

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 WRKY 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

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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.

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 pro-

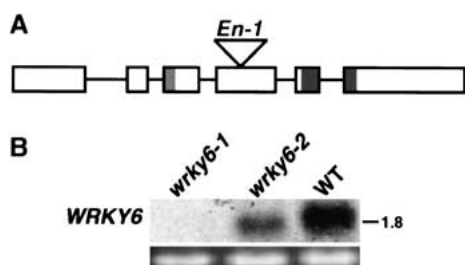


Figure 1. Identification of *wrky6* knockout mutants. (A) Schematic representation of the *Arabidopsis WRKY6* gene. Exons (boxes) and introns (lines) are indicated. The regions of the leucine zipper (light gray) and the WRKY domain (dark gray) as well as the position of the *En-1* insertion (triangle) are shown. (B) Expression analysis of *WRKY6* in senescent leaves of two knockout mutants, *wrky6-1* and *wrky6-2*, compared with wild type (WT). The 28S rRNA band of the ethidium bromide-stained gel is shown for loading control.

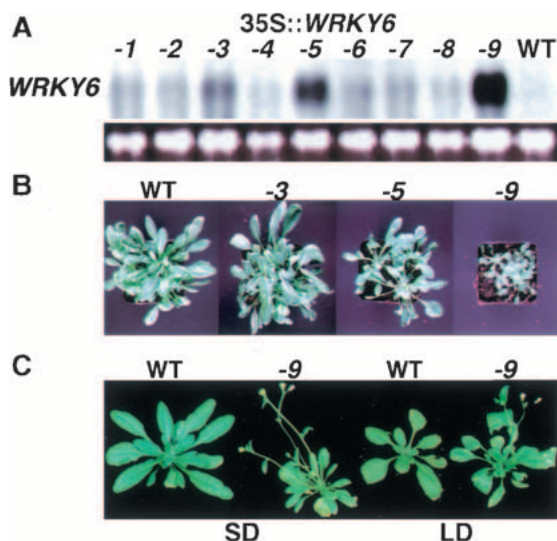


Figure 2. *WRKY6* overexpression lines. (A) Expression of *WRKY6* in nine independent T_2 transgenic plants carrying a *CaMV 35S::WRKY6* construct compared with wild type (WT). The ethidium bromide-stained 28S rRNA band is shown for loading control. (B) Dosage-dependence of the mutant phenotypes. Plants 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.

nounced 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 $MgCl_2$ 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-

