

Expression of *AtWRKY33* Encoding a Pathogen- or PAMP-Responsive WRKY Transcription Factor Is Regulated by a Composite DNA Motif Containing W Box Elements

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WRKY transcription factors regulate distinct parts of the plant defense transcriptome. Expression of many WRKY genes themselves is induced by pathogens or pathogen-mimicking molecules. Here, we demonstrate that *Arabidopsis WRKY33* responds to various stimuli associated with plant defense as well as to different kinds of phytopathogens. Although rapid pathogen-induced *AtWRKY33* expression does not require salicylic acid (SA) signaling, it is dependent on PAD4, a key regulator upstream of SA. Activation of *AtWRKY33* is independent of de novo protein synthesis, suggesting that it is at least partly under negative regulatory control. We show that a set of three WRKY-specific cis-acting DNA elements (W boxes) within the *AtWRKY33* promoter is required for efficient pathogen- or PAMP-triggered gene activation. This strongly indicates that WRKY transcription factors are major components of the regulatory machinery modulating immediate to early expression of this gene in response to pathogen attack.

Additional keywords: *Alternaria*, *Hyaloperonospora parasitica*, pathogen-associated molecular patterns.

WRKY proteins are a class of zinc-finger-containing transcription factors (TFs) encoded in all higher plants by large gene families. In *Arabidopsis thaliana*, the WRKY gene family consists of 74 members, all of which appear to be expressed. Although not solely restricted to plants, their enormous expansion during plant evolution suggests a unique role of these genes within this kingdom (Ülker and Somssich 2004). With few exceptions, genetic studies have failed to uncover specific functions associated with individual WRKY genes. Two *Arabidopsis* developmental mutants, *ttg2* involved in trichome (Johnson et al. 2002) and *mini3* involved in seed development (Luo et al. 2005), have been found to be affected in the genes *AtWRKY44* and *AtWRKY10*, respectively. Another gene, *AtWRKY52* (*RRS1*), was shown to confer resistance of *Arabidopsis* Nd-1 plants toward *Ralstonia solanacearum* strain GMI1000, the casual agent of bacterial wilt (Deslandes et al.

2002). Interestingly, *AtWRKY52* (*RRS1*) also was identified to be the gene affected in the *slh1* mutant showing sensitivity toward low humidity (Noutoshi et al. 2005).

Abiotic and biotic stresses are major external factors influencing the expression of WRKY genes (Eulgem et al. 2000; Ülker and Somssich 2004). The expression of more than 70% of the *Arabidopsis* WRKY genes is influenced by various stresses, particularly by pathogen-related stimuli (Dong et al. 2003; Hahlbrock et al. 2003).

The transcript levels of several parsley WRKY genes were affected by treatment of cells with pathogen-derived elicitors termed pathogen-associated molecular patterns (PAMPs) (Cormack et al. 2002). Virus-induced gene silencing of three tobacco WRKY genes (*NtWRKY1-3*) compromised *N*-gene-mediated resistance toward *Tobacco mosaic virus* (Liu et al. 2004). *AtWRKY22* and *AtWRKY29* were identified as important components of a PAMP-mediated defense signal transduction pathway in *Arabidopsis*, and transient overexpression of *AtWRKY29* reduced susceptibility to the otherwise virulent pathogens *Pseudomonas syringae* and *Botrytis cinerea* (Asai et al. 2002). Likewise, overexpression of *AtWRKY70* in *Arabidopsis* plants resulted in enhanced resistance to two tested virulent bacteria (Li et al. 2004), whereas loss-of-*AtWRKY70* function impaired resistance towards *Erysiphe cichoracearum* and enhanced susceptibility to *Erwinia carotovora* (Li et al. 2006). Recently, functional redundancy between *AtWRKY* factors associated with plant defense was demonstrated by the generation of *Atwrky18Atwrky40* and *Atwrky18Atwrky60* double mutants and *Atwrky18Atwrky40Atwrky60* triple mutant lines (Xu et al. 2006). Whereas the corresponding single mutants showed little altered phenotypic effects upon challenge with *P. syringae* DC3000 compared with wild-type plants, the double and triple mutants clearly were more resistant toward this bacterial strain.

The involvement of MAP kinases in the rapid activation of WRKY gene expression during plant defense has been inferred from several studies. In tobacco, activation of the MAP kinases SIPK and WIPK, by means of an inducible dominant gain-of-function *NtMEK2* transgene, resulted in elevated expression of WRKY genes (Kim and Zhang 2004). In addition, tobacco WRKY1 was shown to be directly phosphorylated by the MAP kinase SIPK and, thereby, to mediate hypersensitive (HR)-like cell death (Menke et al. 2005). In *Arabidopsis*, activation of a MAP kinase pathway, triggered by the interaction of the bacterial PAMP flg22 with the membrane-associated FLS2

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receptor, resulted in enhanced gene expression of *AtWRKY22* and *AtWRKY29* (Asai et al. 2002). Similarly, activation of *AtWRKY33* and *AtWRKY53* was shown to involve a MAP kinase pathway following elicitation of *Arabidopsis* seedlings with chitin, a fungal PAMP (Wan et al. 2004). The *Arabidopsis* MAP kinase 4 mutant, *mpk4*, exhibits constitutive systemic acquired resistance (SAR) and resistance to virulent pathogens (Petersen et al. 2000). MPK4 was shown to interact with MKS1, a nuclear localized protein of unknown function, which can bind two WRKY TFs, namely *AtWRKY25* and *AtWRKY33* (Andreasson et al. 2005). MPK4 is capable of phosphorylating both MKS1 and the WRKY factors and it is hypothesized that MKS1 acts as a coupling protein, modulating the activities of the TFs in MPK4-mediated signaling.

Additional support for a role of WRKY factors in plant defense has come from computational analyses of microarrays to identify regulatory *cis*-acting DNA elements involved in defense gene activation. Applying clustering methods, several groups identified the WRKY factor binding site, referred to as the W box (5'-C/TTGACC/T-3'), often overrepresented within the promoters of coregulated *Arabidopsis* defense response genes (Andreasson et al. 2005; Maleck et al. 2000; Navarro et al. 2004; Petersen et al. 2000). W box elements are often also present within the promoters of WRKY genes and, in the case of parsley *PcWRKY1*, these elements were shown to mediate rapid PAMP-dependent activation of the gene (Eulgem et al. 1999) and to be target sites of WRKY factors *in vivo* (Turck et al. 2004).

Here, we report on the analysis of *AtWRKY33* (At2g38470), the *Arabidopsis* orthologue of parsley *PcWRKY1*. We demonstrate that expression of *AtWRKY33* is induced rapidly by pathogens and by endogenous defense signal molecules. Using transgenic plants carrying various *AtWRKY33* promoter::GUS reporter constructs, as well as parsley protoplasts for transient expression assays, we show that this strong induced expression is dependent on a promoter region containing W box elements and that rapid pathogen- and PAMP-mediated activation of *AtWRKY33* is highly dependent on these W box elements.

RESULTS

Expression of *AtWRKY33* in plants and its response to UV-light and cycloheximide.

