The WRKY70 Transcription Factor: A Node of Convergence for Jasmonate-Mediated and Salicylate-Mediated Signals in Plant Defense

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Cross talk between salicylic acid (SA)- and jasmonic acid (JA)-dependent defense signaling has been well documented in plants, but how this cross talk is executed and the components involved remain to be elucidated. We demonstrate that the plant-specific transcription factor WRKY70 is a common component in SA- and JA-mediated signal pathways. Expression of WRKY70 is activated by SA and repressed by JA. The early induction of WRKY70 by SA is NPR1-independent, but functional NPR1 is required for full-scale induction. Epistasis analysis suggested that WRKY70 is downstream of NPR1 in an SA-dependent signal pathway. Modulation of WRKY70 transcript levels by constitutive overexpression increases resistance to virulent pathogens and results in constitutive expression of SA-induced pathogenesis-related genes. Conversely, antisense suppression of WRKY70 activates JA-responsive/COI1-dependent genes. The effect of WRKY70 is not caused by subsequent changes in SA or JA levels. We suggest that WRKY70 acts as an activator of SA-induced genes and a repressor of JA-responsive genes, integrating signals from these mutually antagonistic pathways.

INTRODUCTION

The need of plants to cope with potential pathogens has led to the evolution of complex adaptive responses involving protective physical barriers and production of a diverse array of antimicrobial metabolites and proteins. A key step in induced plant resistance is the timely recognition of the invading pathogen. Plants have the ability to recognize pathogen-derived molecules, exogenous elicitors, or molecules released from the plant by the action of the pathogen (Yang et al., 1997; Van der Biezen and Jones, 1998; Montesano et al., 2003). This recognition can trigger a local response (hypersensitive response), a form of programmed cell death at the site of infection, which may contain the invading pathogen. Hypersensitive response is often associated with the development of a plant immune response and systemic acquired resistance (SAR) (Feyns and Parker, 2000). SAR is characterized by an increase in endogenous salicylic acid (SA), transcriptional activation of pathogenesis-related (PR) genes (e.g., PR1, BGL2 [PR2], and PR5), and enhanced resistance to a broad spectrum of virulent pathogens (Uknes et al., 1992; Ryals et al., 1996). SA is necessary for SAR, and a series of studies demonstrated that SA signaling is mediated by an ankyrin repeat protein, NPR1/NIM1 (Cao et al., 1994; Delaney et al., 1995; Li et al., 1999; Fan and Dong, 2002), but also that an NPR1-independent pathway does exist (Clarke et al., 1998, 2000; Shah et al., 1999).

In addition to SA, other signaling molecules such as jasmonic acid (JA) and ethylene (ET) regulate various plant defense responses through signaling pathways that are distinct from the classic SA-mediated SAR pathway (Glazebrook, 2001; Kunkel and Brooks, 2002; Turner et al., 2002). For example, concomitant activation of the JA and ET response pathways is required for expression of PDF1.2 encoding an antimicrobial defensin (Penninckx et al., 1998). Arabidopsis thaliana mutants that are impaired in JA production (e.g., fa3 fad7 fad8 triple mutant) or perception (e.g., coi1 and jar1) exhibit enhanced susceptibility to a variety of virulent fungal and bacterial pathogens (Kunkel and Brooks, 2002).

In the complex network of regulatory interactions during plant resistance responses, an antagonistic relationship between SA and JA signaling pathways is evident (Kunkel and Brooks, 2002). The inhibitory effect of SA on JA signaling in Lycopersicon esculentum (tomato) is well established (Doares et al., 1995), and recent studies using A. thaliana mutants provide genetic evidence for this antagonism. The eds4 and pad4 mutants deficient in SA accumulation as well as the npr1 mutant with impaired response to SA exhibit enhanced induction of JA-responsive genes (Penninckx et al., 1996; Clarke et al., 1998; Gupta et al., 2000). New evidence indicates that cellular localization of NPR1 might play a crucial role in modulating the SA-mediated suppression of JA-responsive genes (Spoe et al., 2003). Moreover, studies with Nicotiana tabacum (tobacco) and A. thaliana reveal that JA- and SA-mediated defense responses can be mutually antagonistic (Vidal et al., 1997; Norman-Setterblad et al., 2000). Several genetic studies provide evidence that JA signaling negatively regulates the expression of SA-responsive genes in A. thaliana (Petersen et al., 2000; Kachroo et al., 2001; Kloek et al.,...
2001). It is likely that these two regulatory networks controlling plant defense signaling contain several nodes of interaction, but the identity of those nodes remains to be elucidated. One of the open questions is whether transcription factors are involved in cross talk between SA and JA signaling.

Transcriptional regulation of defense gene expression appears central in induced disease resistance, and >1500 transcription factors (Riechmann et al., 2000) may be involved in plant defense. These include the plant-specific WRKY family of transcription factors defined by a DNA binding domain that contains the highly conserved amino acid sequence WRKYGQK (Eulgem et al., 2000). WRKY factors have been implicated in plant defense, plant senescence, and response to various environmental stresses (Yang et al., 1999; Du and Chen, 2000; Robatzek and Somssich, 2001; Yu et al., 2001; Chen and Chen, 2002; Chen et al., 2002) as well as in developmental processes (Pepperman et al., 2001; Johnson et al., 2002). The involvement of WRKY factors in plant defense is well documented; some of these factors have been shown to confer disease resistance (Deslandes et al., 2002), trigger expression of defense-related genes in SAR (Eulgem et al., 1999; Maleck et al., 2000; Robatzek and Somssich, 2002), and control A. thaliana innate immunity activated by bacterial flagellin (Asai et al., 2002).