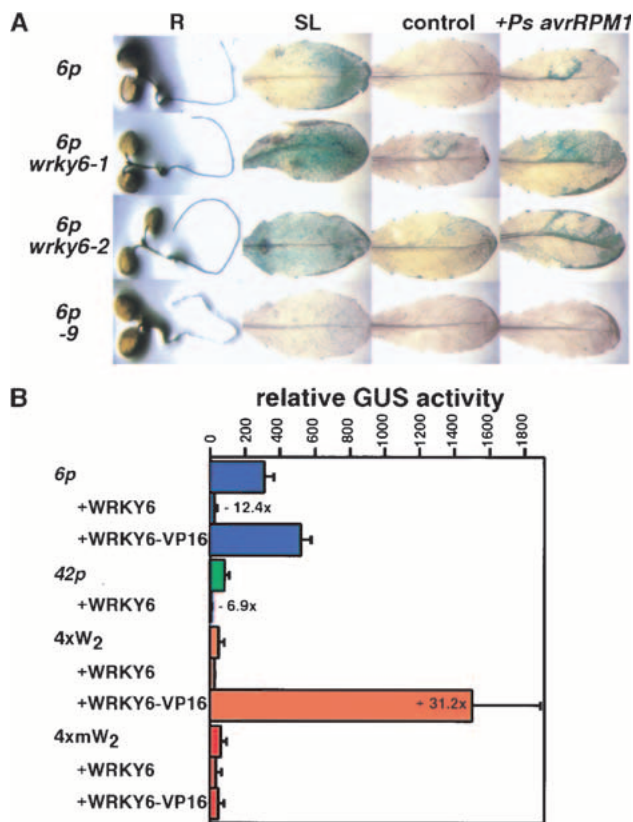


Figure 3. Repressor activity of WRKY6. (A) *WRKY6* promoter-driven GUS reporter gene activity (*6p*) was monitored in wild-type plants, in the knockout mutants *wrky6-1* and *wrky6-2*, as well as in the overexpressor line *CaMV 35S::WRKY6-9* (-9). Shown are GUS-stained roots (R), senescing leaves (SL), and mature leaves 5 h postinoculation with 10^8 -CFU bacterial solution (+*Ps avrRPM1*) or with 10 mM $MgCl_2$ (control). (B) Transient cotransfection assays with different target gene promoters and WRKY6. Presented are relative activities of *WRKY6* promoter (*6p*), *WRKY42* promoter (*42p*), tetramerized W_2 -box ($4xW_2$), and mutated tetramerized W_2 -box ($4xmW_2$) 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 effector 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 \geq twofold are depicted.

ther address this question, we performed transient transfections in *Arabidopsis* protoplasts. Cotransfections of the *WRKY6* promoter-reporter fusion with *CaMV 35S::WRKY6* resulted in a drastic 12-fold repression activity of WRKY6 on its own transcription (Fig. 3B). Similarly, a sevenfold repression activity was seen using a *WRKY42* promoter-reporter fusion. *WRKY42*, the *WRKY6* homolog (Eulgem et al. 2000), also shares a similar promoter architecture of certain regulatory modules with *WRKY6* (Robatzek and Somssich 2001).

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 W_2 -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.

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 $MgCl_2$, 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-

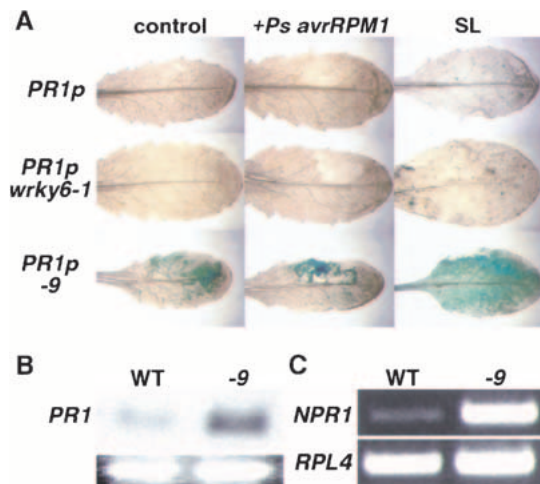


Figure 4. Effect of *WRKY6* overexpression on *PR1* and *NPR1*. (A) Activation of *PR1*-promoter-driven reporter gene activity by *WRKY6*. GUS activity observed in representative leaves of transgenic plants harboring the *PR1* promoter-driven reporter gene in wild type (*PR1p*), *wrky6-1* mutant, and *WRKY6-9* (-9) overexpressor. GUS-stained mature leaves are shown either 5 h postinoculation with 10^8 -CFU bacterial solution (+*Ps avrRPM1*) or with 10 mM $MgCl_2$ (control), as well as senescing leaves (SL). (B) RNA blot analysis of *PR1* in mature leaves of wild-type (WT) and *WRKY6* overexpression lines (-9). The 28S rRNA ethidium bromide-stained band is shown as loading control. (C) RT-PCR analysis of *NPR1* in mature leaves of wild-type (WT) and *WRKY6* overexpression lines (-9). For control *RPL4* (ribosomal protein L4) transcript was amplified.

gest that *WRKY6* action on *PR1* seems to be indirect and likely involves *NPR1* function.

Potential genes regulated by *WRKY6*

To isolate additional candidate target genes, we applied a cDNA-AFLP-based differential display approach (Durrant 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 $\geq 12,000$ 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 $\sim 44\%$ of the R-clones and $\sim 59\%$ 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 Ca^{2+} -, 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 *as1*-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, it 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.