RNA blot analysis was performed using RNA derived from different tissues of the plant and from cultured *Arabidopsis* cells. *AtWRKY33* expression was clearly detectable in mature green leaves, roots, shoots, and inflorescences (Fig. 1A). Little or no expression was observed at the seedling stage and in the siliques. However, the strong *AtWRKY33* expression in mature leaves of plants grown in the greenhouse was not observed when the plants were grown under controlled aseptic conditions in a phytochamber (Fig. 1B), indicating that environmental factors or soil contaminations were partly responsible for the initially observed high expression levels. When seedlings grown in the dark were exposed to UV-containing white light, they showed high levels of *AtWRKY33* transcript after 24 h, indicating that light quality influences *AtWRKY33* expression (Fig. 1C). This response was already detectable after 4 to 6 h of UV light treatment, albeit at rather low levels (compare Fig. 1C and D). Pretreatment of dark-grown *Arabidopsis* suspension cells for 30 min with the protein synthesis inhibitor cycloheximide (CHX) resulted in a very rapid increase in *AtWRKY33* transcript accumulation. This derepression suggests that *AtWRKY33* expression is actively being suppressed by a negative regulator with a rather short half-life. This finding is consistent with recent whole-genome-array studies performed on plants treated with CHX (GEO Dataset GDS515) (Navarro et al. 2004).

Based on various prediction programs, the location of the *AtWRKY33* protein has been reported to be the chloroplast (Mahalingam et al. 2005). However, nuclear localization of *AtWRKY33* recently was demonstrated in transient assays employing the heterologous onion epidermal cell system (Zheng et al. 2006). Using *AtWRKY33*cDNA-GFP fusion constructs driven by the *Cauliflower mosaic virus (CaMV) 35S* promoter for transient expression in *Arabidopsis* epidermal cells revealed consistent protein localization to the nucleus (Fig. 1E). Whether the protein also can reside in chloroplasts was not investigated.

Induction of *AtWRKY33* by pathogens and by defense-signaling molecules.

Global expression data suggest that *AtWRKY33* is induced under various abiotic and biotic stress conditions. We tested the response of this gene to two well-established endogenous signal molecules, namely salicylic acid (SA) and methyl jasmonate (MeJA). Exogenous application of SA to *Arabidopsis* seedlings already resulted in a rapid increase of *AtWRKY33* mRNA 2 h post treatment (Fig. 2A). In contrast, MeJA treatment did not

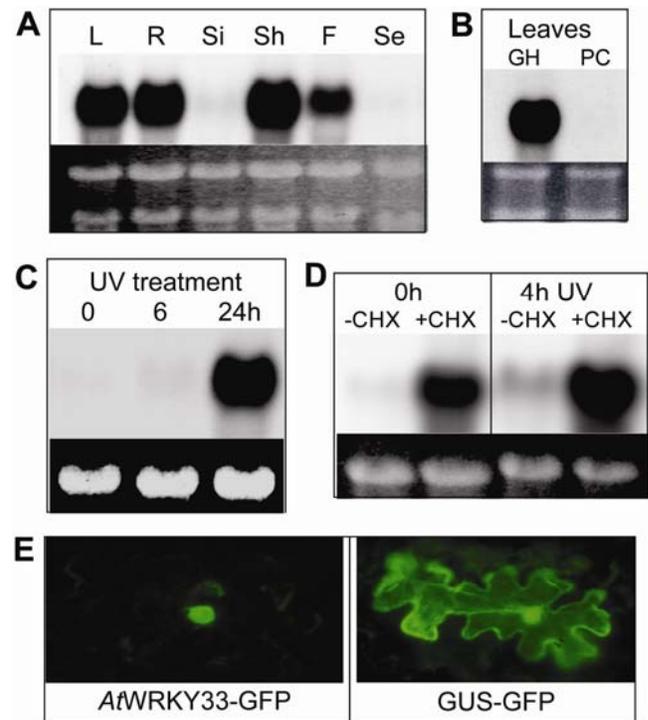


Fig. 1. Expression of *AtWRKY33* and cellular localization of the protein. **A**, *AtWRKY33* mRNA levels in different tissues of *Arabidopsis* greenhouse-grown plants. L, 4-week-old mature leaves; R, roots; Si, siliques; Sh, shoots; F, flowers; Se, seedlings grown in the phytochamber. **B**, *AtWRKY33* mRNA levels in mature leaves of plants grown in the greenhouse (GH) and under aseptic conditions in a phytochamber (PC). **C**, Accumulation of *AtWRKY33* mRNA in *Arabidopsis*-etiolated seedlings upon treatment with UV-containing white light for the time periods indicated. **D**, Accumulation of *AtWRKY33* mRNA upon 30-min pretreatment of *Arabidopsis*-cultured suspension cells with cycloheximide (+CHX) and subsequent 4-h treatment with UV-containing white light. Blots from gels loaded with 20 μ g of total RNA per lane were hybridized with radioactively labeled *AtWRKY33* cDNA and visualized by autoradiography in C and D. Ribosomal rRNA bands of the ethidium bromide-stained gels were used as control for equal loading in A to D. Nuclear localization of *AtWRKY33*-GFP fusion protein in E, Nuclear localization of *AtWRKY33*-GFP fusion protein in *Arabidopsis* epidermal cells. Image using a green fluorescent protein (GFP) filter is shown for the *AtWRKY33*-GFP expression construct. As control construct 35S::GUS-GFP was used showing distribution of GUS-GFP protein within the cell.

significantly increase *AtWRKY33* mRNA levels (Fig. 2B). We next tested whether *AtWRKY33* is induced when seedlings are challenged with the biotrophic oomycete pathogen *Hyaloperonospora parasitica*. Resistance toward this pathogen has been shown to be mediated via the SA-dependent defense signaling pathway (Glazebrook 2005). *Arabidopsis* ecotype Col-0 plants are resistant to the *H. parasitica* isolates Cala2 and Emoy2, whereas ecotype Ler-1 is resistant to the isolate Noks1. *AtWRKY33* expression was induced in both ecotypes by the tested avirulent isolates (Fig. 3A to C). Increased *AtWRKY33* transcript levels were detected readily within 2 to 4 h in Col-0 seedlings challenged by the avirulent strains Cala2 and Emoy2 (Fig. 3A and B). The elevated level of *AtWRKY33* transcript detected in the 10-h water control (Fig. 3B) was not consistently

observed and most likely was due to contamination of some plants in this sample. In Ler-1 seedlings challenged with the avirulent isolate Noks1, strong expression of *AtWRKY33* was detected 12 h postinoculation (Fig. 3C). Expression of *AtWRKY33* also was upregulated rapidly in seedlings infiltrated with avirulent or virulent bacterial *P. syringae* strains (data not shown), consistent with previous findings (Dong et al. 2003). Activation of *AtWRKY33*, however, was not observed in seedlings challenged by the barley pathogen *Blumeria graminis* f. sp. *hordei*, to which *Arabidopsis* is a nonhost (Fig. 3D).

