Targets of AtWRKY6 regulation during plant senescence and pathogen defense

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In Arabidopsis, WRKY factors comprise a large gene family of plant-specific transcriptional regulators controlling several types of plant stress responses. To understand the regulatory role of WRKY proteins during such processes, we identified targets of the senescence- and defense-associated WRKY6 factor. WRKY6 was found to suppress its own promoter activity as well as that of a closely related WRKY family member, indicating negative autoregulation. On the other hand, WRKY6 positively influenced the senescence- and pathogen defense-associated PR1 promoter activity, most likely involving NPR1 function. One novel identified target gene, SIRK, encodes a receptor-like protein kinase, whose developmental expression is strongly induced specifically during leaf senescence. The transcriptional activation of SIRK is dependent on WRKY6 function. Senescing leaves of wrky6 knockout mutants showed a drastic reduction, and green leaves of WRKY6 overexpression lines showed clearly elevated SIRK transcript levels. Furthermore, the SIRK gene promoter was specifically activated by WRKY6 in vivo, functioning very likely through direct W-box interactions.

[Key Words: cDNA-AFLP; WRKY transcription factor; receptor kinase; SIRK; autoregulation; PR1]

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In plants, as in other organisms, many developmental processes and responses to different stress stimuli underlay complex regulatory mechanisms operating at the level of gene expression (Lemon and Tjian 2000). Consistent with this regulatory complexity, nearly 6% of the total genes within the Arabidopsis genome code for transcription factors (Riechmann et al. 2000). One major family of plant-specific transcriptional regulators in Arabidopsis is represented by the WRKY gene family, comprising 74 members. WRKY factors belong to the zinc-finger-type class of proteins [Eulgem et al. 2000]. Although still poorly studied, WRKY factors have been implicated in the regulation of certain plant processes, such as pathogen defense, wound response, and senescence [Eulgem et al. 2000]. To understand the biological significance of WRKY6 factors during such processes, their in vivo target genes must be identified. Potential WRKY target genes have been suggested based on the general binding activity of WRKY factors to their recognized cis-element, TGACC/T, or W box [Eulgem et al. 2000; Yu et al. 2001]. Almost nothing is known concerning trans-regulating activities of defined WRKY proteins on different target gene promoters, although transactivating capabilities of WRKY factors have been shown [de Pater et al. 1996; Eulgem et al. 1999; Hara et al. 2000].

Recently, we characterized one member of the Arabidopsis WRKY family, designated WRKY6, in more detail [Robatzek and Somssich 2001]. The strongest WRKY6 expression was observed during leaf senescence but was also found in certain other tissues including floral organ abscission zones. In addition, expression of WRKY6 was influenced by several external and internal stimuli often associated with senescence and plant defense. Based on inhibitor studies, WRKY6 could be classified as an immediate-early-type gene not requiring de novo protein synthesis for its activation [F. Turck and I.E. Somssich, pers. comm.). Therefore, WRKY6 function is most likely involved in regulating certain early steps of these processes. Consistent with its function as a transcriptional regulator, the WRKY6 protein was found to be exclusively localized to the plant cell nucleus.

Here, we report the use of Arabidopsis wrky6 knockout mutants and a WRKY6 overexpression line to monitor WRKY6 trans-regulation activity on individual gene promoters and to screen for target genes. Several putative targets were identified. Our studies reveal that WRKY6 can function both as a positive and negative regulator of transcription, and in particular we identified one potential direct target gene very likely encoding an important signaling component of leaf senescence and defense response.

Results

To study WRKY6 function and to isolate candidate target genes, we took advantage of a stable wrky6 knockout
mutant, wrky6-2, which is derived from the En-1 insertion line wrky6-1 [Fig. 1A]. The wrky6-1 line still carries an En-1 transposon inserted in the fourth exon of the WRKY6 gene, resulting in a total loss of WRKY6 transcript accumulation [Fig. 1B]. In contrast, the wrky6-2 line carries a frame-shift mutation leading to a stop codon, owing to incorrect excision of the En-1 transposon, resulting in a deletion of 56 bp within the WRKY6 ORF. Although WRKY6 transcript was detectable in the wrky6-2 line, the translation product lacks 290 amino acids of the protein including its DNA-binding domain [data not shown].

In addition, we used previously generated transgenic lines ectopically overexpressing WRKY6. Three lines, CaMV 35S::WRKY6-3, CaMV 35S::WRKY6-5, and CaMV 35S::WRKY6-9, showed clearly elevated levels of WRKY6 transcript in mature leaves, whereas no WRKY6 expression was observed in wild-type plants [Fig. 2A]. The severity of the mutant phenotypes of the lines CaMV 35S::WRKY6-3, CaMV 35S::WRKY6-5, and CaMV 35S::WRKY6-9 strongly correlated with increasing expression levels of WRKY6 [Fig. 2B]. The highest expressing line, CaMV 35S::WRKY6-9, was most strongly affected, showing a complex stress-related mutant phenotype [Fig. 2C]. The plants were dwarfed with partly necrotic leaves, early flowering, and a reduction in their apical dominance.

Figure 2. WRKY6 overexpression lines. [A] Expression of WRKY6 in nine independent T2 transgenic plants carrying a CaMV 35S::WRKY6 construct compared with wild type (WT). The ethidium bromide-stained 28S rRNA band is shown for loading control. [B] Dosage-dependence of the mutant phenotypes. Plants of the overexpressor lines CaMV 35S::WRKY6-3,-5, and -9 showing increasing levels of WRKY6 transcript are compared with wild type. [C] Comparison of the strongest overexpressor line CaMV 35S::WRKY6-9 with wild type. Plants were grown either under short-day (SD) or long-day (LD) conditions.

