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The Antagonist Function of *Arabidopsis* WRKY53 and ESR/ESP in Leaf Senescence Is Modulated by the Jasmonic and Salicylic Acid Equilibrium

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Crosstalk between salicylic acid (SA) and jasmonic acid (JA) signaling is well-studied but not during leaf senescence. We found that the senescence-specific WRKY53 transcription factor interacts with the JA-inducible protein EPITHIOSPECIFYING SENESCENCE REGULATOR (ESR/ESP). The expression of these genes is antagonistically regulated in response to JA and SA, respectively, and each negatively influences the other. Leaf senescence is accelerated in *ESR* knockout plants (ESR-KO) but retarded in *ESR* overexpressors (ESR-OE), with the reverse true for *WRKY53*. ESR-OE showed higher resistance than ESR-KO to bacterial and fungal pathogens. However, pathogen resistance was not altered in WRKY53 overexpressors or knockouts (W53-KO), suggesting that ESR has a greater impact on WRKY53 function in senescence than WRKY53 on ESR function in pathogen resistance. ESR inhibits WRKY53 DNA binding in vitro, and their interaction is localized to the nucleus in vivo; however, ESR is exclusively in the cytoplasm in W53-KO cells, indicating that ESR is brought to the nucleus by the interaction. Therefore, ESR has dual functions: as cytoplasmic epithiospecifier and as negative regulator of WRKY53 in the nucleus. These results suggest that WRKY53 and ESR mediate negative crosstalk between pathogen resistance and senescence, which is most likely governed by the JA and SA equilibrium.

INTRODUCTION

Leaf senescence is a complex process that is accompanied by a massive change in the transcriptome. An estimated number of 12 to 16% of the *Arabidopsis thaliana* genes are up- or down-regulated during leaf senescence, clearly indicating a high activity of transcription factors during this process (Buchanan-Wollaston et al., 2003; Guo et al., 2004; Zentgraf et al., 2004). Many transcription factors are themselves induced on the transcriptional level during leaf senescence (Chen et al., 2002; Buchanan-Wollaston et al., 2003, 2005; Guo et al., 2004). Expression profiling revealed that many WRKY factors are strongly upregulated and that WRKY factors constitute the second largest group of transcription factors of the senescence transcriptome (Guo et al., 2004). During dark-induced senescence, 21 out of 59 WRKY factors are induced (Lin and Wu, 2004). However, the biological function of individual WRKY factors expressed during leaf senescence is still unclear. Target gene analyses of WRKY53 revealed that WRKY53 acts upstream of many other WRKY factors, but WRKY factors rather act in a regulatory network influencing transcription of each other than in a linear signal transduction pathway (Robatzek and Somssich, 2002; Dong et al., 2003; Li et al., 2004; Miao et al., 2004).

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The signaling molecules salicylic acid (SA), jasmonic acid (JA), and ethylene have been implicated in complex interconnecting pathways that control gene expression in plant pathogen responses as well as in stress response (reviewed in Turner et al., 2002; Wang et al., 2002). Since there is a considerable overlap between pathogen-related and senescence-related genes (Weaver et al., 1998; Quirino et al., 1999), these pathways may also be involved in regulating gene expression during senescence. Morris et al. (2000) showed that the expression of certain genes during leaf senescence is dependent on the presence of an active SA pathway. On the other hand, senescence appears to occur normally in SA-deficient and ethylene-deficient plants, indicating that SA and ethylene are not essential for senescence but that senescence-related factors are required (Grbic and Bleecker, 1995; Morris et al., 2000).

JA and related compounds also play an important role in regulating a number of plant responses, such as response to wounding, pathogen infection, or senescence (reviewed in Turner et al., 2002). It was shown that exogenous treatment of barley (*Hordeum vulgare*) leaves with JA or methyl jasmonate (MeJA) led to a loss of chlorophyll and reduced levels of ribulose-1, 5-bisphosphate carboxylase/oxygenase, indicating that senescence was induced (Parthier, 1990). He et al. (2002) demonstrated that treatment of wild-type *Arabidopsis* with JA resulted in typical premature senescence symptoms that did not occur on the JA-insensitive mutant *coi1*. In addition, JA levels were shown to increase during senescence, and several enzymes involved in JA biosynthesis showed senescence-enhanced expression (Buchanan-Wollaston et al., 2003). Expression of *SAG12* and *SEN4* (Nam, 1997) was increased by JA treatment in wild-type plants, severely reduced in the *coi1* mutant, and restored in the