Although *AtWRKY33* expression is strongly and positively affected by SA treatment, SA is not required for its activation by *H. parasitica* Cala2, because transgenic *Arabidopsis* plants expressing the SA-degrading bacterial enzyme salicylate hydroxylase (*NahG*) responded in a way similar to wild-type plants (Fig. 4A). Cala2-dependent *AtWRKY33* activation also was observed in seedlings of *npr1* mutant plants that also are affected in SA signaling downstream of SA (Fig. 4B). In contrast to this, Cala2-dependent expression of *AtWRKY33* was dramatically delayed in *pad4* mutant plants (Fig. 4C). The lipase-like protein PAD4 is a key upstream component of the SA-mediated signaling defense pathway (Glazebrook 2005). Mutant *pad4* plants are susceptible to *H. parasitica* Cala2. This temporal delay in *AtWRKY33* transcript accumulation also was observed in the *Arabidopsis* ecotype Ler-1, which is also highly susceptible to Cala2 (Fig. 4D). Application of SA to *pad4* or Ler-1 seedlings resulted in a very rapid induction of *AtWRKY33* expression (Fig. 4E) consistent with the position of SA in the signaling pathway downstream of PAD4.

AtWRKY33 promoter regions mediating expression during defense signaling.

In order to pinpoint regions of the *AtWRKY33* promoter allowing activation of the gene during the plant defense response, we generated transgenic *Arabidopsis* Col-0 plants expressing the *uidA* (GUS reporter) gene under the control of *AtWRKY33* promoters of varying length. The longest promoter fragment, designated P1, contained 1,230 bp of upstream sequence relative to the transcriptional start site (Supplementary Figure 1). Based on GUS activity staining of different tissues derived from transgenic plants carrying the *AtWRKY33P1::GUS* reporter construct, the P1 promoter proved sufficient to mediate the observed endogenous *AtWRKY33* expression patterns

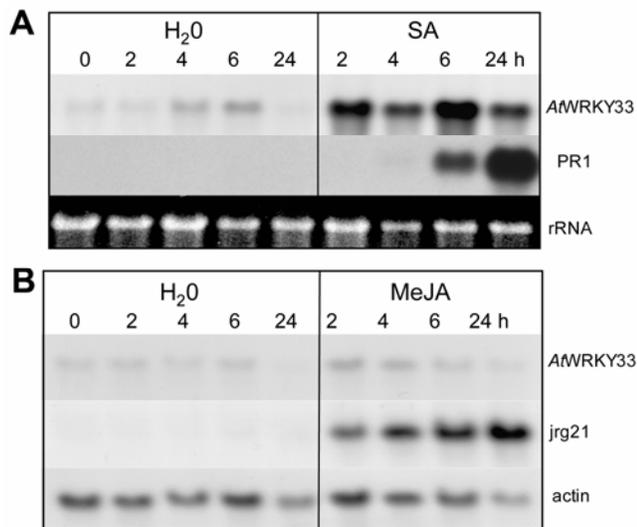


Fig. 2. *AtWRKY33* is induced by salicylic acid (SA) but not by methyl jasmonate (MeJA). **A**, *Arabidopsis* seedlings were sprayed with 5 mM SA or **B**, 45 μ M MeJA solution and total RNA extracted at the time periods indicated. Blots from gels loaded with 15 μ g of RNA per lane were hybridized with radioactively labeled *AtWRKY33* and visualized by autoradiography. The same blots were re-hybridized with a *PR1* probe, a SA-responsive marker gene (**A**), or *jrg21* cDNA representing an MeJA-induced gene (**B**). As controls for equal RNA loading, ribosomal rRNA of the ethidium bromide-stained gel (**A**) or hybridization with an actin cDNA (**B**) were used.

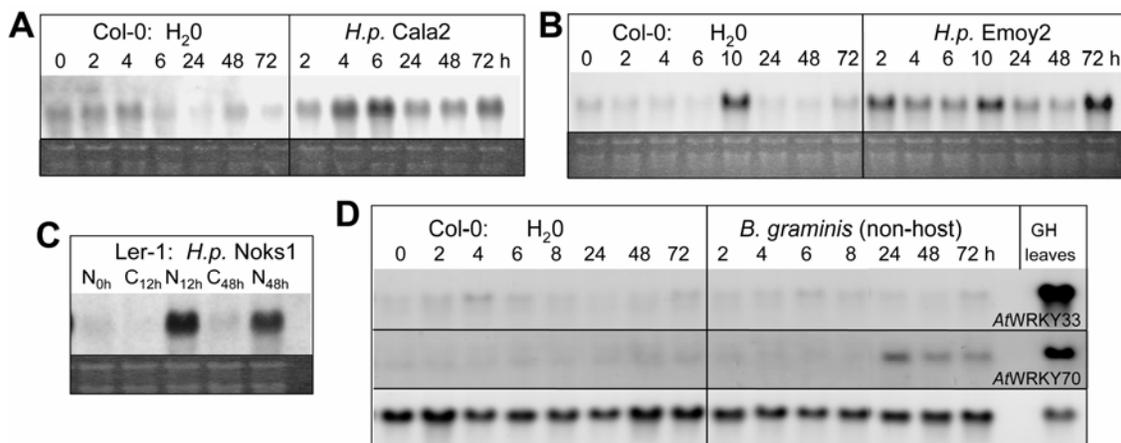


Fig. 3. Response of *AtWRKY33* to pathogen challenge of *Arabidopsis* seedlings. **A** to **C**, *Arabidopsis* seedlings of the indicated ecotypes were sprayed with water or with the respective *Hyaloperonospora parasitica* (*H.p.*) isolates at 2×10^5 spores/ml and harvested at the indicated timepoints. N, Noks1. C, Untreated control. **D**, Col-0 *Arabidopsis* seedlings were treated with spores of the nonhost *Blumeria graminis* and harvested at the indicated timepoints. Blots from gels loaded with 10 μ g of total RNA per lane were hybridized with radioactively labeled *AtWRKY33* cDNA or *AtWRKY70* cDNA (positive control for *B. graminis* infection) and visualized by autoradiography. **A** to **C**, Ribosomal rRNA bands of the ethidium bromide-stained gels or **D**, hybridization with an actin probe served as controls for equal loading. The elevated senescence-dependent levels of *AtWRKY33* and *AtWRKY70* transcripts in leaves of 5-week-old greenhouse (GH)-grown plants are shown to the right.

that were detected by RNA blot analyses (Fig. 5A). Moreover, in floral organs, it revealed a developmentally controlled spatial expression pattern for *AtWRKY33* (Fig. 5A). No GUS staining was detected in young inflorescences or in anthers (Fig. 5A2 to 3). During further floral development, strong staining was observed in stigmatic papillae and the style (Fig. 5A3 to 4). At later stages post fertilization, GUS staining was restricted to the top part and to the abscission zone of fully elongated siliques (Fig. 5A5 to 6). In situ RNA hybridization experiments with longitudinal sections of siliques, including flower base and distal part of the petioles, revealed clear signals at the flower base around the abscission zones of the petals, sepals, and stamens (Fig. 5A7).

Wounding *AtWRKY33P1*::GUS plants, by cutting or squashing the cotyledons, locally activated the reporter gene (Fig. 5B). Exogenous spray application of SA or of the SA functional analog 2,6-dichloroisonicotinic acid (INA) also strongly induced expression of the reporter gene in the cotyledon assay (Fig. 5B). In contrast, spraying plants with MeJA did not result in an increase in GUS activity (data not shown; Fig. 5C), consistent with our RNA data (Fig. 2B) and demonstrating that this signal hormone does not induce *AtWRKY33*.