In this study, we identified WRKY70 as a key factor controlling plant response to Erwinia carotovora subsp. carotovora, a bacterial necrotroph that can trigger both SA- and JA-dependent defense signaling (Kariola et al., 2003). Resistance to this pathogen can be induced by activation of either SA-mediated (Palva et al., 1994; Kariola et al., 2003) or JA/ET-mediated (Norman-Setterblad et al., 2000; Kariola et al., 2003) defense pathways. We characterized the expression of WRKY70 in response to plant hormones and pathogen elicitors and generated WRKY70 transgenic plants to identify target genes controlled by WRKY70. Expression profiling in transgenic plants suggests that WRKY70 can function either as a positive or a negative regulator of PR gene expression, and we identified subsets of genes divergently controlled by WRKY70. Our studies indicate that WRKY70 is an important node of convergence integrating SA and JA signaling during plant defense responses.

RESULTS

WRKY70, an Uncommon Member of the WRKY Transcription Factor Family

To elucidate the spectrum of plant gene activation that occurs during infection by the plant pathogenic enterobacterium E. c. carotovora, we identified A. thaliana genes that increase in expression in response to elicitor preparations (culture filtrates [CF]) from E. c. carotovora SCC3193 using suppressive subtractive hybridization. A subtracted cDNA library enriched for sequences preferentially expressed in CF-treated leaves was established (Brader et al., 2001). One of the isolated clones, corresponding to a CF-induced gene, showed complete match with the putative coding region fragment (437 to 768 bp downstream of ATG) from AT5S19_50 (accession number AL163972). Using PCR, a 956-bp full-length fragment for this gene encoding a DNA binding protein of the WRKY family was acquired from a cDNA library of CF-treated A. thaliana plants. Recently, the gene was defined as WRKY70 (accession number AF421157). The deduced amino acid sequence shares the closest similarity to that of WRKY54 (~53% identity), suggesting that the protein is not very closely related to other members of this transcription factor family. The two proteins have been classified into group III of the WRKY family (Kalide et al., 2003).

WRKY70 Expression Is Induced by Defense Signals

Plant response to pathogens is regulated by multiple signal transduction pathways, in which SA, JA, and ET function as key signaling molecules (Glazebrook, 2001). To elucidate the potential involvement of WRKY70 in plant defense, we characterized expression of the WRKY70 gene by RNA gel blot hybridization in A. thaliana wild-type plants in response to the exogenous signal molecules SA, 1-aminocyclopropane-1-carboxylic acid ([ACC], a natural precursor of ET), methyl jasmonate (MeJA), and E. c. carotovora elicitors (CF). To monitor the treatment procedure, we examined, in parallel, expression of the SAR marker PR1 and a JA-inducible/COI1-dependent gene AtCOR1 (Benedetti et al., 1998). In accordance with previous results (Norman-Setterblad et al., 2000), CF induced a delayed accumulation of PR1 transcripts (Figure 1A), whereas AtCOR1 was rapidly induced with a maximal transcript accumulation 2 h after CF treatment. The antagonistic effect of SA on JA signaling was evident in the following transient decrease of AtCOR1 expression after SA treatment (Figure 1A). WRKY70 displayed a basal expression that showed some temporal variation (Figure 1A). WRKY70 was strongly induced by CF and SA but not by ACC or MeJA (Figure 1A). By contrast, MeJA treatment appeared to repress WRKY70 expression at 8 h post-treatment (hpt). To correlate the changes in CF-induced gene expression with possible alterations in hormone levels, we characterized accumulation of SA and JA in CF-treated leaves (Figure 1B). JA content in untreated mature leaves was low, whereas CF-treated leaves exhibited rapid but transient accumulation of JA. By contrast, there was a significant and stable increase in free SA levels in response to CF after an initial lag period. These data indicate that CF-induced expression of WRKY70 is correlated with elevated levels of endogenous SA and suggest that the increased expression is initially repressed by the transient increase in JA.

WRKY70 Expression Involves SA Signaling

To elucidate plant defense signal transduction networks involved in expression of WRKY70, we employed transgenic NahG plants that fail to accumulate SA and mutants that affect the SA-dependent pathway (npr1-1), the ethylene-dependent pathway (ein2-1), or the JA-dependent pathway (coi1-1). ET signaling does not appear to be required for WRKY70 expression. By contrast, altering of SA (NahG and npr1) or JA (coi1) signaling led to significant changes in WRKY70 transcript levels (Figure 1C). The complete block in basal WRKY70 expression in NahG plants but only a reduction in npr1 plants suggested that endogenous
SA but not functional NPR1 is essential for the basal expression of WRKY70. This is contrasted by enhanced accumulation of WRKY70 transcripts in coi1 plants, supporting the hypothesis that JA signaling represses WRKY70 expression.

SA signaling also appears essential for elicitor-induced WRKY70 expression, as shown by the lack of WRKY70 transcripts in CF-treated leaves of NahG plants and drastically reduced levels of these transcripts in npr1 plants (Figure 1D). Similarly, SA-induced expression of WRKY70 was completely abolished in NahG plants (Figure 1D). However, the SA induction of WRKY70 in npr1 plants (Figure 1D) was only abolished at the later time points, suggesting that induction of WRKY70 is partially
NPR1-independent. The results indicate that WRKY70 is likely to be associated with the SA- and NPR1-mediated SAR response in plants.

**Overexpression or Antisense Suppression of WRKY70 Modulates Plant Disease Resistance**

To elucidate the putative function of WRKY70 during plant defense to pathogens, we generated *A. thaliana* overexpression or antisense suppression lines with WRKY70. From >50 independent overexpression or antisense suppression lines, homozygous progenies containing a single insert were used for further experiments. As shown in Figure 2A, five overexpression lines exhibited substantially elevated levels of WRKY70 transcripts, and eight antisense suppression lines expressed no detectable WRKY70 transcripts after CF treatment. The five overexpression lines displayed distinct morphological traits; they were smaller in size than control plants, exhibited changes in morphology with lancet shaped and slightly twisted leaves (Figure 2B), and were delayed in flowering (data not shown). By contrast, antisense suppression lines were slightly larger in size than control plants (Figure 2B) and showed an early flowering phenotype (data not shown).