Transient transfections of green leaves were used to monitor *WRKY6*-dependent activation of the W-box-rich *SIRK* gene promoter, which in stable transgenic *Arabidopsis SIRKp*::GUS lines was shown to mediate leaf senescence-inducible expression (data not shown). Cobombardments of an *SIRK* promoter fusion to the GUS reporter gene with *CaMV* 35S::*WRKY6* in either *wrky6-2* knockout mutants or wild-type plants resulted in strong GUS activities, whereas the promoter-reporter construct

Table 1. cDNA-AFLP fragments and homologies to sequences in the Arabidopsis database

Clone ^a	Expression ^b	Accession no. ^c	Similarity	W	as1 ^d
R11 ^e	+++	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 ^e	--	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 ²⁺ -transporting ATPase-like protein	4	2
R62 ^e	+++	S66346	SEN1	5	1
R64	+++	X89866	glutathione peroxidase	0	0
R67 ^e	---	AC006551	alcohol dehydrogenase-like	2	0
R68	---	T02156	glucosidase homolog	4	1
R72	---	AC007651	putative glutathione transferase	3	1
R74 ^e	--	T05493	thaumatin-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 ^e	+++	AC006931	putative lipase	0	1
R105	-	AF217546	calmodulin-binding protein	1	0
R129	++	AC009519	MAP kinase-like protein	1	2
R140 ^e	+++	2924653	heat shock protein HSP81-2	4	0
R143	+	AC020579	putative disease resistance protein	5	3
R144 ^e	++	Y14590	class IV chitinase	6	1
P2	+++	AC005275	putative xyloglucan endotransglycosylase	1	2
P4	+++	AL162506	fructose-bisphosphate aldolase-like protein	5	1
P7 ^e	--	AC002339	zinc protease-like protein	2	2
P24 ^e	+++	T00540	receptor-like protein kinase	9	0
P27 ^e	+++	AC002336	putative expansin	3	0
P29	-	S51478	drought-induced protein Di19	1	0
P34 ^e	++	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 ^e	---	AC010676	putative porin	2	0
P57	-	AC006592	homeobox factor HB6	2	0
P58	-	T02644	ABC-type transport protein homolog	2	1

^aDesignated cDNA-AFLP fragments.

^bDifferential expression of the cDNA-AFLP fragments detected in the null-mutant or the overexpression line compared to wild type.

^cGenBank accession numbers of identified genes corresponding to the cDNA-AFLP fragments.

^dNumber of W boxes and *as1*-like elements present within 1 kb putative promoter sequence of corresponding genes.

^eConfirmed by RNA blot or RT-PCR analysis (others not tested).

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 ($\Delta 3$) containing only two of the nine W boxes and one TGACA motif. Mutations within these three elements ($\Delta 3m1/2/3$) 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 ($\Delta 3m2$) 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).