WRKY6 negatively influences its own promoter function

To follow the effects of WRKY6 on its own promoter activity, transgenic lines carrying a WRKY6 promoter-reporter fusion were crossed with wrky6-1 and wrky6-2 knockout mutants as well as with the CaMV 35S::WRKY6-9 line. The WRKY6 promoter function, monitored by GUS activity, was analyzed with respect to tissue-specific and pathogen-triggered expression. Whereas wild-type plants showed strong GUS activity in roots and senescing leaves, this effect was even more pronounced in the wrky6 knockout mutants [Fig. 3A]. The opposite effect was observed in the WRKY6 overexpressor. Only very faint GUS signals could be detected in roots, and no signals were present in senescing leaves. This indicates that WRKY6 is negatively regulating its own promoter-mediated expression, which occurs in a broad spectrum of cell types.

The repression effect was also seen under inducing conditions, namely, upon inoculation with the avirulent bacterial strain Pseudomonas syringae pv. tomato DC3000 [Ps avrRPM1]. In contrast to wild-type plants, infected leaves of WRKY6 overexpression lines showed no inducible WRKY6 promoter-dependent GUS activity [Fig. 3A]. On the other hand, loss of function of WRKY6 caused a clear enhancement of the WRKY6 promoter-mediated reporter gene activity. In these mutants, bacterial challenge as well as control treatments with MgCl2 resulted in increased GUS signal. In addition, the observed local restriction of GUS activity to infection sites in wild-type plants was clearly relaxed. The spread of GUS activity into noninoculated leaf areas of the wrky6 knockout mutants suggests that WRKY6 may be required for down-regulating its own expression once a certain threshold level has been achieved. Thus, WRKY6 may directly or indirectly function in limiting certain plant responses to a specific cell layer surrounding the site of pathogen ingress.

The enhancement of WRKY6 promoter-mediated GUS activity in the wrky6 knockout mutants points in the direction of WRKY6 showing repressor activity. To fur-
The WRKY6 repression activity may be due to competition with other transcriptional activators or interference with coactivators. To clarify this point, we modulated the activity of WRKY6 by fusion to the strong activation domain of VP16, and observed diminishment of the repressor activity of WRKY6. The WRKY6–VP16 protein negated the negative effect on WRKY6 promoter activity, showing, instead, a slight induction above background values [Fig. 3B]. The VP16 fusion to WRKY6 was not affecting nuclear targeting nor specific promoter-binding capability, because strong activation is observed for WRKY6–VP16 when a tetramerized W2-box element was used to drive expression of the reporter gene, resulting in a 31-fold induction of GUS activity. No such increase was seen when a block mutation was introduced into the W-box motif. This strongly suggests that WRKY6 binds to W-box elements, which is in perfect agreement with all previous reports about cognate binding sites of WRKY factors [Eulgem et al. 2000]. Whether W boxes are the only recognized cis-acting element of WRKY6 needs further elucidation.

Figure 3. Repressor activity of WRKY6. (A) WRKY6 promoter-driven GUS reporter gene activity (6p) was monitored in wild-type plants, in the knockout mutants wrky6-1 and wrky6-2, as well as in the overexpressor line CaMV 35S:WRKY6-9 [-9]. Shown are GUS-stained roots (R), senescing leaves (SL), and mature leaves 5 h postinoculation with 10^8 CFU bacterial solution (+Ps avrRPMI) or with 10 mM MgCl₂ (control). (B) Transient cotransfection assays with different target gene promoters and WRKY6. Presented are relative activities of WRKY6 promoter (6p), WRKY42 promoter (42p), tetramerized W2-box (4xW2), and mutated tetramerized W2-box (4xmW2) driven GUS reporter gene constructs after transfection of cell culture-derived Arabidopsis protoplasts. Transient transfections were done either with reporter constructs alone or combined with an effecter construct containing a CaMV 35S-driven WRKY6 cDNA [WRKY6] or a CaMV 35S-driven fusion of the WRKY6 cDNA to the VP16 activation domain [WRKY6–VP16]. Each bar represents the median of four independent transfections. Normalized GUS values were obtained using a control luciferase plasmid for standardization. Relative fold induction or repression values >twofold are depicted.

Positive WRKY6 activity on PR1 promoter function

PR-type genes were previously described to be potential WRKY target genes [Eulgem et al. 2000]. Given that PR1 contains several W boxes within its promoter [Maleck et al. 2000], including one involved in negatively regulating expression during systemic acquired resistance [SAR; Lebel et al. 1998], we tested whether PR1 gene expression is influenced by WRKY6. For this, we crossed PR1 promoter–reporter transgenic lines with the wrky6-1 knockout mutant and the CaMV 35S:WRKY6-9 line [Fig. 4A]. Upon local infiltration of mature leaves with avirulent bacteria, the PR1-promoter-mediated level of GUS activity was strikingly high in the WRKY6 overexpression line, whereas in the wrky6 knockout mutant background, GUS activities similar to wild type were found. This pathogen-inducibility was detectable at much earlier time points [3–5 h] than in wild-type plants [24–48 h, Fig. 4A; data not shown]. Clear GUS activities were also present in control inoculations with MgCl₂, whereas only low GUS activities were observed in completely untreated control leaves. Leaf senescence slightly induces PR1 gene expression [Robatzek and Somssich 2001], which is drastically increased in the WRKY6 overexpression line [Fig. 4A; SL]. In contrast, no other plant tissue showed such an up-regulation of the PR1 promoter activity [data not shown]. Increased basal PR1 gene expression was confirmed by RNA blot analysis [Fig. 4B]. Together these data indicate that WRKY6 overexpression causes a general up-regulation of PR1, but more importantly, mediates a stronger and faster response under stress inducing conditions [Fig. 4A]. Recently, Yu et al. [2001] showed that WRKY factors can activate NPR1 via W boxes present within its promoter. NPR1 is a key regulator of the SAR-dependent signal pathway leading to PR1 expression [Cao et al. 1997]. As shown in Figure 4C, overexpression of WRKY6 also results in elevated NPR1 transcript levels. This would sug-
Potential genes regulated by WRKY6