To determine the contribution of WRKY70 to disease resistance in *A. thaliana*, we examined the resistance of the transgenic plants to two virulent bacterial pathogens, strain SCC1 of the necrotroph *E. c. carotovora* and *Pseudomonas syringae* pv *tomato* strain DC3000. Antisense suppression of WRKY70 appeared to sensitize the plants to SCC1 infection, leading to more rapid spreading of disease, maceration of nascent and young leaves, and dehydration of mature leaves (Figure 2C). Furthermore, the survival of antisense suppression plants was drastically reduced. By contrast, most WRKY70 overexpression plants did not exhibit spreading maceration to systemic leaves and displayed clearly enhanced survival of SCC1 infection. In agreement with this result, overexpression of WRKY70 led to enhanced resistance to *P. s. tomato* DC3000 (Figure 2C). These data suggest that the constitutive activation of the WRKY70-controlled defense mechanisms directly contributes to the enhanced disease resistance.

**WRKY70 Does Not Affect Endogenous Levels of JA, ET, and SA**

SA is a central mediator of plant defenses and is required for the SAR response. One possibility to explain the mode of action of WRKY70 is that this transcription factor might be involved in an amplification loop or signaling cascade that modulates SA biosynthesis. This is supported by the strong morphological antisense lines were tested in comparison to vector control plants with similar results. Growth of *P. s. tomato* DC3000 in planta was monitored 24 and 72 h after inoculation. Data represents the mean bacterial titer of at least six plants per time point. Error bars represent 95% confidence limits of log-transformed data (Mann-Whitney U test). At least two independent homozygous lines from each construct were tested with similar results. Ecc, *E. c. carotovora*; Pst, *P. s. tomato.*
phenotypes observed in overexpression and antisense plants, suggesting that the endogenous hormone balance might be altered. To test this possibility, we determined whether the endogenous levels of hormones implicated in plant defense were affected in WRKY70 transgenic plants. The basal levels of free SA, ET, and JA were not significantly different between the different transgenic lines (Figure 3). Interestingly, the content of conjugated salicylate glucoside was clearly reduced in WRKY70 overexpression plants (Figure 3A). We conclude that overexpression or antisense suppression of the WRKY70 gene does not cause major changes in endogenous levels of free SA, ET, or JA in A. thaliana leaves and argue that the modulation in disease resistance is not a result of alterations in the balance of these hormones.

**WRKY70 Overexpression Plants Show Constitutive Expression of PR Genes**

Enhanced disease resistance in A. thaliana is often accompanied by the accumulation of elevated levels of transcripts of PR genes associated with the SA-mediated defense pathway (PR1, PR2, and PR5; Uknes et al., 1992). Because exogenous SA and E. c. carotovora elicitors triggered expression of WRKY70, we sought to determine whether WRKY70 is directly involved in controlling expression of these PR genes. RNA gel blots demonstrated that PR2 and PR5 were constitutively expressed in WRKY70 overexpression plants (Figure 4A). By contrast, no PR1 band of the normal size could be detected, but a larger size transcript (~1.5 kb) hybridizing to a PR1-specific DNA probe was constitutive (Figure 4B). Consistent with this observation, reverse transcriptase (RT)-PCR analysis showed the presence of a much stronger PR1-specific band in the overexpression line S55 than in control plants under noninduced conditions (Figure 4C). The identity of this RT-PCR product was confirmed by sequencing (data not shown). Interestingly, the larger transcript disappeared rapidly when plants were treated with SA and was replaced by the PR1 transcript, possibly suggesting a precursor–product relationship. Furthermore, overexpression of WRKY70 promoted SA-induced accumulation of PR1 mRNA, whereas antisense suppression of WRKY70 resulted in delayed and reduced accumulation of these transcripts (Figure 4B). A more detailed examination of SA-inducible PR1 expression at 8 hpt at lower SA concentrations revealed that the levels of PR1 transcripts were remarkably reduced in antisense suppression plants, suggesting reduced sensitivity to SA (Figure 4B). Together, these results indicate that WRKY70 is one of the coactivators of SAR-associated PR genes (PR1, PR2, and PR5) and a limiting factor for full-scale SA-induced expression of PR1.

**WRKY70 Acts Downstream of NPR1 in an SA-Dependent Signal Pathway**

NPR1 functions downstream of SA in the SA-dependent signaling pathway and is required for the activation of some of the SA-dependent defense responses (Cao et al., 1994). Yu et al. (2001) suggest that some WRKY proteins act upstream of NPR1 and positively regulate its expression during the activation of plant defense responses. The reduced expression of WRKY70 in the npr1-1 mutant (Figure 1D) suggested that WRKY70 could be downstream of NPR1. To determine whether the effect of WRKY70 on PR gene expression is mediated by NPR1, we first examined NPR1 expression in both overexpression and antisense suppression plants. RNA gel blots revealed that overexpression or antisense suppression of WRKY70 did not cause any change in NPR1 transcript accumulation (Figure 4A). Furthermore, epistasis analysis was used to investigate the relative position of WRKY70 and NPR1 in the signal transduction pathway leading to PR gene expression. To achieve this, WRKY70 transgene from the overexpression line S55 was crossed into NahG and npr1-1 plants, respectively. The expression of the WRKY70 transgene was not affected in npr1-1 and NahG backgrounds (Figure 5A). The constitutive expression of PR2 or the putative PR1 precursor because of WRKY70 overexpression was still evident under noninduced conditions in the NahG or npr1-1 background, albeit at a somewhat reduced level (Figure 5A). These observations indicate that WRKY70 acts downstream of SA and NPR1.