***AtWRKY33P1*::GUS plants challenged with spores of *H. parasitica* Cala2 showed a marked increase in GUS activity staining at and around infection sites (Fig. 5D1).** A similar local activation of the reporter gene was observed in several independent transgenic lines and with different pathogens, including *P. syringae* DC3000 (virulent), *P. syringae* DC3000 carrying *avrRpt4* (avirulent), *Sclerotinia sclerotiorum* (Fig. 5D2), *Alternaria brassicicola* (Fig. 5D3), *Pythium sylvaticum* (Fig. 5D4), and *A. alternata* (Fig. 6B) (*AtWRKY33*-P1::GUS). In the case of the root oomycete pathogen *P. sylvaticum*, massive GUS staining was observed primarily in secondary root initiation zones, secondary roots, and root hairs (Fig. 5D4).

Several independent F2 and F3 transgenic lines harboring different 5' *AtWRKY33* promoter deletion-reporter gene constructs (Fig. 6A and Supplementary Figure 1, P1 to P6) were tested for their response to different pathogens. As an initial standard assay, we applied defined-sized droplets of *A. brassicicola* fungal mycelium suspensions onto leaves of the plants and assayed for GUS activity staining 5 and 6 days postinoculation (Fig. 6B). Strong, local, and very similar staining was observed around infection sites of all tested lines that harbored the P1, P2, P3, and P4 *AtWRKY33* promoter reporter constructs (Fig. 6B; data not shown). In lines carrying the P5 construct, local responsiveness to *A. brassicicola* was still observed; however, GUS activity staining was significantly reduced and confined to fewer cells around the infection site (Fig. 6B). All these lines also were subsequently tested using *H. parasitica* Cala2, *P. sylvaticum*, and *Pseudomonas syringae* DC3000 (\pm *avrRpt4*) and very similar results were obtained, with the exception that the P5 lines showed no clear GUS staining when challenged by *H. parasitica* Cala2 (data not shown). Thus, these results suggest that elements residing within the 38-bp stretch at position -333 to -295 are responsible for mediating the strong pathogen-responsiveness of *AtWRKY33*, but that additional elements downstream of -295 also contribute. The 38-bp sequence contains two W box elements (W1 and W2) (Fig. 7A) known to mediate pathogen responsiveness of plant defense genes by interaction with WRKY TFs (Ülker and Somssich 2004). Interestingly, two similarly spaced functionally critical W box elements ($W_{A/B}$) also are present in the promoter of *PcWRKY1*, the parsley orthologue of *AtWRKY33* (Fig. 7A) (Eulgem et al. 1999). Furthermore, a construct (Fig. 7B, P4_{m1+2}) significantly lowered overall GUS activity and elicitor-mediated inducibility to approximately four- to fivefold. Simultaneous mutation of W1, W2, and W3 (Fig. 7B, P4_{m1-3}) nearly abolished overall PAMP inducibility,

additional W box element (W3) embedded in DNA sequences similar to those found for the parsley W_C element is present between positions -270 and -246 (Fig. 7A). This element or an additional W box at position -211 (W4) may be responsible for the weaker but clear pathogen responsiveness observed with the P5 transgenic lines. The pathogen responsiveness of the P5 transgenic lines was more obvious when bacterial suspensions of an avirulent strain of *P. syringae* DC3000 (+*avrRpt4*) were directly injected at high density into the apoplastic space of leaves via a needleless syringe (Supplementary Figure 2). In contrast to this, P6 transgenic lines showed no GUS activity staining above basal levels with any of the treatments used (Fig. 6B).

PAMP-triggered responsiveness of the *AtWRKY33* promoter is mediated via W boxes.

Apart from two potential W boxes, database searches in PLACE or AtTFDB failed to reveal any other known *cis*-acting DNA elements implicated in defense gene activation within the P5 promoter. The functionality of the W boxes within the -333 *AtWRKY33* promoter in PAMP-triggered activation was tested by transient expression assays employing the well-established parsley protoplast system and the oomycete-derived peptide Pep25 (Hahlbrock et al. 2003). PAMP-dependent activation of the reporter gene was observed with the *AtWRKY33* P4 construct (Fig. 7B). Overall, this promoter was capable of 11-fold elicitor-stimulated expression of the reporter gene. Mutating both upstream W boxes (W1 and W2; 5'-C/TTGAC C/T-3' to 5'-C/TTGGAC/T-3') in the context of the P4 promoter

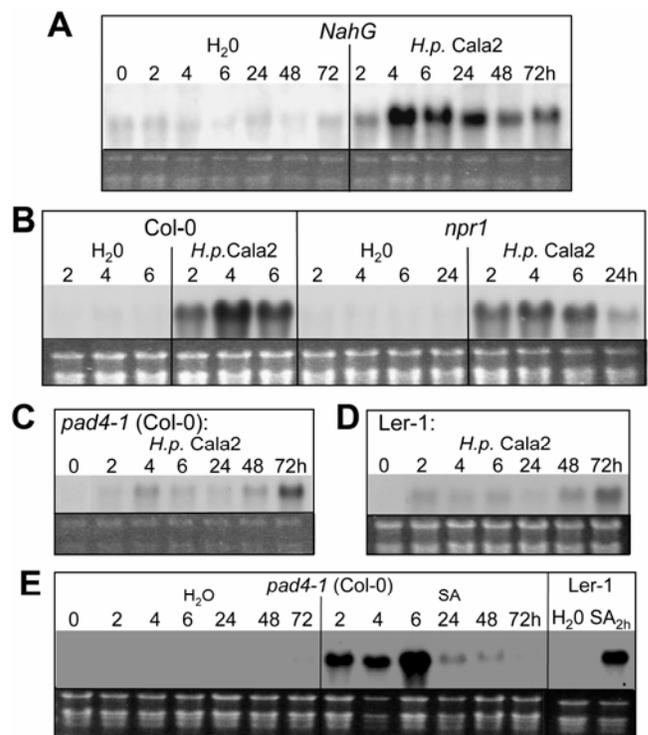


Fig. 4. Rapid activation of *AtWRKY33* by *Hyaloperonospora parasitica* Cala2 does not require salicylic acid (SA) or *NPRI* function but is dependent on *PAD4*. **A** through **E**, *AtWRKY33* mRNA levels detected at the indicated timepoints from *Arabidopsis* **A**, *NahG* and **B**, Col-0 wild-type and *npr1* mutant seedlings sprayed with water or with *H. parasitica* Cala2 at 2×10^5 spores/ml; *Arabidopsis* **C**, *pad4-1* and **D**, ecotype Ler-1 seedlings sprayed with *H. parasitica* Cala2 at 2×10^5 spores/ml; and **E**, *Arabidopsis pad4-1* and Ler-1 seedlings sprayed with water or with 5 mM SA solution. Blots from gels loaded with **A** and **C**, 10 or **B**, **D**, and **E**, 20 μ g of total RNA per lane were hybridized with radioactively labeled *AtWRKY33* cDNA and visualized by autoradiography. Ribosomal rRNA bands of the ethidium bromide-stained gels served as equal loading.

suggesting a functional role of W3 in this response. The values obtained for P4_{m1-3} were nearly identical to P4_{m1-4} (Fig. 7B), in which the W4 element at position -211 also was mutated, indicating that W4 did not function as an independent W box element in these assays. Consistent with these results, GUS reporter gene expression driven by the shorter *AtWRKY33* P5 fragment also showed PAMP inducibility with values similar to those of P4_{m1+2} (Fig. 7B). Furthermore, mutations of W3 or W3 + W4 in the context of the P5 construct yielded nearly identical GUS activity values and nearly no Pep25 inducibility (P5_{m3} and P5_{m3+4}) (Fig. 7B). Thus, PAMP-triggered activation of *AtWRKY33* is mediated mainly by a set of three W box elements.