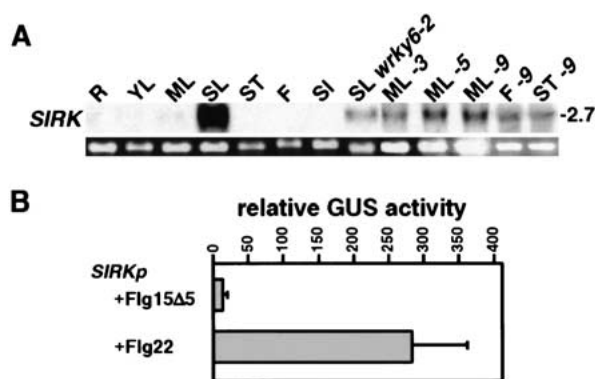


Figure 5. Dependence of *SIRK* gene expression on WRKY6. (A) Expression analysis of *SIRK* transcripts in different tissues, including roots (R), young leaves (YL), mature leaves (ML), senescing leaves (SL), stems (ST), flowers (F), or siliques (SI), derived from wild-type plants (WT), the *wrky6-2* knockout mutant, or from the overexpressor lines -3, -5, and -9. The 28S rRNA band of the ethidium bromide-stained gel is shown as loading control. (B) Effect of the bacterial-derived elicitor flagellin 22 (Flg22) on *SIRK* promoter activity in transient transfection assays. Presented are relative activities of the *SIRK* promoter (*SIRKp*) driven GUS reporter gene after addition of the active (Flg22) and inactive (Flg15Δ5) forms of the elicitor. Each bar represents the median of four independent transfections. Normalized GUS values were obtained using a control luciferase plasmid.

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

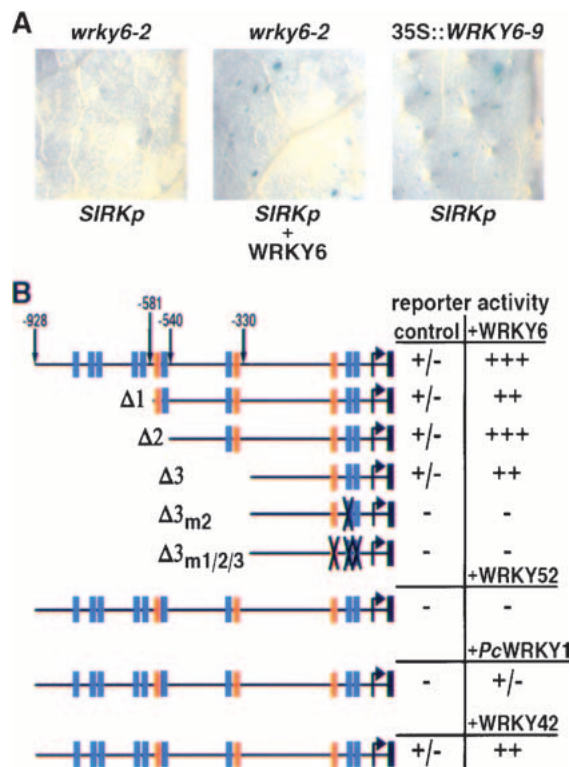


Figure 6. Specificity of the WRKY6-*SIRK* promoter interaction. (A) Representative leaves of four independent biolistic-mediated transient transfection assays using a 0.9-kb *SIRK* promoter-driven GUS reporter gene (*SIRKp*). Bombardments of detached mature leaves of *wrky6-2* knockout mutants and the 35S::*WRKY6-9* overexpressor line were done either with *SIRKp* alone or in combination with a *CaMV* 35S-driven *WRKY6* cDNA construct (*WRKY6*). (B) Results of bombardment assays using various *SIRK*-promoter deletion/mutation GUS constructs as depicted schematically on the left. The respective sizes relative to the transcription start site (bent arrow) as determined by 5'RACE (O. Noubibou, pers. comm.; GenBank accession no. AF486619) are indicated by arrows. W boxes (TGACC/T) are marked by blue, TGACA motifs by orange rectangles, and mutated elements by crosses. *SIRK* promoter deletion constructs, $\Delta 1$, $\Delta 2$, $\Delta 3$, and mutation constructs, $\Delta 3m2$ and $\Delta 3m1/2/3$, were bombarded in detached mature wild-type leaves either alone (control) or in combinations with the effectors (+*WRKY6*, +*WRKY52*, +*PcWRKY1*, +*WRKY42*). Activation of the *SIRK* promoter constructs is indicated by relative values ranging from - (no GUS staining), +/- (1-5 GUS-positive cells in total), ++ (>20 GUS-positive cells per leaf), to +++ (>50 GUS-positive cells per leaf). Three independent experiments were performed.