**WRKY70 Acts as a Repressor of JA-Inducible Genes**

Considerable effort has been directed toward elucidating the regulatory network controlling expression of JA-inducible genes...
involved in plant defense responses. It has been well documented that increased SA levels antagonize JA signaling (Felton et al., 1999; Gupta et al., 2000). For example, AtVSP induction in response to E. c. carotovora exoenzyme elicitors in A. thaliana appears to be strictly dependent on the JA pathway but negatively regulated by the presence of SA (Norman-Setterblad et al., 2000). Similarly, our results show a transient induction of AtCORI expression by E. c. carotovora elicitors but a transient reduction in its constitutive expression by SA (Figure 1A). It is tempting to speculate that WRKY70 acts as a repressor controlling a subset of JA-responsive genes antagonized by SA. To test this hypothesis, we examined the effect of WRKY70 levels on the constitutive expression of AtCORI by RNA gel blot analysis (Figure 1A). It is tempting to speculate that WRKY70 acts as a repressor controlling a subset of JA-responsive genes antagonized by SA. To test this hypothesis, we examined the effect of WRKY70 levels on the constitutive expression of the JA-inducible genes AtCOR1 and AtVSP. As shown in Figure 4A, RNA gel blots reveal that antisense suppression of WRKY70 led to expression of both AtVSP and AtCOR1, indicating that WRKY70 is a repressor of these JA-inducible genes. In accordance with these results, expression of WRKY70 suppressed the MeJA-induced expression of PDF1.2, another marker for JA response (Figure 5B). Interestingly, this suppression was abolished in S55/npr1-1 plants, suggesting that NPR1 is required for this effect.

Differential Regulation of Defense-Related Genes by WRKY70

Our results (Figure 4A) suggest that WRKY70 might differentially control subsets of defense-related target genes. To learn more about the function of this transcription factor in A. thaliana defense responses, we wanted to identify potential WRKY70 regulon genes at genome-scale level using microarray technology. To this aim, we compared global gene expression in transgenic WRKY70 and vector control plants by using the Affymetrix AtGenome1 GeneChip containing ~8300 probe sets. Of >4500 probes that were accurately detectable, 331 (see supplemental data online) exhibited more than twofold differential expression in overexpression or antisense plants compared with their expression in control plants. Among them, 42 genes with >2.5-fold differential expression previously implicated in plant defense could be assigned to four major groups (Table 1). Although this list is not comprehensive, it clearly illustrates different patterns of gene expression.

The first two groups contain genes that are positively controlled by WRKY70, including 24 genes upregulated in WRKY70 overexpression plants. Among these are pathogen-responsive genes (e.g., WRKY60, PR2, and At1g75040 encoding a thaumatin-like protein) as well as genes related to oxidative stress responses (e.g., GST11 and At3g49120 encoding a peroxidase). There are also at least three disease resistance genes encoding LRR proteins (e.g., At2g32680, At4g04220, and At3g48080) in group I. GST11 and the senescence-related gene SAG21 have been characterized as upregulated genes during A. thaliana SAR (Maleck et al., 2000). Notably, the upregulated genes also include those potentially involved in cell wall modification, such as XTR3 and CaS1. The second group includes five genes downregulated in antisense suppression plants. Their products contain the transcription factor MYB71 and receptor-like protein kinases probably involved in signal transduction during pathogen infection.

The other two groups consist of genes negatively regulated by WRKY70. These include the JA-inducible genes AtVSP1 and AtVSP2 upregulated in antisense plants as well as genes downregulated in overexpression plants. This latter group contains additional JA-inducible genes, like OPR1, but also several genes encoding proteins with putative signaling and regulatory functions, like the cell wall–associated receptor-like

Figure 4. Differential Expression of SAR Markers and JA-Responsive Genes.

(A) Effect of WRKY70 on constitutive expression of SA-inducible and JA-responsive genes in transgenic A. thaliana by RNA gel blot analysis. To ensure equal loading of RNA, one representative filter was subsequently stripped and rehybridized with a probe against rRNA (rDNA).

(B) Effect of WRKY70 on induction of PR1 by SA.

(C) RT-PCR analysis of PR1 constitutive expression in overexpression transgenic line and control plants. The levels of actin were assayed as controls.
suggest that the WRKY70 ones that are positively controlled by WRKY70. These data group includes several defense-related genes distinct from the kinase WAK4, and some transcription factors. Moreover, the group includes several defense-related genes distinct from the ones that are positively controlled by WRKY70. These data suggest that the WRKY70 regulon consists of two subsets of defense-related genes, one activated by this transcription factor and the other repressed by it.

To elucidate the extent of the WRKY70 control on JA- and SA-responsive genes, we compared our data with published microarray data of Glazebrook et al. (2003). The clusters of upregulated or downregulated genes in WRKY70 overexpression line S55 in comparison to the antisense suppression line A18 were compared with data sets in which coi1, NahG, and sid2 plants were compared with their respective controls after treatment with P. syringae pv maculicola. The data demonstrate that WRKY70 clearly controls a substantial portion of SA- or JA-(COI1-dependent) responsive genes (Table 2). A substantial portion (61.4%) of the genes found in both data sets, which are upregulated in the WRKY70 overexpression plants, is also upregulated in the coi1 mutant. Accordingly, 52.4% of the WRKY70 downregulated genes are also downregulated in the coi1 plants. On the other hand, more than half of the genes downregulated in WRKY70 overexpression plants are upregulated in NahG plants, and 40.9% of the genes upregulated in WRKY70 overexpression plants are downregulated in NahG plants. Similar results can be found for sid2 mutants, demonstrating an inverse trend for plants deficient in SA signaling, and for the coi1 mutant deficient in the reception of jasmonate (Table 2).

**DISCUSSION**

Induced disease resistance in plants relies on the ability of the host to recognize the potential pathogen and trigger an appropriate response. Plants often employ distinct recognition mechanisms and signaling pathways for different pathogen elicitors. These pathways are not necessarily linear, but the signals mediated by the major endogenous signal molecules SA, JA, or JA/ET appear to form a network of synergistic and antagonistic interactions (Glazebrook, 2001; Kunkel and Brooks, 2002; Spoel et al., 2003). Some pathogens, like the Gram-negative bacterial necrotroph E. c. carotovora, appear to be capable of triggering both SA- and JA- or JA/ET-mediated defense responses (Kariola et al., 2003), and E. c. carotovora hence provides an excellent model for studies of the interaction between different signal pathways. Furthermore, induced resistance to this pathogen can be obtained either by SA-mediated or JA/ET-mediated defenses (Palva et al., 1994; Norman-Setterblad et al., 2000; Kariola et al., 2003). Here, we show that E. c. carotovora elicitors indeed cause alterations in the endogenous SA/JA balance and trigger accumulation of mRNAs for both the SAR marker PR1 and the JA-responsive gene Acor1 (Figure 1B). Previous studies indicate that the mutual antagonism between SA- and JA-dependent pathways involves a common regulatory element acting downstream of SA in the SA-mediated response (Vidal et al., 1997; Norman-Setterblad et al., 2000). To elucidate the identity of such nodes of interaction, we screened a subtractive library of A. thaliana for early E. carotovora−induced genes (Brader et al., 2001). In this study, we provide strong evidence that one of the genes identified, WRKY70, regulates defense-related gene expression via integrating SA- and JA-signaling events (Figure 6).