Increased association of WRKY factors to the *AtWRKY33* promoter upon benzothiadiazole *S*-methyl ester treatment.

We used chromatin immunoprecipitation (ChIP) to determine if there is an increase in WRKY factor abundance associated with the *AtWRKY33* promoter region encompassing the relevant W box elements upon activating plant defenses. Fragmented chromatin, isolated from *Arabidopsis* wild-type plants sprayed with benzothiadiazole *S*-methyl ester (BTH), a chemical inducer of systemic acquired resistance (Maleck et al. 2000), was immunoprecipitated using an anti-all-WRKY antibody that detects the majority of the WRKY factor family (Turck et al. 2004). Polymerase chain reaction (PCR) was performed on DNA before immunoprecipitation (INPUT) or on immunoprecipitated DNA using primers that amplify the -399 to -187 region of the *AtWRKY33* promoter or, as a control, the *AtWRKY33* open reading frame region spanning intron 1 (Fig. 7C). The ChIP experiment revealed a BTH-dependent *in vivo* increase in

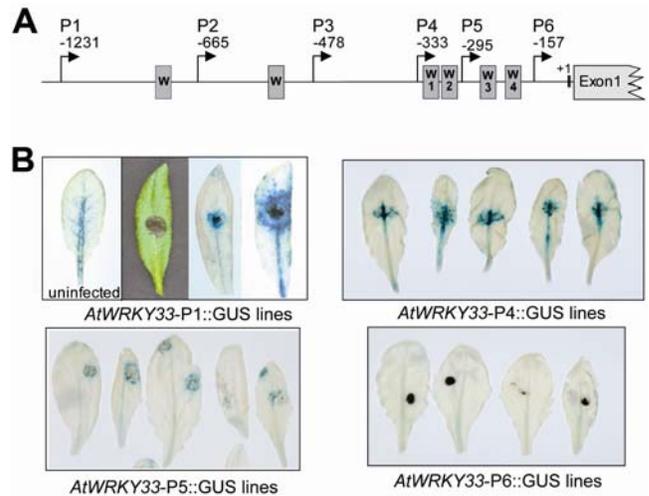


Fig. 6. Pathogen-induced expression of the GUS reporter gene under the control of different derivatives of the *AtWRKY33* promoter. **A**, Schematic diagram of the 5' upstream region of the *AtWRKY33* gene. Bent arrows marked with P1 to P6 represent the different 5' ends of the *AtWRKY33* promoter used to generate transgenic *Arabidopsis AtWRKY33::GUS* expression lines. The transcription start site is marked with +1, and the first exon indicated. Putative W box DNA elements are boxed and those relevant for this publication are marked W1 to W4. **B**, Histochemical GUS activity staining in leaves of different *AtWRKY33promoter::GUS* transgenic reporter lines challenged with *Alternaria brassicicola* or *A. alternata* (mid two leaves in b, *AtWRKY33*-P1::GUS lines). Fungal suspension was applied (25- to 50- μ l droplets per leaf) and staining performed 5 and 6 days postinoculation. Representative leaves from both T2 and T3 transgenic lines harboring the indicated *AtWRKY33::GUS* reporter constructs are shown. Three independent repetitions, each using leaves from several transgenic lines, were performed.

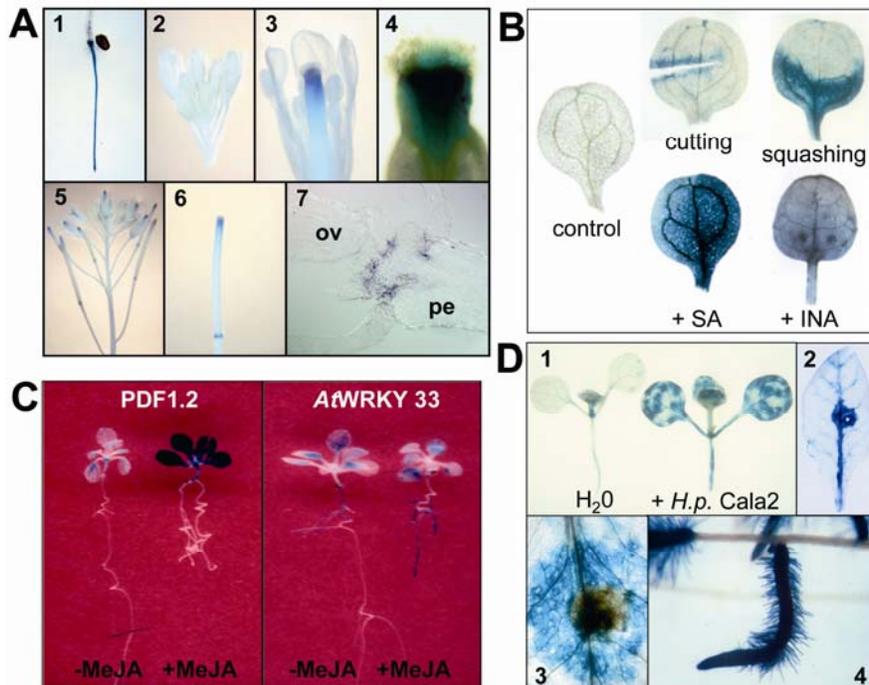


Fig. 5. Detection of GUS reporter gene expression driven by the 1.23-kb *AtWRKY33* promoter in transgenic *Arabidopsis* plants. **A**, Histochemical localization of GUS activity of *AtWRKY33::GUS* transgenic plants in etiolated roots (1), young inflorescences and anthers (2 and 3), stigmatic papillae and the style (3 and 4), and floral abscission zones (5 and 6). *AtWRKY33* transcripts (in situ hybridization) in sections made from siliques including flower base and distal part of petioles (7); ov, ovary; pe, petal. **B**, Histochemical localization of GUS activity detected in cotyledons of transgenic *AtWRKY33::GUS* reporter plants upon wounding or upon spraying with 5 mM salicylic acid (SA) or 300 μ M 2,6-dichloroisonicotinic acid (INA) solution 6 h post treatment. **C**, Histochemical localization of GUS activity of transgenic *AtWRKY33::GUS* reporter lines grown on plates and sprayed with 45 μ M methyl jasmonate (MeJA). The transgenic line PDF1.2 contains the GUS reporter gene under the control of the nearly 1.2-kb-long *PDF1.2a* gene promoter and served as a positive control for jasmonic acid responsiveness. **D**, Histochemical localization of GUS activity of transgenic *AtWRKY33::GUS* reporter lines upon challenge of seedlings with *Hyaloperonospora parasitica* Cala2 (1), of fully expanded leaves with *Sclerotinia sclerotiorum* (2) and *Alternaria brassicicola* (3), and of roots with *Pythium sylvaticum* (4).

WRKY factors associated with the promoter region. However, it also showed that there is already significant WRKY protein binding to this DNA region in untreated leaf tissue, which is in agreement with previous findings that W box elements often are constitutively occupied by WRKY TFs (Turck et al. 2004).