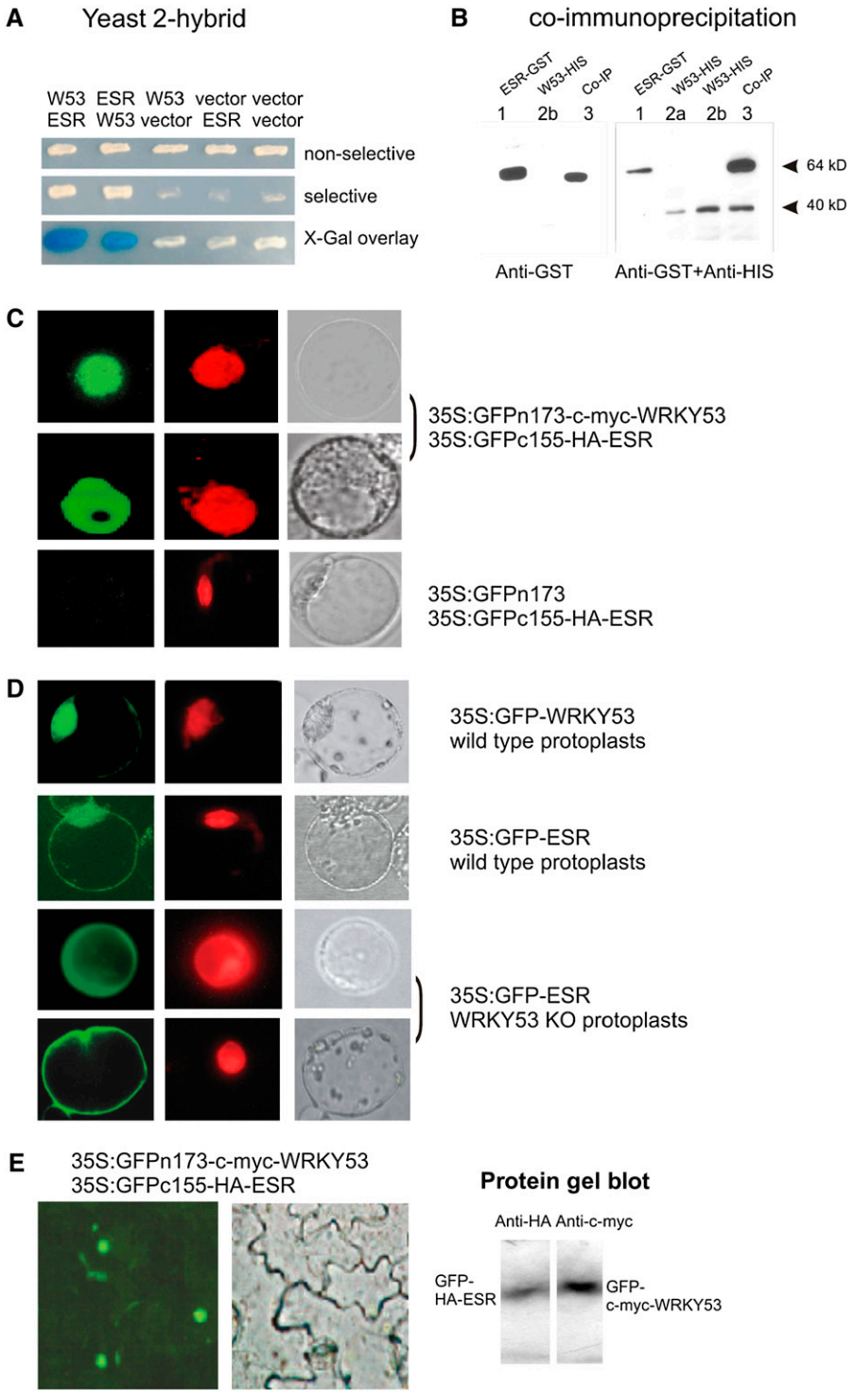

Figure 1. Interaction of WRKY53 and ESR in Vivo and in Vitro.

(A) Yeast-two hybrid system. Growth selection and X-Gal agarose overlay assay to visualize β-galactosidase activity of *LacZ* reporter gene of yeast strains that were cotransformed with full-length cDNA sequences encoding *ESR* and *WRKY53* (W53) cloned into prey and bait vectors, respectively. If *WRKY53* was inserted in the bait construct, the truncated version of *WRKY53* lacking the activated domain was used.