To isolate additional candidate target genes, we applied a cDNA-AFLP-based differential display approach (Durarrant et al. 2000). We compared transcript populations either derived from roots of wild-type plants and wrky6-2 mutants, because roots are tissues of high WRKY6 expression (Robatzek and Somssich 2001), or derived from all aerial parts of wild-type and CaMV 35S::WRKY6-9 plants. Screening of ≥12,000 different cDNA fragments resulted in the identification of 154 differentially expressed clones from root transcripts, designated R1–R154, and 63 clones from aerial part transcripts, designated P1–P63. The expression of ~44% of the R-clones and ~59% of the P-clones was up-regulated in the wrky6-2 and in the CaMV 35S::WRKY6-9 mutants, respectively.

Sequence analysis of the cDNA-AFLP fragments revealed, in 33% of the cases, homologies to only hypothetical ORFs. A number of candidates showed strong similarities to Ca2+--, defense-, and senescence-related genes, as well as different types of kinases, including receptor-like protein kinases (Table 1). To confirm the cDNA-AFLP results, we selected clones based on their sequence homologies and differential expression pattern.

RT–PCR studies using independent RNA preparations verified 70% of the tested R-clones and 50% of the P-clones (data not shown).

Because W boxes, TGACC/T, are the cognate binding sites of WRKY factors (Eulgem et al. 2000), we searched 1-kb putative promoter sequences of these candidate target genes for their presence. In addition, we checked for ap2-like elements (Rushton and Somssich 1998), which also contain the highly conserved TGAC core motif. Although a single W box within a promoter is sometimes sufficient to mediate WRKY-dependent gene expression, a clustering of W boxes is often observed (Eulgem et al. 1999, Maleck et al. 2000). Indeed, some of the isolated potential WRKY6 target genes contained numerous W boxes within their promoters (Table 1). Based on these data, the most promising WRKY6 target gene was chosen for further investigations.

The receptor-like protein kinase SIRK is a WRKY6 target

The gene (GenBank accession no. T00540) corresponding to the cDNA-AFLP fragment P24, showing induced expression in CaMV 35S::WRKY6-9 plants, encodes a typical leucine-rich repeat receptor-like protein kinase (Shiu and Bleecker 2001). Expression profiling using different plant tissues revealed a strong association of P24 with the process of senescence, being highly induced in senescent leaves but not detectable in any of the other tested organs (Fig. 5A). Based on its expression pattern, we renamed P24 to AtSIRK for Arabidopsis thaliana senescence-induced receptor-like kinase. In contrast to the wild-type situation, the level of SIRK transcript detected in senescent leaves of the wrky6-2 knockout mutant was drastically reduced. Furthermore, elevated SIRK expression was also detected in mature leaves, stems, and flowers of WRKY6 overexpression lines. Taken together, these results strongly imply that high SIRK expression is dependent on WRKY6. Because the developmental expression patterns of WRKY6 and SIRK are only partly overlapping, transcriptional activation of SIRK by WRKY6 seems to be leaf senescence-specific.

In addition, WRKY6 expression is induced by bacterial pathogen infection (Fig. 3A). We therefore analyzed the responsiveness of the SIRK promoter to the bacterial elicitor flagellin (Felix et al. 1999). Transient transfection assays in protoplasts revealed an 18-fold increase of GUS reporter activity using the active versus the inactive elicitor (Fig. 5B). WRKY6 may therefore also play a role in this response.
on its own showed only faint background activities (Fig. 6A). Furthermore, bombardments of the promoter–reporter construct alone in WRKY6 overexpression lines showed strong GUS activities. Therefore, WRKY6 is able to transactivate SIRK gene expression in vivo.

Because the SIRK promoter contains nine W boxes, and WRKY6 could function through one or several of these, we analyzed a SIRK promoter–reporter deletion series (Fig. 6B). WRKY6 was still capable of activating the shortest deletion construct (3m3) containing only two of the nine W boxes and one TGACA motif. Mutations within these three elements (3m1, 2, 3) completely abolished its ability to activate the reporter gene. Interestingly, a single block mutation within the second W box between positions −43 and −49 bp (3m2) equally led to total loss of function. This shows that, indeed, at least one W box is important for WRKY6 recognition. Furthermore, cobombardment with an SIRK promoter-derived construct spanning the four W boxes within region −581 to −736 bp did not lead to a significant increase of GUS activities above background values (data not shown).

Therefore, WRKY6–SIRK-promoter interactions rely on more than just the presence of W-box motifs.

To show specificity on the protein side, we investigated cobombardments with two defense-associated WRKY factors, namely, WRKY52 and PcWRKY1 (Fig. 6B). In both cases, no obvious GUS activities were detected, indicating a specific requirement for WRKY6. However, WRKY42, the closest WRKY6 family homolog, was capable of activating the SIRK promoter (Fig. 6B).

