps of the signaling cascade. Possibly, it is this concerted interplay of involved factors which ultimately results in the activation of the complete plant stress response. Recently, SA-dependent and SA-independent activation of distinct *WRKY* factors was also demonstrated in tobacco following tobacco mosaic virus infection (Yoda et al. 2002).

The described four induction groups do not mirror phylogenetic relationships. Instead, their expression patterns are not predictable from their amino acid sequences. Two of the most closely related proteins in *WRKY* group III are *AtWRKY70* and *AtWRKY54*. *AtWRKY70* has previously been demonstrated to be strongly induced in the *acd11* mutant (Brodersen et al. 2002) and, therefore, is most likely to be involved in the hypersensitive cell-death response, whereas expression of *AtWRKY54* was not observed. Recent group-wide analysis of other gene families, like the *AtCMPG*, glutamate-receptor-like gene family or the *AtHVA22* gene family (Chen et al. 2002a; Chiu et al. 2002; Heise et al. 2002), revealed that a similar primary structure does not necessarily correlate with a similar

gene regulatory response. Still, despite their different regulation, similar physiological roles of these proteins cannot be excluded.

We assume that the large number of *WRKY* factors involved in plant defense helps to orchestrate a broad and spatially controlled response. The apparent loss of the transient expression kinetics for several *WRKY* genes observed in the *AtWRKY54* mutant hints toward an intricate interconnected signaling network in which *WRKY* factors are involved in integrating diverse stress stimuli. In such a scenario, the loss of one function directly or indirectly affects the temporal and perhaps also the spatial expression of numerous other genes. However, as with *AtWRKY54* here and from previous studies (Robatzek and Somssich 2002), single *WRKY* knockouts do not result in obvious altered phenotypes. Thus, functional redundancy must exist between different *WRKY* factors. In fact, in only one reported instance has the disruption of a single *WRKY* gene resulted in an altered phenotype. Interestingly, expression of this gene, *TTG2*, which corresponds to *AtWRKY44*, is not influenced by pathogens nor by various abiotic stresses (Chen et al. 2002b) but rather is involved in trichome and seed coat development (Johnson et al. 2002).

Currently 39 out of the 74 *Arabidopsis* *WRKY* genes have been shown to respond to different biotic and abiotic stresses. Still, the functions of these transcription factors are not restricted to plant defense. Instead, they are involved in numerous processes required for diverse plant functions, such as proper development (Johnson et al. 2002), embryogenesis (Alexandrova and Conger 2002), dormancy and drought tolerance (Pnueli et al. 2002), and also thermal hysteresis (Huang and Duman 2002). It is most likely that the involvement of *WRKY* factors in these diverse processes is as complex as that during plant defense. Defining the role of individual members remains a great challenge for future work. Whole-genome expression profile analysis, particularly in combination with various other signaling mutants and with knockout mutants for all *WRKY* genes, will certainly be useful in this respect. Such studies will help to reveal how interwoven signaling networks are and should provide valuable information how, within such networks, *WRKY* factors contribute to maintain proper plant health and development.

MATERIALS AND METHODS

Plant material and growth conditions.

Experiments were performed with *Arabidopsis thaliana* ecotype Columbia (Col-0) and Landsberg erecta (Ler-1). The *nahG* and *pad4-1* mutants were described previously (Gaffney et al. 1993; Glazebrook et al. 1997). Approximately 200 plants per pot were grown in a phytochamber (Percival Scientific, Boone, IA, U.S.A.) at 20 to 22°C under short day conditions (9 to 10 h light, 14 to 15 h darkness); 12- to 14-day-old plants were used for experiments.

Inoculation with fungal strains and treatment with SA.

Fungal strains *Peronospora parasitica* Emoy2 and Cala2 were supplied by E. Holub (Horticulture Research International, East Malling, U.K.). The fungus *B. graminis* f. sp. *hordei* was provided by V. Lipka (MPIZ, Köln, Germany). Pathogen challenge inoculations were conducted by spraying the seedlings with a *P. parasitica* spore suspension (10^5 spores mL^{-1}) or by dispersing spores of *B. graminis* f. sp. *hordei* on the leaves. Plants infected with *P. parasitica* were incubated under 100% relative humidity. Control plants were treated equally but not infected with fungal spores. For SA treatment, plants were sprayed with 5 mM SA until wet and were incubated similarly to *P. parasitica*-infected plants. As mock control,

Early expression = 2 - 6 h post inoculation
 Delayed expression = 24 - 72 h post inoculation

* not *RPP2* dependent
 + entirely SA dependent

Fig. 8. Summary of the *WRKY* gene expression patterns observed in this study and integration of the data into the *RPP*-dependent signaling pathways. **A**, Early *WRKY* expression is defined as detectable 2 to 6 h postinoculation, whereas delayed expression is detectable 24 to 72 h postinoculation. **B**, *PAD4* (boxed) encodes a lipase-like protein and has been shown to be a key player in the *RPP2/4* defense signaling pathway (van der Biezen et al. 2002). Question mark indicates an unknown pathway not dependent on *RPP2/4* or *PAD4*. Open arrows point towards those group III *WRKY* members whose expression is induced at that hierarchical level along the pathway.

other plants were sprayed with deionized water. RNA was isolated at different times after treatment with SA or fungal infection. For RNA analysis, leaf material was harvested at the indicated timepoints, frozen in liquid N₂, and stored at -70°C. Development of *P. parasitica* inside leaves was monitored by boiling fresh leaves for 3 min with lactophenol-trypan blue, destaining in chloral hydrate overnight, and examination in 70% glycerol under a light microscope.

RNA analysis.

Plant material was ground in liquid N₂, and RNA was isolated using either RNA/DNA Maxi Kit (Qiagen, Hilden, Germany) or TRIZOL-reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. RNA was quantified by UV-spectrometry, and 20 µg was used for RNA blot analysis. RT-PCR was performed using either the OneStep RT-PCR kit (Qiagen) and gene specific primers or Super-script reverse transcriptase (Invitrogen, Karlsruhe, Germany) with Oligo(dT)₁₂₋₁₈ primers (Amersham Pharmacia, Freiburg, Germany) and subsequent PCR reaction with gene specific primers. Depending on the *WRKY* gene, amounts of 5 ng to 0.5 µg RNA (exception: 2 µg for *AtWRKY41*) were used, and 25 to 35 cycles were performed for each reaction. Preliminary experiments verified that PCR conditions were not saturated. For quantification, the PCR-amplified products were separated on agarose gels.

Transgenic plants.

AtWRKY54 promoter::GUS reporter gene constructs containing either the -694-, -512-, or -196-bp promoter fragment, the 138 bp containing the 5' untranslated region, and the first 10 codons were fused in-frame with the *uidA* gene (Fig. 6A) in the binary Ti vector pGPTV (Becker et al. 1992). Plants were transformed using the floral-dip method (Clough and Bent 1998), *Agrobacterium tumefaciens* GV3101. Seeds from transformed plants were surface-sterilized and were germinated on 0.6% agar plates containing kanamycin. Plants from the T2

generation were analyzed for β-glucuronidase (GUS) expression, as described previously (Jefferson et al. 1987), 24 h after the different treatments.

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