DISCUSSION

Global expression profiling has confirmed that pathogen-triggered activation of plant defenses is associated with massive transcriptional reprogramming (Eulgem 2005). Many of the class of “immediate-early” type genes in this program, whose transcriptional activation does not require de novo protein biosynthesis, encode for TFs important in regulating the defense transcriptome. Several genes of the WRKY TF family belong to this class, including *AtWRKY33*. Based on microarray data, expression of *AtWRKY33* is rapidly and strongly induced by PAMPs such as the bacterial flagellin-derived peptide flg22 (Navarro et al. 2004) and by chitin, a structural element of fungal walls (Wan et al. 2004). It also is induced by avirulent strains of *P. syringae* (Bartsch et al. 2006), by H₂O₂, and by ozone oxidative stress (Davletova et al. 2005; Mahalingam et al. 2005).

Our results illustrate that *AtWRKY33* expression also is induced rapidly by various other pathogens, including avirulent and virulent races of the biotrophic oomycete *H. parasitica*, by the necrotrophic fungi *A. brassicicola*, *A. alternata*, and *S. sclerotiorum*, and by the root oomycete *Pythium sylvaticum*. Additionally, *AtWRKY33* responds in seedlings to wounding and rapidly to exogenous application of SA, INA, and BTH, but not to

MeJA. *A. brassicicola*-dependent expression of *AtWRKY33* is not affected in the jasmonic acid (JA)-insensitive mutant *jar1* (Staswick et al. 2002), further indicating that JA or MeJA signaling is not required for its expression (data not shown). Still, *H. parasitica*-mediated *AtWRKY33* expression does not require SA for its activation, as demonstrated by its unaltered response to this pathogen in *NahG* plants. Similarly, pathogen-induced expression of *AtWRKY33* was mostly unaffected in *npr1* plants, suggesting that signaling components downstream of SA also are not needed. However, rapid *H. parasitica*-induced expression of *AtWRKY33* expression does require *PAD4* function. In *Arabidopsis* ecotype Col-0, resistance toward *H. parasitica* Cala2 is determined by the major resistance (R) gene *RPP2* (Aarts et al. 1998). *RPP2*-dependent resistance has been shown to require *PAD4*, an important regulator upstream of SA signaling (Glazebrook et al. 1997). Our results indicate, however, that activation of *AtWRKY33* expression by *H. parasitica* Cala2 occurs within an SA-independent signaling pathway downstream of *PAD4*. This additional pathway appears also to be *RPP2* dependent, because a similar strong temporal delay in *H. parasitica* Cala2-induced *AtWRKY33* expression is observed in *pad4* mutants and in wild-type susceptible Ler-1 plants that lack *RPP2*. The existence of both SA-dependent and SA-independent pathways linked to the two key interacting regulators EDS1 and *PAD4* recently has been demonstrated (Bartsch et al. 2006). In tobacco, activation of an early *Tobacco mosaic virus*-responsive WRKY gene *TIZZ* also was shown to occur through an SA-independent defense signaling pathway (Yoda et al. 2002).

Intriguingly, the biotrophic barley powdery mildew pathogen *B. graminis* did not activate *AtWRKY33* whereas the ne-

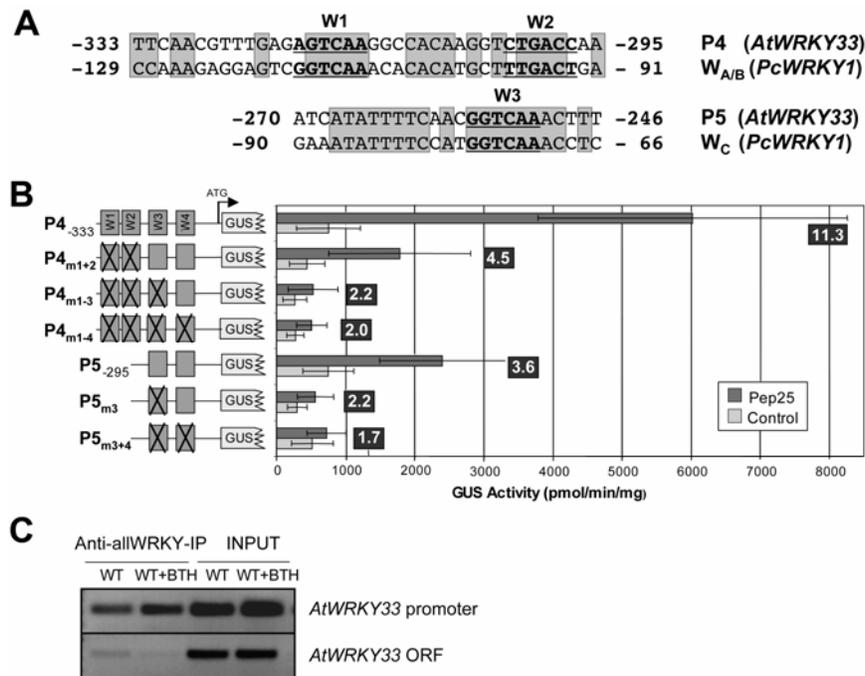


Fig. 7. W box elements mediate pathogen-associated molecular pattern (PAMP)-triggered transient expression of the *AtWRKY33* promoter-driven GUS reporter gene in parsley protoplasts. **A**, Detailed comparison of the 5' regulatory regions that share sequence similarity between the *Arabidopsis AtWRKY33* gene and its parsley orthologue *PcWRKY1* (Eulgem et al. 1999). Sequence numberings are based on their positions within their respective gene promoters relative to the transcription start site. **B**, Transient transfection assays in parsley protoplasts. Protoplasts were transfected with the different *AtWRKY33* promoter::GUS translational fusion constructs schematically shown on the left, and either left untreated (control) or treated with the oomycete-derived PAMP polypeptide Pep25 for 8 h. The average specific GUS activities along with their standard deviation for each construct \pm Pep25 are given. Mutations of the respective W box element (5'CTTGACC/T3' to 5'CTTGAC/T) are marked by a bold X in the left-hand scheme. Specific GUS activities (bars) and the values for fold elicitor inducibility (boxed numbers) each represent the means from 10 measurements derived from five independent transfection experiments. **C**, Chromatin immunoprecipitation (ChIP) was conducted using the anti-all-WRKY serum (Turck et al. 2004) on extracts from untreated and 6-h benzothiadiazole S-methylester (BTH)-treated *Arabidopsis* wild-type leaves. Polymerase chain reaction (PCR) was performed using primers spanning the -399 to -187 region of the *AtWRKY33* promoter or spanning intron 1 of the *AtWRKY33* open reading frame (ORF). INPUT represents PCR products from the same tissue extracts following formaldehyde crosslinking but not subjected to immunoprecipitation.

crotoph *A. alternata* did. *Arabidopsis* is nonhost to these two pathogens and, in both cases, hyphal growth appears to be arrested at a similar early penetration stage (Narusaka et al. 2005; Zimmerli et al. 2004). Although not completely comparable, the microarray data that were performed on plants challenged with these two pathogens did reveal differences in the nonhost plant resistance responses. Most notable was the finding that *B. graminis* induced expression of some prominent marker genes of the JA/ethylene (ET) signaling pathway, whereas expression of these genes was unaffected upon *A. alternata* infection (Narusaka et al. 2005; Zimmerli et al. 2004). Because the JA/ET and SA signaling pathways can be antagonistic (Glazebrook 2005), activation of part of the JA pathway by *B. graminis* may have a negative effect on some component within the signaling pathway downstream of PAD4 that is required for triggering *AtWRKY33* expression.