| Clone<sup>a</sup> | Expression<sup>b</sup> | Accession no.<sup>c</sup> | Similarity | W | as1<sup>d</sup> |
|-------------------|-----------------------|-------------------------|------------|---|---------------|
| R11<sup>e</sup> | ++                    | AC024081                | JA-regulatory protein NAC2 | 2 | 0             |
| R16               | ++                    | AC011698                | NAM-like protein | 2 | 0             |
| R18               | --                    | T06055                  | kinesin domain containing protein | 4 | 2             |
| R40               | ++                    | AB023034                | xylosidase | 2 | 1             |
| R41<sup>e</sup> | --                    | AC010718                | putative calmodulin | 5 | 1             |
| R43               | --                    | AB003590                | sulfate transporter | 2 | 1             |
| R48               | ++                    | AJ270302                | putative β-galactosidase | 3 | 0             |
| R50               | --                    | AC010926                | putative casein kinase | 3 | 1             |
| R52/53            | --                    | AL163818                | Ca<sup>2+</sup>-transporting ATPase-like protein | 4 | 2             |
| R62<sup>e</sup> | ++                    | S66346                  | SEN1 | 5 | 1             |
| R64               | ++                    | X89866                  | glutathione peroxidase | 0 | 0             |
| R67<sup>e</sup> | ++                    | AC006551                | alcohol dehydrogenase-like | 2 | 0             |
| R68               | --                    | T02156                  | glucosidase homolog | 4 | 1             |
| R72               | ++                    | AC007651                | putative glutathione transferase | 3 | 1             |
| R74<sup>e</sup> | --                    | T05493                  | thauatin-like PR protein | 3 | 0             |
| R81               | --                    | AF058919                | putative calmodulin-binding heat shock protein | 3 | 1             |
| R92               | --                    | AC000132                | receptor-like protein kinase | 2 | 2             |
| R96               | --                    | AC011708                | putative pectin esterase | 4 | 1             |
| R102<sup>e</sup> | ++                    | AC006931                | putative lipase | 0 | 1             |
| R105              | --                    | AF217546                | calmodulin-binding protein | 1 | 0             |
| R129              | ++                    | AC009519                | MAP kinase-like protein | 1 | 2             |
| R140<sup>e</sup> | ++                    | 2924653                 | heat shock protein HSP91-2 | 4 | 0             |
| R143              | +                     | AC020579                | putative disease resistance protein | 5 | 3             |
| R144<sup>e</sup> | ++                    | Y14590                  | class IV chitinase | 6 | 1             |
| P2                | ++                    | AC002572                | putative xyloglucan endotransglycosylase | 1 | 2             |
| P4                | ++                    | AL162506                | fructose-biphosphatase aldolase-like protein | 5 | 1             |
| P7<sup>e</sup>  | --                    | AC002339                | zinc protease-like protein | 2 | 2             |
| P24<sup>e</sup>  | ++                    | T00540                  | receptor-like protein kinase | 9 | 0             |
| P27<sup>e</sup>  | ++                    | AC002336                | putative expansin | 3 | 0             |
| P29               | --                    | SS1478                  | drought-induced protein Di19 | 1 | 0             |
| P34<sup>e</sup>  | ++                    | AC005724                | putative calmodulin-binding protein | 2 | 2             |
| P35               | ++                    | AL163812                | fructosidase-like protein | 2 | 0             |
| P38               | --                    | T09930                  | thioredoxin homolog | 0 | 0             |
| P40               | --                    | T04549                  | AP2 domain protein homolog | 2 | 0             |
| P55<sup>e</sup>  | --                    | AC010676                | putative porin | 2 | 0             |
| P57               | --                    | AC006592                | homeobox factor HB6 | 2 | 0             |
| P58               | --                    | T02644                  | ABC-type transport protein homolog | 2 | 1             |

<sup>a</sup>Designated cDNA-AFLP fragments.

<sup>b</sup>Differential expression of the cDNA-AFLP fragments detected in the null-mutant or the overexpression line compared to wild type.

<sup>c</sup>GenBank accession numbers of identified genes corresponding to the cDNA-AFLP fragments.

<sup>d</sup>Number of W boxes and as1-like elements present within 1 kb putative promoter sequence of corresponding genes.

<sup>e</sup>Confirmed by RNA blot or RT-PCR analysis (others not tested).
Regulation of SIRK promoter activity seems to involve functionally redundant members of the WRKY family.

Discussion

Functional redundancy within multigene families often complicates genetic attempts to define the role of individual members (Bouche and Bouchez 2001). This also appears to be the case for the wrky6 knockout mutation, which resulted in no obvious mutant phenotype. In certain cases, overexpression of the respective gene can give clues to its biological function. However, particularly with transcription factors like WRKY6, ectopic expression leading to nonphysiological concentrations of the protein can affect a plethora of regulatory networks and yield multiple mutant phenotypes, thereby negating conclusions derived from inference. Despite such problems, our results using cDNA-AFLP differential display indicate that the single wrky6 knockout does result in altered gene expression profiles. This indicates that functional redundancy is not complete. Furthermore, several putative target genes identified in these comparative analyses (Table 1) corroborate our previous findings that WRKY6 is involved in controlling processes related to senescence and pathogen defense (Robatzek and Somssich 2001). These include genes encoding the senescence-associated protein 1 (SEN1), a protease, the jasmonic acid regulatory protein NAC2, a glutathione transferase (Nam 1997; Dong 1998), as well as several genes encoding defense-related proteins (R22, R74, R143, R144). The SEN1 gene promoter contains five W boxes within the first 1 kb of sequence. Its expression was strongly up-regulated in the wrky6 knockout mutant, indicating that WRKY6 may act as a negative regulator on this promoter. Additional genes identified in our study represent signaling components of calcium and kinase cascades, which also function during senescence and pathogen defense. Interestingly, similar sets of potential WRKY-regulated genes were identified in expression profiling experiments addressing SAR (Maleck et al. 2000; Petersen et al. 2000). Furthermore, chitinases and...
also receptor-like protein kinases have been proposed to be possible WRKY targets [Yang et al. 1999; Du and Chen 2000; Ohtake et al. 2000].

Both senescence and hypersensitive response, a successful defense strategy against numerous pathogens, are forms of programmed cell death (PCD). Because several defense-associated genes are expressed during leaf senescence, and defense-related mutants show alterations in senescence-associated gene expression, cross-talk between distinct PCD pathways do exist [Quirino et al. 1999; Morris et al. 2000].