Our data indicate that increased levels of SA promote WRKY70 expression. Both the basal and elicitor-induced expression of WRKY70 appear to involve SA-dependent defense signaling (Figure 1). This is supported by the lack of expression in NahG transgenic plants, decreased expression in npr1 mutants, and induction of the gene by exogenous SA in the absence of any pathogen elicitor. Moreover, we could show that the mpk4 mutant with constitutively high SA levels (Petersen et al., 2000) accumulates elevated levels of WRKY70 transcripts (data not shown). Our results are consistent with those of Brodersen et al. (2002) showing that WRKY70 is clearly upregulated in the SA-dependent lesion mutant acd11 (Brodersen et al., 2002). The SA responsiveness appears common in related WRKY genes and may indicate their involvement in different steps of the SAR response in plants. For example, two WRKY70 homologs in N. tabacum are similarly induced by SA and pathogen infection (Chen and Chen, 2000). Dong et al. (2003) showed that 49 of the 72 A. thaliana WRKY genes were differentially regulated in response to exogenous SA or infection by a bacterial pathogen. Although WRKY70 expression in leaves was apparently below the detection limit in that study, a more recent analysis demonstrated the SA and pathogen inducibility of this gene (Kalde et al., 2003).

The ankyrin repeat protein NPR1 is essential for activation of PR gene expression in response to SA, and NPR1 itself is positively regulated by some SA-inducible WRKY proteins (Yu
et al., 2001). Our data show that WRKY70 is not a positive regulator of NPR1 expression and indicate that WRKY70 is not upstream of NPR1. First, npr1 mutants exhibit reduced expression of WRKY70 (Figures 1C and 1D). Second, overexpression or antisense suppression of WRKY70 does not change the constitutive expression of NPR1 (Figure 4A). Third, epistasis tests reveal that the constitutive expression of PR2 or the putative PR1 precursor is still activated when a WRKY70 transgene is crossed into the NahG or npr1-1 background (Figure 5). Together, these results strongly indicate that WRKY70 acts downstream of NPR1 in an SA-dependent signal pathway. Notably, the presence of the npr1-1 mutation does not affect the WRKY70 transgene expression level but reduces PR2 transcripts (Figure 5A). The precise mechanism remains to be determined.

### Table 1. Defense-Related Genes Differentially Expressed in WRKY70 Transgenic Plants

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<th>S/C</th>
<th>Function/Similarity</th>
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<th>W-like box (TTGACA)</th>
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<td>4</td>
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<td>20238_g_at</td>
<td>At3g13790</td>
<td>N</td>
<td>6.1</td>
<td>Glycosyl hydrolase family 32, identical to β-fructofuranosidase 1</td>
<td>3</td>
<td>4</td>
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<tr>
<td>19195_at</td>
<td>At2g44380</td>
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<td>6.5</td>
<td>Putative CHP-rich zinc finger protein</td>
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<td>13167_l_at</td>
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<td>Thioredoxin</td>
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<td>9.0</td>
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<tr>
<td>20625_at</td>
<td>At3g48080</td>
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<td>Similar to disease resistance protein EDS1</td>
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<td>At5g57260</td>
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<td>Acidic β-1,3-glucanase 2 (BGL2), PR2</td>
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<td>0</td>
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<td>13.1</td>
<td>Glycosyl hydrolase family 19, similar to type II chitinase</td>
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<td>0</td>
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<tr>
<td>17840_at</td>
<td>At2g43570</td>
<td>1.8</td>
<td>18.4</td>
<td>Glycosyl hydrolase family 19, similar to chitinase class IV</td>
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<td>1</td>
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<td>19267_s_at</td>
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<td>Similar to pectinesterase</td>
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<td>16597_s_at</td>
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<td>19025_at</td>
<td>At1g77280</td>
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<td>−2.3</td>
<td>Similar to receptor-like protein kinase</td>
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<td>16875_at</td>
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<td>Ser/Thr kinase-like protein</td>
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<td>3</td>
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<tr>
<td>16840_at</td>
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<td>N</td>
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<td>2</td>
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<td>20227_s_at</td>
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<td>Myrosinase binding protein MBP1.2</td>
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<td>3</td>
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<td>15141_s_at</td>
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<td>−1.9</td>
<td>Vegetative storage protein VSP2</td>
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<td>18339_at</td>
<td>At3g57710</td>
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<td>−2.6</td>
<td>Wall-associated kinase WAK4</td>
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<td>0</td>
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<tr>
<td>15581_s_at</td>
<td>At2g28190</td>
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<td>−2.8</td>
<td>Copper/zinc superoxide dismutase CSD2</td>
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<td>1</td>
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<tr>
<td>12923_s_at</td>
<td>At1g76680</td>
<td>−2.9</td>
<td>12-Oxophytodienoate reductase OPR1</td>
<td>2</td>
<td>0</td>
<td></td>
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<tr>
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<td>At4g21760</td>
<td>N</td>
<td>−2.9</td>
<td>Glycosyl hydrolase family 1; β-glucosidase</td>
<td>5</td>
<td>1</td>
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<tr>
<td>12251_at</td>
<td>At4g39930</td>
<td>N</td>
<td>−3.0</td>
<td>Similar to Leu-rich repeat disease resistance protein Cl-2.1</td>
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<td>0</td>
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<td>18035_at</td>
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<td>Myb family transcription factor</td>
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<td>1</td>
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<tr>
<td>18156_at</td>
<td>At4g18640</td>
<td>N</td>
<td>−3.4</td>
<td>Putative Leu-rich repeat transmembrane protein kinase</td>
<td>1</td>
<td>2</td>
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<td>14606_at</td>
<td>At2g32990</td>
<td>N</td>
<td>−3.6</td>
<td>Glycosyl hydrolase family 9, similar to endo-β-1,4-glucanase</td>
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<tr>
<td>17517_at</td>
<td>At5g36910</td>
<td>N</td>
<td>−3.7</td>
<td>Thionin THI2.2</td>
<td>2</td>
<td>1</td>
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The table indicates >2.5-fold upregulated or downregulated gene expression in antisense suppression line A18 (A/C) or in overexpression line S55 (S/C) compared to vector control pCP60. W box or W-like box distribution is within 1.5-kb promoter sequences. N, <1.5-fold difference.
The observed SA-induction pattern is independent early response and an NPR1-dependent late response (Figure 1D). The observed SA-induction pattern is consistent with that of two other response (Figure 1D). The observed SA-induction pattern is independent early response and an NPR1-dependent late response. One possible interpretation is that WRKY70 and an SA and NPR1-dependent uncharacterized factor activate synergistically expression of the downstream genes, such as PR2. This activation might act in concert to trigger, even involve, feedback regulation through WRKY70-controlled factors.