A recent publication by Zheng and associates (2006) that appeared after submission of our work confirmed the importance of *AtWRKY33* in plant defense. Two *Atwrky33* loss-of-function mutants showed enhanced susceptibility toward *Botrytis cinerea* and *A. brassicicola*, whereas ectopic overexpression of *AtWRKY33* increased resistance toward these fungi but compromised basal resistance to *Pseudomonas syringae* DC3000 bacteria. Interestingly, however, the *Atwrky33* mutants did not differ in their response to this bacterial strain when compared with wild-type plants. The authors conclude from their study that *AtWRKY33* acts as a positive regulator of JA- and ET-mediated defense signaling but as a negative regulator of SA-mediated responses. They note that pathogen-induced expression of *AtWRKY33* does not require the signaling molecule SA, a finding that is in complete agreement with our data.

Functional dissection of the *AtWRK33* promoter revealed that three closely spaced W box elements are the key enhancers mediating the observed rapid pathogen- or PAMP-triggered activation of transcription. Together with our in vivo findings demonstrating stimulus-dependent enrichment of WRKY factors at this promoter, this strongly implicates WRKY factors as the main regulatory proteins in modulating expression of this gene under biotic stress conditions. W box motifs commonly are detected within the promoters of coregulated *Arabidopsis* defense response genes, implying that WRKY factors play a major role in their regulation (Ülker and Somssich 2004). Reporter gene constructs driven by W box elements in transgenic *Arabidopsis* plants were strongly activated by diverse pathogens (Rushton et al. 2002). A W-box-containing element termed E17-27 was identified in the parsley *CMPG1* promoter that enabled immediate elicitor-induced reporter gene expression in transient assays and pathogen-triggered expression in transgenic *Arabidopsis* plants (Kirsch et al. 2001). An element highly similar to E17-27, present in the *Arabidopsis* *CMPG1* promoter, mediated rapid pathogen responsiveness of a reporter gene in transgenic *Arabidopsis* plants (Heise et al. 2002). *AtCMPG1* recently was shown to be required for activation of defense mechanisms triggered by resistance genes in tobacco and tomato (Gonzalez-Lamothe et al. 2006). Other defense-associated genes for which the involvement of W box elements in their activation has been functionally demonstrated include *Arabidopsis* *NPR1* (Yu et al. 2001), *AtTRXh5* (Laloi et al. 2004) and *SIRK/FRK1* (Asai et al. 2002; Robatzek and Somssich 2002), *Brassica oleracea* *SFR2* (Rocher et al. 2005), maize *PRms* (Raventós et al. 1995), some parsley *PcPR10* genes (Rushton et al. 1996), and tobacco *CHN48* (Yamamoto et al. 2004).

Like *AtWRKY33*, many pathogen-induced *Arabidopsis* WRKY genes contain multiple W box elements within their 5' regulatory regions (Dong et al. 2003), suggesting that they are under positive or negative feedback regulation. In the case of

PcWRKY1, the parsley orthologue of *AtWRKY33*, ChIP analyses revealed elicitor-dependent and transient in vivo binding of parsley *PcWRKY1* to three closely spaced $W_{A/B/C}$ elements within the promoter of *PcWRKY1* (Turck et al. 2004). In addition, the palindromically arranged $W_{A/B/C}$ boxes were shown to be responsible for the particularly rapid response of *PcWRKY1* to the PAMP Pep-25 (Eulgem et al. 1999). A similar structural organization of three functionally important W box elements (W1, W2, and W3) also is present in the *AtWRKY33* promoter (Fig. 7A). Although the spacing of W3 relative to W1 and W2 in the *AtWRKY33* promoter is larger than between the $W_{A/B/C}$ elements of *PcWRKY1*, W1 and the parsley W_C elements both appear embedded in a similar DNA sequence environment (Fig. 7A). Whether this is purely fortuitous or whether it's indicative of a DNA domain that is targeted by common regulatory components remains to be addressed. Although intense database searches have not revealed the presences of additional *cis*-acting DNA elements within the decisive *AtWRKY33* promoter region, we nevertheless cannot completely exclude a contribution of other unknown elements in modulating pathogen-dependent expression of the gene. Still, our data strongly favor the hypothesis that WRKY factors acting via W box binding are the major regulators of *AtWRKY33*. Because activation of *AtWRKY33* occurs independent of de novo protein synthesis, such factors should be present in unchallenged cells and should be post-translationally regulated upon pathogen stimuli, a situation reminiscent of the one observed for *PcWRKY1* activation in parsley (Turck et al. 2004).

Key questions that need to be addressed include identifying the signal molecule or molecules required for pathogen-induced activation of *AtWRKY33* transcription, pinpointing the WRKY factors responsible for the rapid and transient W box-dependent activation of *AtWRKY33*, determining whether these WRKY factors are identical to or are part of the negative regulators controlling *AtWRKY33* as inferred from the protein synthesis inhibitor studies, and discovering what post-translational modifications they undergo upon pathogen challenge of the cell. Finally, elucidation of the direct *AtWRKY33* target genes will be of utmost importance and should reveal vital clues to our understanding of the pathways controlled by this TF.

MATERIALS AND METHODS

Plants, cell suspension culture, and growth conditions.

Arabidopsis thaliana ecotype Col-0 and Ler-1 plants, *NahG* plants (Gaffney et al. 1993), *ndr1* (Century et al. 1997), *npr1* (Cao et al. 1997), and *pad4-1* (Glazebrook et al. 1997) mutants, and PDF1.2::GUS transgenic lines were used in this study. The plants were grown on double-autoclaved soil in phytochambers (Percival Scientific, Boone, IA, U.S.A.) for most purposes under short-day conditions (8 h of light and 16 h of dark) at 20°C. *A. thaliana* (ecotype Col-0, line At7) cells were propagated in continuous darkness as previously reported (Trezzi et al. 1993).

Treatment of plants or cultured cells with SA, INA, MeJA, CHX, UV-light, and wounding.

For INA, MeJA, and SA treatment, 10- to 14-day-old seedlings were sprayed individually with solutions containing either 5 mM Na⁺-salicylate + 0.02% (vol/vol) Silwet L-77 (Lehle Seeds, Round Rock, TX, U.S.A.), 45 μM MeJA in water, or a 0.59-mg/ml (300 μg) solution of INA in water for the indicated time periods. Control plants were sprayed with water or water containing 0.02% Silwet L-77. For UV illumination of *Arabidopsis* cells, four TL 18W/29 bulbs (warm white light; Philips Licht, Hamburg, Germany), three TL18W/18 blue bulbs (Philips) and two Sylvania blacklight blue bulbs (OSRAM Sylvania,

München, Germany) were employed in a dark phytochamber at 26°C. CHX (20 µM) was added to dark-grown cell cultures 30 min prior to UV-light treatment.

For the plate assays shown in Figure 5C, seeds were germinated and kept on 0.5 Murashige-Skoog (MS) plates containing 4.5 µM MeJA.

Treatment of plants with pathogens.