(B) Coimmunoprecipitation. Protein extracts of *Escherichia coli* strains expressing recombinant GST-tagged ESR (lane 1, ERS-GST) and His-tagged WRKY53 (lane 2, W53-His) were separated on SDS-PAGEs. Both extracts were coincubated and immunoprecipitated with anti-GST antibodies (lane 3,

coi1 suppressor1 mutant (Xiao et al., 2004). However, progression of senescence appears not to be impaired in the JA-insensitive mutant *coi1* or in plants, which produce low levels of JA due to a knockout mutation in *OPR3* (Stintzi and Browse, 2000; He et al., 2002). Positive and negative signaling pathways have been broadly documented (Turner et al., 2002; Rojo et al., 2003; Gfeller and Farmer, 2004), and MPK4, NPR1, and WRKY70 have been identified as the key regulators of the crosstalk in SA and JA signaling during *Arabidopsis* defense (Petersen et al., 2000; Spoel et al., 2003; Li et al., 2004). It is likely that these two regulatory networks controlling plant defense signaling contain several nodes of interaction. However, how the crosstalk between SA and JA signaling is executed during senescence and which components are involved remains to be elucidated. The WRKY70 transcription factor was shown to acts as an activator of a subset of SA-induced genes and a repressor of a subset of JA-responsive genes, integrating signals from these mutually antagonistic pathways (Li et al., 2004). Here, we used the yeast two-hybrid system to identify interacting partners of the senescence-related WRKY53 transcription factor. We could characterize a JA-inducible protein (EPITHIOSPECIFYING SENESCENCE REGULATOR [ESR/ESP]) to interact with WRKY53 on the protein level connecting natural plant senescence and JA-mediated signaling. Expression analyses of *WRKY53* and *ESR* after JA and SA treatment of wild-type plants and *npr1*, *coi1*, and *jar1* mutant plants suggest that *WRKY53* is positively regulated by SA and negatively regulated by JA-mediated signaling. Bacterial and fungal infection and gas chromatography–mass spectrometry (GC-MS) analysis of glucosinolate hydrolysis products were performed to analyze the function of ESR and WRKY53 according to pathogen resistance. In vivo localization of the protein complexes in the nucleus and an additional function in the nucleus for ESR could be predicted.

RESULTS

WRKY53 Interacts with a Putative JA-Inducible Protein

To find out whether WRKY53 interacts with partner proteins to connect different signaling pathways, the yeast two-hybrid system was used. The *WRKY53* full-length cDNA was deleted for the activation domain (33 amino acids at the C terminus) and was fused to the c-myc tag of the bait vector. This construct was transformed to the Y187 yeast strain. A cDNA expression library was prepared from leaves of 7-week-old plants exhibiting maximum expression of *WRKY53* during plant development. The cDNAs were introduced into the prey vector and transformed to the AH109 yeast strain. After mating, colonies were selected and sequenced. The full-length cDNAs of selected candidate proteins were isolated, and the interaction with WRKY53 was tested in the yeast two-hybrid system. For 10 proteins, the interaction with WRKY53 could be confirmed for the full-length c-DNA clones also including putative transcription factors. One of the proteins (At1g54040) was an epithiospecifying protein (ESP) (de Torres Zabala et al., 2005) and a putative jasmonate-inducible protein (Figure 1) containing 341 amino acids and four kelch repeat domains. To characterize the function of this protein as a candidate for crosstalk between senescence and JA signaling in senescence, we analyzed it in more detail. To specify its function more precisely, we termed it ESR/ESP. First, we confirmed the interaction in vitro by coimmunoprecipitation. Anti-glutathione *S*-transferase (GST) antibodies were able to pull down not only the recombinant GST-tagged version of ESR with the size of ~64 kD but also the recombinant His-tagged version of WRKY53 with the size of ~40 kD (Figure 1B), clearly indicating a protein–protein interaction between ERS and WRKY53. Since it was not predictable how much His-tagged WRKY53 can be precipitated by the interaction with GST-tagged ESR with the anti-GST antibodies, two different concentrations of the bacterial extracts were loaded on the gel for the detection with both antibodies.