Furthermore, we show that the SA-induced expression of WRKY70 actually includes two different responses: an NPR1-regulated expression through WRKY70-controlled factors. One possible interpretation is that WRKY70 and an SA and NPR1-dependent uncharacterized factor activate synergistically expression of the downstream genes, such as PR2. This activation might act in concert to trigger, even involve, feedback regulation through WRKY70-controlled factors.

Furthermore, we show that the SA-induced expression of WRKY70 actually includes two different responses: an NPR1-dependent early response and an NPR1-dependent late response (Figure 1D). The observed SA-induction pattern is consistent with that of two other WRKY genes, WRKY18 and WRKY53 (Yu et al., 2001), and supports the idea that NPR1 is involved in an amplification loop of SA-induced expression of WRKY70. The NPR1-independent SA-induced expression of WRKY70 may coincide with the SA-mediated NPR1-independent pathway leading to PR gene expression and resistance involving additional signal such as reactive oxygen species, cell wall fragments, or nitric oxide in the cpr6 mutant (Clarke et al., 1998, 2000).

Evidence presented here shows that WRKY70 controls plant disease resistance. By overexpressing WRKY70, we were able to generate enhanced resistance to two virulent pathogens with different virulence strategy, E. c. carotovora SCC1 and P. s. tomato DC3000. By contrast, antisense suppression of WRKY70 led to enhanced susceptibility to E. c. carotovora SCC1. The spectrum of resistance established in overexpression plants can be attributed partially to constitutive activation of a subset of defense-related genes. Several of these genes have been shown to be responsive to SA and associated with SAR, including PR genes like PR2 and PR5. Thus, a set of SAR marker genes likely to be involved in pathogen defense is upregulated in the overexpression plants and may account for the enhanced resistance phenotype. Some of these genes are also upregulated in mutants with increased SA levels exhibiting constitutive SAR (Clarke et al., 1998, 2000; Petersen et al., 2000). Interestingly, overexpressing WRKY70 caused constitutive expression of PR2 and PR5 but did not lead to accumulation of PR1 mRNA (Figures 4A and 4B). Several lines of evidence have hinted to the absence of coordinate regulation between the SA genes PR1 and PR2/PR5 (Rogers and Ausubel, 1997; Nawrath and Métraux, 1999; Clarke et al., 2000). Our findings provide the direct genetic evidence for separate regulation of subsets of SA-mediated PR genes and support the notion that SA might activate several proteins that act in synergy to induce the expression of PR1.

The observed repression of WRKY70 expression by JA and enhancement of its basal expression in coi1 mutant plants indicate that the endogenous JA levels play an important role in controlling the expression of the WRKY70 gene. It is possible that the regulation is mediated by a JA-responsive factor acting as a repressor of WRKY70. The results presented here argue for a model (Figure 6) in which recognition of a pathogen or a pathogen-derived elicitor would change the endogenous levels of SA and JA and alter the balance between these two hormones. We show that an increase in SA levels activates the WRKY70 gene expression, and conversely, an increase in JA levels represses its expression. Hence, the level of WRKY70 transcripts and presumably WRKY70 protein would reflect the cellular SA/JA balance. The cellular WRKY70 levels would in turn affect the expression of downstream target genes. This effect of WRKY70 in transgenic lines is not the result of an alteration in the levels of free SA or JA but appears to be controlled more through the regulation of SA/JA balance.

Table 2. Comparison of Gene Clusters Differentially Expressed in WRKY70 Transgenic Lines, and Mutants or Transgenic Plants Impaired in Jasmonate or Salicylate Signaling

<table>
<thead>
<tr>
<th>Line</th>
<th>coi1</th>
<th>coi1</th>
<th>NahG</th>
<th>NahG</th>
<th>sid2</th>
<th>sid2</th>
</tr>
</thead>
<tbody>
<tr>
<td>S55/A18</td>
<td>61.4%</td>
<td>9.1%</td>
<td>15.9%</td>
<td>40.9%</td>
<td>4.5%</td>
<td>27.3%</td>
</tr>
<tr>
<td>S55/A18</td>
<td>14.3%</td>
<td>52.4%</td>
<td>52.4%</td>
<td>9.5%</td>
<td>28.6%</td>
<td>0</td>
</tr>
</tbody>
</table>

S55/A18 indicates the percentage of genes upregulated (1) and downregulated (−) in the WRKY70 overexpression line S55 in relation to the antisense suppression line A18, which are also upregulated or downregulated in coi1, NahG, and sid2 plants in relation to their respective controls. The source of the data sets was Glazebrook et al. (2003). The leaves of mutant or transgenic plants were compared to the wild type (Columbia 0) 30 h after treatment with 10^6 cfu P. syringae pv maculicola strain ES4326 per cm^2 leaf. | and . = 1.5-fold different from control.