### WRKY6 activator and repressor function

All studied WRKY proteins have been shown to act as positive transcriptional regulators [de Pater et al. 1996; Eulgem et al. 1999; Har a et al. 2000]. A negative function for WRKY factors was merely derived from inference [Eulgem et al. 1999; Hara et al. 2000]. A negative function between distinct PCD pathways do exist [Quirino et al. 1999; Morris et al. 2000].

Although we cannot exclude the possibility that the negative autoregulation of WRKY6 is mediated via W boxes, transcriptional repression of WRKY42 by WRKY6 points to another mechanism. The putative WRKY42 promoter sequence contains no W-box consensus motifs, indicating either an indirect WRKY6 effect or the involvement of other cis-acting elements. Such elements may be modifications of the W-box consensus, because several TGAC-core motifs of the TGACG [Rushton and Hansen 1996] or of the TGACA type [Desveaux et al. 2000] are present within the WRKY42 promoter.

WRKY6 acts as a positive regulator on *PR1* expression. Most likely, this is because of an activation rather than a competition mechanism caused by ectopic WRKY6 expression, given that, apart from leaves, no such effect was observed in other tissues. Direct involvement of WRKY6 in *PR1* transcription is supported by the presence of several W boxes within the *PR1* promoter, and by the fact that elevated NPR1 levels alone are insufficient to induce *PR1* [Cao et al. 1998]. On the other hand, the further substantial increase of *PR1* expression in the overexpression line under stress conditions favors a more indirect role of WRKY6. The *PR1* upstream regulator *NPR1* has been shown to be a WRKY target gene [Yu et al. 2001], and WRKY6 may be one of its activators or alternatively impinge on the function of a specific WRKY factor. Despite these elevated levels for both *NPR1* and *PR1* in leaves of the WRKY6 overexpression lines, we could not detect a significant enhancement of resistance or increased cell death toward compatible and incompatible strains of *Pseudomonas syringae pv toma*to DC3000 [lacking or carrying *avrRPM1*; data not shown]. It should be noted that elevated levels of endogenous NPR1 and PR1 need not necessarily lead to resistance [Greenberg et al. 2000]. One likely explanation is that the observed levels are insufficient, because it has been shown that NPR1 confers pathogen resistance in a dosage-dependent fashion [Cao et al. 1998].

Dual activities of transcription factors can be dependent on the cell environment and the type or level of signal input [Hoecker et al. 1995]. Concentration-dependence is one mechanism of dual functionality by which transcription factors can act as activators or repressors [Ogbourne and Antalis 1998; Rushlow et al. 2001]. Differing expression levels of WRKY6 may therefore determine whether target gene transcription is stimulated or repressed. Protein interactions and the abundance of interacting partners within different cell types or upon stress conditions contribute as well to the mechanism of dual functionality [Motohashi et al. 2000]. This may also be valid for WRKY6, because it contains a leucine zipper capable of mediating dimerizations [S. Robatzek and I.E. Somssich, unpubl.].

The senescence-induced receptor kinase *SIRK*

Our data strongly imply that WRKY6 acts upstream of *SIRK* in the process of leaf senescence. This interaction appears to be direct, acting through at least one W box present within the *SIRK* promoter, and involving a specific requirement for WRKY6 function. We cannot, however, completely exclude alternative possibilities, for example, that WRKY6 induces other WRKY genes whose products interact with the W-box element. To date, *SIRK* is the only identified plant receptor kinase developmentally expressed solely during leaf senescence. One other receptor kinase, *PvSARK* from bean, has been associated with senescence, but is also detected in roots [Hajouj et al. 2000]. The senescence-signaling pathway is often linked to pathogen defense [Quirino et al. 1999], and *SIRK* and WRKY6 are targets of both programs. Interestingly, the 1-kb *SIRK* promoter is capable of perceiving signals from these two cascades. Consistent with *SIRK* being a WRKY6 target gene, the temporal accumulation of *SIRK* mRNA upon Flg22 stimulation followed the rapid and transient increase of WRKY6 transcript in a slightly delayed manner [C.B. Zipfel and S. Robatzek, pers. comm.]. Furthermore, preliminary results show that W-box elements are also required for flagellin responsiveness of this promoter (O. Nouhibou, P. Rushton, and I.E. Somssich, pers. comm.). Whether common or distinct W boxes and WRKY factors mediate the signals from both pathways remains to be determined.

A connection between WRKY proteins and other receptor-like kinases as potential targets has been suggested based on the clustering of W boxes within their promoter regions and the ability of WRKY factors to bind to such elements in vitro [Du and Chen 2000; Ohtake et al. 2000]. The expression of these receptor-like kinases was shown to be inducible upon treatment with salicylic acid [Ohtake et al. 2000], and the expression of one gene,
RLK3, was also induced by pathogen attack (Czernic et al. 1999). Whether W boxes mediate these responses was not shown. Nevertheless, because WRKY6 expression is also up-regulated by salicylic acid and by bacterial infection (Fig. 3; Robatzek and Somssich 2001), WRKY6 may participate in their transcriptional control as well. Interestingly, the expression pattern of HAESA, another kinase gene with W-box clusters in its promoter (Ohtake et al. 2000), shows a strong overlap with that of WRKY6. Expression of both genes is highly activated in floral organ abscission zones (Jinn et al. 2000). Robatzek and Somssich (2001), suggesting another possible link between WRKY6 and a potential target gene in such cells. All of these receptor-like kinases show only ~30% amino acid identity to SIRK; therefore, they most likely act in different signal perception/transduction pathways. WRKY6 could be a transcriptional regulator of distinct receptor kinase genes functioning in specific cells and during certain developmental stages in response to different external and internal signaling cues.