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; Hara et al. 2000). A negative function for WRKY factors was merely derived from inference (Lebel et al. 1998; Li et al. 1999). In this report, we showed that WRKY6 clearly acts as a negative regulator on its own and on *WRKY42* expression, but the mechanism remains unknown. The WRKY6 protein does contain regions homologous to known trans-activation domains (Robatzek and Somssich 2001), but lacks obvious similarities to trans-repression domains (Hanna-Rose and Hansen 1996). Therefore, WRKY6 repressor activity may be direct, functioning via a novel type of repressor domain, or its action could be indirect through interaction/interference with other proteins.

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 Somssich 1998) 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 overexpressor 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* overexpressor lines, we could not detect a significant enhancement of resistance or increased cell death toward compatible and incompatible strains of *Pseudomonas syringae* pv *to-*

mato 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. Noubibou, 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 F27F23.1 and F27F23.3 show 60.6% and 59.9% identity, respectively. The F27F23.3 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, *BRI1* in brassinolide signaling, *FLS2* in flagellin signaling, *HAESA* in floral organ abscission, and *BrSRK1* in self-incompatibility (Shiu and Bleeker 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 initiated by binding of a senescence-triggered ligand to *SIRK*. Concomitant to this, expression of *WRKY6* is induced. *SIRK* function activates a downstream kinase cascade resulting in modification of *WRKY6* protein, thereby enabling it to activate the transcription of several genes including *SIRK* and to down-regulate *WRKY6* expression.

As for other multigene families, unraveling the biological role of individual *WRKY* transcription factors and how they contribute to the establishment of the complex plant regulatory network remains a challenging endeavor. The identification of *WRKY6* target genes, especially ones involved in the process of leaf senescence, is of particular importance given that, to date, nearly no

regulatory components of leaf senescence are known (Nam 1997; Woo et al. 2001). Isolation of *SIRK* knockout mutants via reverse genetics or dsRNAi and their combination with other mutants affecting senescence and/or defense response will surely facilitate the molecular dissection of this important process as will the identification of additional components influencing or being 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 6AAK₆₇ 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 *XhoI* 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-

GEN). In all cases, 10 μ g 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 - 32 P]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 TTG ATG GAC ACC ACC ATT GAT GG-3' and 5'-GTC TGC GCA TTC AGA AAC TCC TTT AGG C-', or the *RPL4*-specific primers 5'-GTG ATA GGT CAG GTC AGG GAA CAA C3-' and 5'-CCA CCA CCA CGA ACT TCA CCG CGA GTC-', 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 μ g 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 Mse-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 *Hind*III and *Bam*HI sites of the pUC9-GUS reporter construct (van de Löcht 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 (Δ 3m2 and Δ 3m1/2/3), as indicated in Figure 6B, using the megaprimer method (Landt et al. 1990). The pBT8 construct containing the *CaMV* 35S-driven *WRKY6* cDNA was used as the *WRKY6* effector. *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 *Xho*I and *Pin*AI sites, replacing the *bZIP* sequence of a pBT8 derivative (Feldbrügge et al. 1994). The 4xW₂ GUS reporter contained a tetramer of the hexameric TTGACC W-box motif (Eulgem et al. 1999), and the 4xmW₂ GUS construct contained the tetramer of CATTGT (Rushton et al. 2002).

Particle bombardments were done as previously described (Shirasu et al. 1999). For each combination 15–20 mature leaves were transfected 4 h after detachment with 3 μ g of *SIRKp*::GUS reporter variants together with 3 μ g of empty vector or effector plasmids *WRKY6*, *WRKY42*, *WRKY52*, (Deslandes et al. 2002), and the parsley *PcWRKY1* (Eulgem et al. 1999). Bombardments were done at 900 psi with a 7 \times 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 Löcht et al. 1990; Hartmann et al. 1998; Jin et al. 2000). For each assay, 2 \times 10⁶ protoplasts were transfected with 10 μ g of promoter-GUS reporter together with 5 μ g of empty vector, *WRKY6*, or *WRKY6-VP16* effectors along

with 5 μ g 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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Targets of *At*WRKY6 regulation during plant senescence and pathogen defense

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