Figure 6. Working Model Showing WRKY70-Mediated Cross Talk between SA- and JA-Dependent Defense Signaling.

Recognition of a particular pathogen or pathogen-derived elicitor may trigger the synthesis of SA or JA (or both) and lead to subsequent activation of the corresponding signal pathways. The balance between the two pathways determines the level of WRKY70 expression. As a consequence, WRKY70 level determines which type of response is favored. High WRKY70 levels activate expression of SAR-related genes while repressing JA-responsive gene expression. Conversely, low WRKY70 levels favor JA-responses over SAR. Thus, WRKY70 acts directly or indirectly by integrating signals from both pathways, the outcome being dependent on the initial signal strength.
directly by WRKY70. We show that high WRKY70 transcript levels promote activation of a subset of SA-responsive PR genes, whereas low levels favor expression of JA-responsive genes. In conclusion, the evidence presented here suggests that WRKY70 is a pivotal integrator of SA and JA signals in the regulation of the plant defense responses. This model would at least partly explain the mutual antagonism between SA- and JA-mediated defense signaling and identify WRKY70 as the postulated node of interaction between these pathways. This antagonistic interaction, executed by the common regulatory component WRKY70 for both pathways, possibly signifies the evolution of a mechanism designated to prioritize different resistance responses.

How does the WRKY70 transcription factor act as a positive regulator of a subset of genes, including several known SA-responsive genes (e.g., PR1)? Several lines of evidence suggest that SA-responsive WRKY proteins might activate expression of downstream genes by binding to the cognate W or W-like boxes (Maleck et al., 2000; Yu et al., 2001). The presence of W or W-like boxes in the promoters of the genes upregulated or downregulated by WRKY70 (Table 1) suggests that such genes might be under direct control of this transcription factor (Figure 6). However, we cannot exclude the possibility that WRKY70 indirectly regulates these genes through yet uncharacterized regulatory factors, which are the primary targets of WRKY70. For example, WRKY60 with several W boxes in its promoter is upregulated by overexpression of WRKY70 and might act as direct regulator for a subset of WRKY70-controlled genes.

The close inverse correlation of WRKY70 and SA-responsive gene expression and bioinformatics analysis of available data sets (Table 2) strongly suggest that WRKY70 can act as a negative regulator of a subset of JA-responsive genes. How does WRKY70 control the SA-mediated suppression of JA-responsive gene expression? One possibility is that WRKY70, as a DNA binding protein, is inactivated or removed from promoters of the JA-responsive gene upon JA induction. It has recently been proposed that COI mediates the removal of transcription factors tagged by JA-dependent phosphorylation (Turner et al., 2002). Thus, WRKY70 could be released from its target genes (e.g., AtVSP) by such a mechanism. However, not all WRKY70-repressed genes (e.g., PDF1.2) contain W or W-like boxes (Spoel et al., 2003). One possible explanation for WRKY70-mediated downregulation of such genes is that WRKY70 activates another negative regulator (Figure 6). Recently, it has been proposed that SA-mediated suppression of JA-responsive gene expression is controlled by hypothetical cytosolic NPR1 (Spoel et al., 2003). Therefore, we speculate that the fate of the WRKY70-controlled negative regulator of JA-responsive genes is likely dependent upon cytosolic NPR-mediated protein modification. In the absence of the functional NPR1 protein, the putative negative regulator in the cytosol would be nonfunctional. In support of this assumption, the epistasis test indeed shows that the WRKY70-mediated suppression of JA-induced PDF1.2 expression is released in the npr1-1 background (Figure 5B).

In conclusion, WRKY70 could form a master switch for regulatory cascades that control distinct subsets of defense-related genes, integrating signals from two defense pathways. Further studies with WRKY70 transgenic plants in combination with pathway-specific mutants will help to refine the model.

METHODS

Plant Materials and Growth Conditions

Seeds of A. thaliana wild type and mutants were kept at 4°C at least 2 d before placement in a growth environment to aid uniform germination. Seedlings were transferred to pots (7 × 7 cm) after 1-week germination on MS plates (Murashige and Skoog, 1962) or soil. Plants were grown on a 1:1 mixture of vermiculite:peat (Finnppeat B2, Kekkilä Oy, Tuusula, Finland) in a 22°C growth room under a 12 h photoperiod and a light intensity of 40 μmol·m⁻²·sec⁻¹. The A. thaliana mutants ein2-1 and npr1-1 were obtained from the Arabidopsis Biological Resource Center (accession numbers CS3071 and CS3726). The coii-1 mutant and the transgenic line expressing the NahG gene were kindly provided by J. Turner (University of East Anglia, Norwich, UK) and J. Ryals (Ciba Geigy, Research Triangle Park, NC), respectively. All plants were derived from the Columbia 0 ecotype.

Cloning of WRKY70, Vector Construction, and Plant Transformation

A 956-bp full-length fragment for WRKY70 was cloned from a cDNA library of A. thaliana plants treated with CF by PCR using the following primer pairs: 5'-TAGATACACACCTCACAAAAACTTCTCAA-3' and 5'-CTCAT3GTCCTTTATGCTTAGGTG-3'. The full-length PCR fragment was cloned into a pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced to verify the sequence. The plasmid harboring the 956-bp full-length fragment for WRKY70 was digested with SpeI and NotI and then subcloned into the corresponding sites XbaI and NotI of the binary vector pCP60, which is derived from pBIN19 containing the 35S promoter of Cauliflower mosaic virus, multiple cloning sites, and NOS, resulting in the fusion construct S-pCP60-WRKY70, with the 35S promoter directing expression in the sense orientation of the full-length WRKY70. A 332-bp cDNA clone from a substracted cDNA library (CF minus control; Brader et al., 2001) specific for WRKY70 was cloned into the Smal-EcoRI sites of pCP60, resulting in the fusion construct A-pCP60-WRKY70, with the 35S promoter directing expression in the antisense orientation. The fidelity of all constructs was confirmed by restriction and sequence analysis. A. thaliana transformation was performed as described previously (Clough and Bent, 1998). Transgenic progeny lines with single insertion loci were selected on MS plates containing kanamycin and carried to homozygosity. The empty vector pCP60 was used to generate transgenic control plants in a similar manner.