Database searches identified two additional receptor-like kinases with high homologies to SIRK. The proteins encoded by the genes P27F23.1 and P27F23.3 show 60.6% and 59.9% identity, respectively. The P27F23.3 gene contains six W boxes within its first 1 kb of promoter sequence, indicating that at least one other SIRK-related receptor kinase could be under the control of WRKY factors.

Receptor-like kinases serve as receivers and transducers of external and internal stimuli. Various input signals are transmitted through phosphorylation/dephosphorylation cascades, which lead to changes in gene expression patterns. To date, only a few receptor-like kinases have been linked to certain plant processes. These include CLV1 in meristem organization, ERECTA in organ shape, BR1 in brassinolide signaling, FLS2 in flagellin signaling, HAESA in floral organ abscission, and BrSRK1 in self-incompatibility (Shiu and Bleecker 2001). WRKY proteins are expected to be substrates of kinases and/or phosphatases (Eulgem et al. 2000). This is consistent with recent identification of a set of specific potential WRKY effector genes being constitutively expressed in a MAP kinase mutant, mpk4, which negatively regulates SAR (Petersen et al. 2000). A hypothetical model derived from our results would suggest a dual function for WRKY6 during some stage of leaf senescence, which is influenced by WRKY6 function.

Materials and methods

Plant growth and treatments

Plant growth conditions for obtaining plant material, bacterial growth and infections, and histochemical GUS staining were performed as described by Robatzek and Somssich (2001).

Knockout mutants

A knockout mutation of the WRKY6 gene (GenBank accession no. AF331712) was identified by a PCR-based screen of an En-1 insertion population as described previously (Baumann et al. 1998). The combination of the WRKY6-specific primer 5'-ATC CCG TCG TGA CTA GAC ATT GAC-3' and the En-1-specific primer 5'-GAG CGT CGG TCC CCA CAC TTC TAT AC-3' led to the isolation of the line 6AAK-1, as a wrky6 mutant. The En-1 insertion in the mutant [wrky6-1] was confirmed by Southern analysis, and its exact position following codon 263 determined by sequencing. The footprint within the wrky6-2 mutant was detected using WRKY6-specific primers flanking the original En-1 insertion site. Both mutants contain three additional En-1 insertions after twice back-crossing to wild-type plants. Homozygous plants for the wrky6 mutation were used for expression analysis.

Transgenic plants

WRKY6 cDNA was amplified by RT–PCR and introduced behind the CaMV 35S promoter into the Xhol and SacI sites in the pBT8 construct, a derivative of pBT2 (Weisshaar et al. 1991). Following digestion with ClaI and SacI, the CaMV 35S::WRKY6 fragment was introduced into the binary vector pGPTV (Koncz and Schell 1986). In addition to the WRKY6 coding region, the construct carries 37 bp of the 5' untranslated region (UTR) and 64 bp of the 3' UTR. The correctness of the constructs was verified by sequencing. Stable A. thaliana Col-0 transgenic lines were generated using the Agrobacterium tumefaciens-mediated gene-transfer procedure involving infiltration of inflorescences (Clough and Bent 1998). Independent transgenic lines were selected for kanamycin resistance and confirmed by Southern analysis. Plants of the T2 generation were used in detailed molecular and phenotypic studies.

Promoter reporter lines

6p::GUS (Robatzek and Somssich 2001) and PR1p::GUS (Lebel et al. 1998) were crossed into wrky6-1, wrky6-2 knockout mutants and the CaMV 35S::WRKY6-9 overexpressing line. Transgenic plants were selected for kanamycin resistance, and by Southern and PCR analysis. Expression studies were done using homozygous wrky6 mutants, and heterozygous CaMV 35S::WRKY6-9 lines.

Northern/RT-PCR analysis

Different tissues of A. thaliana plants ecotype Col-0 were used for total RNA extraction with the RNA/DNA-maxi kit (QIA-
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GENEs. In all cases, 10 µg of total RNA was loaded per lane, and the gels were blotted using standard molecular procedures (Sambrook et al. 1989). DNA probes were radioactively labeled by random priming using [α-32P]dCTP (Amersham) and the Ready-To-Go kit (Pharmacia).

RT-PCR was performed with 50 ng of total RNA, the NPr1-specific primers 5′-CTG TTT AGT GAC ACC ACC ATT GAT GG-3′ and 5′-GTC TGC GCA TTA AGA AAC TCC TTC TTT AGG C-3′, or the RPL4-specific primers 5′-GTC ATA GGT CAG TGC AGG GAA CAA C-3′ and 5′-CCA CCA CCA CGA ACT TCA CCG CGG GTC-3′, using the Ready-To-Go RT–PCR beads (Amersham) according to the manufacturer’s instructions.

cdNA-AFLP differential display

The method of cdNA-AFLP differential display was done as previously described (Durrant et al. 2000). For template construction, 1 µg of double-purified poly(A)+ mRNA derived from sterile-grown root tissue (wrky6-2 knockout mutants and wild type) and all aerial plant tissue (CaMV 35S::WRKY6-9 and NPTII gene) transgenic wild type germinated under selective conditions) was used. Selective amplifications were done in primer combinations of Apo-WD10, 11, 12, 22, 58, and 63 with Msc-WD31 to WD46 (Durrant et al. 2000). Identified differential signals were re-amplified, cloned into the TOPO vector (Stratagene), and sequenced.