H. parasitica Emoy2 and Cala2 were provided by E. Holub (Warwick HRI, Wellesbourne, England), Noks1 was provided by E. Kombrink and J. Ehling, (Max Planck Institut für Züchtungsforschung [MPIZ], Köln, Germany), and *Blumeria graminis* f. sp. *hordei* by V. Lipka (MPIZ). Pathogen challenge inoculations were conducted by spraying the seedlings (10 to 14 days old) or plant leaves with an *H. parasitica* spore suspension (2×10^5 spores/ml in H₂O containing 10⁻⁶% [vol/vol] Tween 20) or by dispersing *B. graminis* spores on the leaves.

P. syringae pv. *tomato* strain DC3000 (±avrRpt4) was infiltrated into leaves at 10⁸ CFU ml⁻¹ in 10 mM MgCl₂.

Alternaria brassicicola strain MUCL20297 (Broekaert et al. 1990) was grown for 1 week in potato dextrose (PD) liquid culture under agitation at 28°C. The mycelium subsequently was homogenized by an Ultra-thurax or Polytron and 25- to 50-µl droplets of fungal suspension were applied per leaf depending on their size. *A. alternata* and *Pythium sylvaticum* were obtained from DSMZ, the German National Resource Centre for Biological Material (Braunschweig, Germany), and *S. sclerotiorum* was kindly provided by W. Gieffers (MPIZ). Growth conditions of *S. sclerotiorum* were at 25°C in PD liquid media under slow agitation on a shaker. The mycelium was processed as describe for *Alternaria* spp. above.

RNA isolation and analysis.

Plant material was ground in liquid N₂, and RNA was isolated using the RNA/DNA Maxi Kit (Qiagen, Hilden, Germany) or TRIZOL-reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. In all, 10 to 20 µg was used for RNA blot analysis using standard molecular procedures (Sambrook et al. 1989). DNA probes were radioactively labeled by random priming using [α -³²P]dCTP and the Ready-To-Go Kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's instructions.

Construction of transgenes and generation of transgenic plants.

For each reporter construct, the relevant *AtWRKY33* promoter containing the 5' ATG upstream regions, 1,248 bp (P1), 682 bp (P2), 495 bp (P3), 350 bp (P4), 312 bp (P5), and 174 bp (P6), were amplified by PCR and introduced into the *Hind*III-*Bam*HI sites of pUC9-GUS (van de Löcht et al. 1990). The entire promoter-GUS cassette than was excised from the vector and cloned into the binary Ti vector pGPTV (Becker et al. 1992), replacing the endogenous GUS cassette. Col-0 plants were transformed using the floral-dip method (Clough and Bent 1998) and the *Agrobacterium tumefaciens* GV3101 (Koncz et al. 1994). The *PDF1.2::GUS* transgenic line contained 1,178 bp of promoter sequence of the *PDF1.2a* gene (At5g44420) and was generated as described for the *AtWRKY33* promoter reporter constructs above.

Transient transfection experiments.

The *AtWRKY33* P4 and P5 DNA fragments in the pUC9-GUS reporter vector were used for transient expression analyses with parsley cell culture protoplasts as previously described (van de Löcht et al. 1990). The P4_{m1-4} and P5_{m3-4} promoter constructs (Fig. 6B) in which the core sequence of the respective W box, 5'-TGAC-3', was altered by a 2-bp block mutation to

5'-TGGA-3', was achieved by the PCR-based megaprimer method (Landt et al. 1990). In each assay, 2×10^6 protoplasts were transfected with 5 µg of GUS reporter plasmid linearized with *Sca*I. PAMP treatment was carried out with Pep-25 at 333 ng/ml originally derived from the cell wall of the oomycete *Phytophthora sojae* (Nürnberger et al. 1994). Suspensions of transfected protoplasts were split, and one-half was treated for 8 h with the elicitor while the other half remained untreated. Aliquots subsequently were harvested and treated as described previously (Rushton et al. 1996).

For transient expression in single cells, the *AtWRKY33* cDNA was cloned into the Gateway destination vector pPAM-KAN-35S-GWY-GFP according to the protocol provided by Invitrogen. This allowed the generation of an N-terminal fusion protein to green fluorescent protein (GFP). The control GFP construct was made by deletion of the Gateway cassette, resulting in a vector expressing *GFP* directly from the 35S CaMV promoter. These constructs were used to bombard leaves of leek and 3-week-old *Arabidopsis* Col-0 plants. DNA coating of gold particles and bombardments were performed as previously described (Panstruga et al. 2003). To allow for protein accumulation, the bombarded leaves were placed in a light chamber at 18°C for 20 h prior to analysis by fluorescence microscopy using a Zeiss Axiophot 1 (Carl Zeiss, Jena, Germany) and appropriate filters for GFP (excitation: 470/40; emission: 525/50). Fluorescence and brightfield micrographs were taken with a digital imaging system (Diskus, Technisches Büro Hilgers, Königswinter, Germany).

Chromatin immunoprecipitation.

Col-0 wild-type and *pad4.1* plants were grown for 28 days on MS media plates under long-day conditions. Plants were sprayed with BTH solution (126 mg/liter = 300 µM) and harvested together with untreated control plants after 6 h. Whole-plant material (2 g per assay) was processed for chromatin immunoprecipitation according to the protocol of Gendrel and associates (2005) and using the previously described anti-all WRKY serum (Turck et al. 2004). The final immunoprecipitated DNA was resuspended in 50 µl of Tris-EDTA buffer and 1.5 µl was used for PCR amplification in the presences of 0.3 µM each primer, 200 µM deoxynucleotide triphosphate, and *Taq* polymerase (Ampliqon) for 33 cycles of 94, 58, and 72°C steps with 30 s at each temperature. The primer pair 5'-ATCATACGTGTCAGAACGAGACAT-3' and 5'-TTCGTTTTTATTTTTAGGGTCAACA-3' was used to amplify 212 bp of the *AtWRKY33* promoter region encompassing the W box DNA elements. Primer pair 5'-CTCCTTCTCTGTCTCTCTCTCC-3' and 5'-TTGTGATTAAAGCTCCTGTGGTT-3' was used to amplify 368 bp of the *AtWRKY33* open reading frame spanning intron 1.

GUS staining.

Histochemical staining of plant tissue was performed by vacuum infiltration (three times for 1 to 2 min each) with X-gluc solution (100 mM NaPO₄, 2 mM K₃Fe(CN)₆, 0.1% Triton-X-100, and X-gluc at 0.5 mg/ml) followed by incubation at 37°C overnight, and subsequent destaining of the tissue for several days in 70% ethanol.

In situ RNA hybridization.

In situ hybridization was performed according to Fletcher and associates (1999). Briefly, tissue pieces, including half of the silicles and the distal part of the petioles, were harvested and fixed in 4% formaldehyde for 4 h on ice. After dehydration in a graded series of ethanol, the tissue was embedded in paraffin. Longitudinal semithin sections were prepared and hybridized in situ with digoxigenin-labeled sense and antisense transcripts. Signal development was by using anti-digoxigenin alkaline

phosphatase. Micrographs were taken using a Zeiss Axiophot 1 microscope and DIC optics and a digital imaging system (Diskus, Technisches Büro Hilgers, Königswinter, Germany).

DNA sequencing.

The correctness of all reported DNA constructs was determined by the MPIZ DNA core facility ADIS using BigDye terminator chemistry on Applied Biosystems 377 sequencers. Oligonucleotides were purchased from GIBCO (Life Technologies, Gaithersburg, MD, U.S.A.).

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