RNA Gel Blot Analysis

Isolation of total RNA, labeling of DNA probes with digoxigenin (DIG), and RNA gel blot analysis were performed as described previously (Kariola et al., 2003). Twenty micrograms of total RNA was transferred to a nylon membrane (Boehringer Mannheim, Basel, Switzerland) and hybridized with DIG-labeled DNA or RNA probes, as indicated. DIG RNA labeling, hybridization, and detection were performed according to manufacturer’s instructions (Roche, Basel, Switzerland). A 332-bp cDNA fragment cloned to pBluescript II SK+ (Stratagene, La Jolla, CA) was used as a template for WRKY70-specific DNA and RNA probes. The other DNA probe templates were amplified by PCR from PR1 (gene identifier [GI]: 3810599), PR2 (GI: 166636), PR5 (GI: 2435405), NPR1 (GI: 1773294), AICOR1 (GI: 2460202), and AtVSP (GI: 14994298). Equal loading was confirmed by hybridization of the filters with an rDNA probe or running agarose-formaldehyde gels with ethidium bromide.
RT-PCR Analysis

RT-PCR analysis was performed with 1 μg of total RNA treated with DNaseI (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. With SuperScript II RNaseH- and Oligo(dT)16 RT (Invitrogen), the reverse transcription was performed according to the manufacturer’s instructions. Subsequent PCR was performed for 30 cycles under the following conditions: 94°C for 45 sec, 58°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Samples were visualized on 1.2% agarose gels. To confirm the RT-PCR specificity for the PR1 gene, the generated RT-PCR product was inserted into a PCR 2.1 vector (Invitrogen), and positive clones were subsequently sequenced. The following primer pairs were used: 5′-CTCTTGTAGGTGCTTGTTCTCC-3′ and 5′-CTTCATTAGTATGGCTTCTGTC-3′ for PR1, and 5′-GACATGGAAGGATAGTGCCATC-3′ and 5′-AGATCGTCTCAGATCAGACATGAC-3′ for actin.

Preparation of E. carotovora Elicitors and Application of Chemicals

For E. carotovora elicitor (CF) preparation, a bacterial culture filtrate was prepared as described previously (Vidal et al., 1997). CF, SA (5 mM in water), and MeJA (100 μM in 0.1% [v/v] ethanol) were applied at the indicated concentrations as 5 μL droplets on four fully expanded leaves for each soil plant (five drops per leaf). MeJA-treated plants were immediately placed to a tray with a transparent lid. Control plants were treated identically with water containing 0.1% (v/v) ethanol. Leaves were collected at time points indicated.

Pathogen Infections

E. c. carotovora SCC1 inoculation was done by applying 10 μL drops of suspension (10^6 cells/mL) in 0.9% NaCl on local leaves (one leaf per plant). After inoculation, plants were incubated for the indicated period of time under high humidity conditions. Leaf maceration and plant survival ratio were examined. Inoculation with P. s. tomato DC3000 was performed by infiltration of mature leaves (one leaf per plant; ~5 μL suspension of 10^6 cells/mL in 10 mM MgCl₂). Inoculated leaves were harvested 24 or 72 h after infiltration and homogenized in 10 mM MgCl₂. Diluted leaf extracts were used to titer the bacteria. Colony-forming units (cfu) of 6–8 individual plants were determined from each time point. At least two independent lines for each construct were tested. All experiments were repeated three times.

Quantification of JA and SA

JA and SA were extracted and quantified with (±)-9,10-Dihydro-jasmominic acid and 14C-SA as internal standards using the protocol of Baldwin et al. (1997). Conjugated SA was determined after hydrolyzing with hydrochloric acid (final concentration 2 M) at 80°C for 1 h and proceeding after that as with free SA and JA.

Expression Profiling

Leaves from a pool of 12 different 4-week old plants were harvested. Fifteen micrograms of total RNA from pCP60 vector control, antisense suppression line A18, and overexpression line S55 were submitted to the Finnish DNA Microarray Center, where synthesis of biotin-labeled cRNA, microarray hybridization to AtGenome1 GeneChips (Affymetrix, Santa Clara, CA), and scanning procedures were performed (http://www.bbk.utu.fi/Genomics). Experiments and hybridizations were performed twice and averaged, and Gene Spring software (Silicon Genetics, Redwood City, CA) was used for normalization and further analyses. Expression data were normalized globally by taking the 50th percentile of all measurements as a positive control for each sample, with the bottom 10th percentile as test for correct background subtraction. The measurement for each probe set in each sample was divided by the corresponding averaged values of pCP60 (set to 1) when this expression value was >0.5. Normalized averaged expression values <0.05 were adjusted to 0.05. Probe sets with a detection P value <0.06 in at least two independent samples were defined as accurately detectable (57% of all probe sets) and corresponded to expressed genes. Genes with a more than twofold change in expression level in any transgenic plant compared with the two others (7% of the expressed genes; see supplemental data online) were clustered by K-means analysis.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AL163972, AF421157, CS3071, and CS3726.

ACKNOWLEDGMENTS

We thank the following for materials: J. Turner (coi1-1), J. Ryals (NahG), A. Hegedus (pCP60 plasmid), M. Romantschuk (virulent Pst DC3000), and the Arabidopsis Biological Resource Center (npr1-1 and ein2-1). We thank Turku Center for Biotechnology for Affymetrix service. This work was supported by the Academy of Finland (Grants 38033, 42180, 49905, 44252, and 44883; Finnish Center of Excellence Programme 2000–2005), Biocentrum Helsinki, and Helsinki Graduate School in Biotechnology and Molecular Biology.

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The WRKY70 Transcription Factor: A Node of Convergence for Jasmonate-Mediated and Salicylate-Mediated Signals in Plant Defense
Jing Li, Günter Brader and E. Tapio Palva
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