Transient transfections

For each reporter construct, the relevant promoter containing 5′-ATG upstream regions, 1315 bp [6p], 1132 bp [42p], and 928 bp [SIRKp] were amplified by PCR and introduced into the HindIII and BamHI sites of the pUC9-GUS reporter construct (van de Locht et al. 1990). The promoter regions were fused translationally to the Escherichia coli uidA gene (Jefferson et al. 1987). These constructs therefore also contained 21 bp [6p], 21 bp [42p], and 9 bp [SIRKp] of the respective ORFs. The core TGAC motifs of the DNA elements were changed to ATTG within the SIRK-promoter deletion constructs (Δ3m2 and Δ3m1/2/3), as indicated in Figure 6B, using the megaprimmer method (Landt et al. 1990). The pBT8 construct containing the CaMV 35S-driven WRKY6 cDNA was used as the WRKY6 effecto. WRKY6 fusion to the transactivation domain of VP16 (derived from Herpes simplex virus protein 16) was achieved by cloning a PCR-amplified WRKY6 cDNA into the Xhol and PvuAI sites, replacing the bZIP sequence of a pBT8 derivative (Feldbrugge et al. 1994). The 4xWRKY6 GUS reporter contained a tetramer of the hexameric TGTGAC W-box motif (Eulgem et al. 1999), and the 4xmWRKY6 GUS construct contained the tetramer of CATTGT (Rushion et al. 2002).

Particle bombardments were done as previously described (Shirasu et al. 1999). For each combination 15–20 mature leaves were transected 4 h after detachment with 3 µg of SIRKp::GUS reporter variants together with 3 µg of empty vector or effector plasmids WRKY6, WRKY42, WRKY52, [Deslandes et al. 2002], and the parsley P::WRKY1 [Eulgem et al. 1999]. Bombardments were done at 900 psi with a 7× diffuser in a vacuum chamber (Bio-Rad). GUS staining was performed 16 h after incubation under long-day conditions. Efficiency of the bombardments was monitored using a strong constitutive 35S::GUS construct.

Protoplast isolation derived from cultured Arabidopsis cells, and transient cotransfection experiments were performed as previously described (van de Locht et al. 1990; Hartmann et al. 1998; Jin et al. 2000). For each assay, 2 × 10⁶ protoplasts were transfected with 10 µg of promoter–GUS reporter together with 5 µg of empty vector, WRKY6, or WRKY6-VP16 effectors along with 5 µg of 35S::LUC reference plasmids. Protein, LUC, and GUS activity measurements were carried out 20 h after incubation in the dark. LUC expression was used to normalize for specific GUS activities. For assays using the active and inactive forms of the bacterial elicitor flagellin (Felix et al. 1999), transfected protoplasts were incubated in the presence of 1 nM elicitor.

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References

Baumann, E., Lewald, J., Saudler, H., Schulz, B., and Wisman, E. 1998. Successful PCR based reverse genetic screens using an En-1 mutagenised Arabidopsis thaliana population generated via single-seed descent. Theor. Appl. Genet. 97: 729–734.

Bouche, N. and Bouchez, D. 2001. Arabidopsis gene knockout: Phenotypes wanted. Curr. Opin. Plant Biol. 4: 111–117.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. 1997. The Arabidopsis NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57–63.

Cao, H., Li, X., and Dong, X. 1998. Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. 95: 6531–6536.

Clough, S.J. and Bent, A.F. 1998. Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735–743.

Czernic, P., Visser, B., Sun, W., Savoure, A., Deslandes, L., Marco, Y., Van Montagu, M., and Verbruggen, N. 1999. Characterization of an Arabidopsis thaliana receptor-like protein kinase gene activated by oxidative stress and pathogen attack. Plant J. 18: 321–327.

de Pater, S., Greco, V., Pham, K., Memelink, J., and Kijne, J. 1996. Characterization of a zinc-dependent transcriptional activator from Arabidopsis. Nucleic Acids Res. 24: 4624–4631.

Deslandes, L., Olivier, J., Theulière, T., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J., and Marco, Y. 2002. Resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes. Proc. Natl. Acad. Sci. 99: 2404–2409.

Desveaux, D., Despres, C., Joyeux, A., Subramaniam, R., and Brisson, N. 2000. PBF-2 is a novel single-stranded DNA bind-
ing factor implicated in PR-10a gene activation in potato. *Plant Cell* **12**:1477–1489.

Dong, X. 1998. SA, JA, ethylene, and disease resistance in plants. *Curr. Opin. Plant Biol.* 1:316–323.

Du, L. and Chen, Z. 2000. Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid-induced WRKY DNA-binding proteins in *Arabidopsis*. *Plant J.* **24**:837–847.

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., and Jones, J.D.G. 2000. cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. *Plant Cell* **12**:963–977.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K., and Somssich, I.E. 1999. Early nuclear events in plant defence signalling: Rapid gene activation by WRKY transcription factors. *EMBO J.* **18**:4689–4699.

Eulgem, T., Rushton, P.J., Robatzek, S., and Somssich, I.E. 2000. The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* **5**:199–206.

Feldbrügge, M., Sprenger, M., Dinkelbach, M., Yazaki, K., Harter, K., and Weisshaar, B. 1994. Functional analysis of a light-responsive plant bZIP transcriptional regulator. *Plant Cell* **6**:1607–1621.

Felix, G., Duran, J.D., Volko, S., and Boller, T. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J.* **18**:265–276.

Greenberg, J.T., Silverman, P.F., and Liang, H. 2000. Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the *Arabidopsis* mutant *acd5*. *Genetics* **156**:341–350.

Hajjou, T., Michielis, R., and Gepstein, S. 2000. Cloning and characterization of a receptor-like protein kinase gene associated with senescence. *Plant Physiol.* **124**:1305–1314.

Hanna-Rose, W. and Hansen, U. 1996. Active repression mechanisms of eukaryotic transcription repressors. *Trends Genet.* **6**:229–234.

Hara, K., Yagi, M., Kusano, T., and Sano, H. 2000. Rapid systemic accumulation of transcripts encoding a tobacco WRKY transcription factor on wounding. *Mol. Gen. Genet.* **263**:30–37.

Hartmann, U., Valentine, W.J., Hays, J., Jenkins, G.I., and Weisshaar, B. 1998. Identification of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Mol. Biol.* **36**:741–754.

Hoecker, U., Vasil, I.K., and McCarty, D.R. 1995. Integrated control of seed maturation and germination programs by activator and repressor functions of Viviparous-1 of maize. *Genes & Dev.* **9**:2459–2469.

Jefferson, R.A., Kavanagh, T.A., and Bevan, M.W. 1987. GUS fusions: β-Glucuronidase a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6:3901–3907.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., and Martin, C. 2000. Transcriptional repression by AtMYB4 controls production of UV/blue light-response elements in the *Arabidopsis thaliana* chalcone synthase promoter using a homologous protoplast transient expression system. *Plant Mol. Biol.* **36**:741–754.

Jin, H., Cominelli, E., Bailey, P., Parr, A., Mehrtens, F., Jones, J., Tonelli, C., Weisshaar, B., and Martin, C. 2000. Transcriptional repression by AtMYB4 controls production of UV/protection screens in *Arabidopsis*. *EMBO J.* **19**:6150–6161.

Jinn, T.-L., Stone, J.M., and Walker, J.C. 2000. *HAESA*, an *Arabidopsis* leucine-rich repeat kinase, controls floral organ abscission. *Genes & Dev.* **14**:108–117.

Koncz, C. and Schell, J. 1986. The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**:383–396.

Landt, O., Grunert, H.-P., and Hahn, U. 1990. A general method for rapid site-directed mutagenesis using the polymerase chain reaction. *Gene* **96**:125–128.

Lebl, E., Heftetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. 1998. Functional analysis of regulatory sequences controlling PR-1 gene expression in *Arabidopsis*. *Plant J.* **16**:223–233.

Lemon, B. and Tijan, R. 2000. Orchestrated response: A symphony of transcription factors for gene control. *Genes & Dev.* **14**:2551–2569.

Li, X., Zhang, Y., Clarke, J.D., Li, Y., and Dong, X.I. 1999. Identification and cloning of a negative regulator of systemic acquired resistance, SN1H, through a screen for suppressors of *appr1-1*. *Cell* **98**:329–339.

Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L., and Dietrich, R.A. 2000. The transcriptional program of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* **26**:403–410.

Morris, K., MacKerness, S., Page, T., John, C.F., Murphy, A.M., Carr, J.P., and Buchanan-Wollaston, V. 2000. Salicylic acid has a role in regulating gene expression during leaf senescence. *Plant J.* **23**:677–685.

Motohashi, H., Katsuoka, F., Shavit, J.A., Engel, J.D., and Yamamoto, M. 2000. Positive or negative MARE-dependent transcriptional regulation is determined by the abundance of small Maf proteins. *Cell* **103**:865–875.

Nam, H.G. 1997. The molecular genetic analysis of leaf senescence. *Curr. Opin. Biotech.* **8**:200–207.

Nakamura, S. and Antalis, T.M. 1998. Transcriptional control and the role of silencers in transcriptional regulation in eukaryotes. *Biochem. J.* **331**:1–14.

Ohtake, Y., Takahashi, T., and Komeda, Y. 2000. Salicylic acid induces the expression of a number of receptor-like kinase genes in *Arabidopsis thaliana*. *Plant Cell Physiol.* **41**:1038–1044.

Petersen, M., Broderson, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., et al. 2000. *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. *Cell* **103**:1111–1120.

Quirino, B.F., Normany, J., and Amasino, R.A. 1999. Diverse range of gene activity during *Arabidopsis thaliana* leaf senescence includes pathogen-independent induction of defense-related genes. *Plant Mol. Biol.* **40**:267–278.

Riechmann, J.L., Heard, J., Martin, G., Reuber, L., Jiang, C.-Z., Keddie, J., Piccida, O., Ratcliffe, O.J., Samaha, R.R., Creelman, R., et al. 2000. *Arabidopsis* transcription factors: Genome-wide comparative analysis among eukaryotes. *Science* **290**:2105–2110.

Robatzek, S. and Somssich, I.E. 2001. A new member of the *Arabidopsis* WRKY transcription factor family, AtWRKY6, is associated with both senescence- and defense-related processes. *Plant J.* **28**:123–133.

Rushlow, C., Colosimo, P.F., Lin, M.-C., Xu, M., and Kirov, N. 2001. Transcriptional regulation of the *Drosophila* gene zen by competing Smad and Brinker inputs. *Genes & Dev.* **15**:340–351.

Rushton, P.J. and Somssich, I.E. 1998. Transcriptional control of plant genes responsive to pathogens. *Curr. Opin. Plant Biol.* **1**:311–315.

Rushton, P.J., Reinstädler, A., Lipka, V., Lippok, B., and Somssich, I.E. 2002. Pathogen-inducible synthetic plant promoters. *Plant Cell* [in press].

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Shirasu, K., Nielsen, K., Pifanelli, P., Oliver, R., and Schulze-Lefert, P. 1999. Cell-autonomous complementation of mlo resistance using a biolistic transient expression system.
Shiu, S.-H. and Bleecker, A.B. 2001. Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc. Natl. Acad. Sci. 98: 10763–10768.

van de Locht, U., Meier, I., Hahlbrock, K., and Somssich, I.E. 1990. A 125 bp promoter fragment is sufficient for strong elicitor-mediated gene activation in parsley. EMBO J. 9: 2945–2950.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. 1991. Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J. 10: 1777–1786.

Woo, H.R., Chung, K.M., Park, J.-H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K., and Nam, H.-G. 2001. ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. Plant Cell 13: 1779–1790

Yang, P., Wang, Z., Fan, B., Chen, C., and Chen, Z. 1999. A pathogen- and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of tobacco class I chitinase gene promoter. Plant J. 18: 141–149.

Yu, D., Chen, C., and Chen, Z. 2001. Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. Plant Cell 13: 1527–1539.