Moreover, we tested the direct interaction between WRKY53 and ESR in vivo. Therefore, we used a technique designated as bimolecular fluorescence complementation. Protoplasts prepared from leaves of $35S_{pro}$:*GFPc155*-HA-*ESR* transgenic *Arabidopsis* plants were transiently transformed with plasmids encoding a $35S_{pro}$:*GFPn173*-c-myc-*WRKY53* fusion protein. If the WRKY53 and ESR proteins are able to interact directly in plant cells, green fluorescent protein (GFP) should be reconstituted and should then be able to emit green fluorescence. Whereas cells transformed with empty vectors produced no or only background fluorescence, a strong signal was observed in the nucleus when *GFPc155*-HA-*ESR* was coexpressed with *GFPn173*-cmyc-*WRKY53* (Figure 1C). These results indicated that WRKY53 and ESR proteins are colocalized in the nucleus and interact on the protein level. If the *WRKY53* coding sequence was fused to full-length *GFP* and transformed into protoplasts of wild-type plants, green fluorescence could be observed in the nucleus. If the *ESR* coding sequence was fused to *GFP* and transformed into protoplasts of wild-type plants, green fluorescence could be observed in the cytoplasm and in the nucleus.

Figure 1. (continued).

Co-IP). Lane 2b (W53-His) represents the 10-fold amount of proteins of lane 2a. The molecular mass of the detected proteins is indicated at the right (64 kD for ESR-GST and 40 kD for W53-His)

(C) Bimolecular fluorescence complementation assay. *Arabidopsis* protoplasts prepared from $35S_{pro}$:*GFPc155*-HA-*ESR* transgenic plants were transformed with $35S_{pro}$:*GFPn173*-c-myc-*WRKY53*. Green fluorescence can be detected if ESR and WRKY53 interact on the protein level (left), with ethidium bromide staining of the nucleus (middle), or in bright field (right).

(D) *Arabidopsis* wild-type protoplasts were transformed with $35S_{pro}$:*GFP-ESR* or $35S_{pro}$:*GFP-WRKY53*, and *Arabidopsis* W53-KO protoplasts were transformed with $35S_{pro}$:*GFP-ESR.* Green fluorescence (left), ethidium bromide staining of the nucleus (middle), and bright field (right).

(E) *Arabidopsis* epidermal cells are transiently transformed by injection with $35S_{pro}$:*GFPc155*-HA-*ESR* and $35S_{pro}$:*GFPn173*-c-myc-*WRKY53*. Green fluorescence (left), bright field (middle), and analyses of the tagged proteins on a protein gel blot (right) using either anti-HA or anti-c-myc antibodies.

However, if the same construct was transformed into protoplast prepared from *WRKY53* knockout plants (W53-KO), green fluorescence was excluded from the nucleus, indicating that ESR is brought into the nucleus via protein–protein interaction with WRKY53 (Figure 1D).

To show direct interaction between WRKY53 and ESR in plant tissue, we also transformed the constructs into leaf epidermal cells of *Arabidopsis* by *Agrobacterium tumefaciens* infiltration. A strong GFP fluorescence was observed in the nuclei of the epidermal cells when *GFPn173*-c-myc-*WRKY53* and *GFPc155*-HA-*ESR* were coexpressed (Figure 1E). Expression of *GFPn173*-c-myc-*WRKY53* and *GFPc155*-HA-*ESR* alone induced no or only weak fluorescence signals. Expression of both fusion proteins could be demonstrated in the leaf tissue using HA- and c-myc–specific antibodies (Figure 1E). Taken together, it can be concluded that the WRKY53 protein can directly interact with the ESR protein in vivo, that the interaction takes place in the nucleus, and that ESR is directed to the nucleus by the interaction with the WRKY53 protein.

ESR Is a JA-Inducible Protein Involved in Plant Response to Pathogens

To identify whether ESR and WRKY53 are targets of JA and SA signaling, wild-type plants, JA-insensitive mutants *jar1-1* and *coi1-1*, and SA-insensitive mutant *npr1-1* were treated with 80 μM JA or 2 mM SA and were analyzed for mRNA levels of *WRKY53* and *ESR*. The result of the RNA gel blot analyses revealed that expression of *ESR* is reduced by SA and activated by JA; conversely, expression of *WRKY53* is activated by SA and is reduced by JA in wild-type plants (Figure 2A). For SA and JA treatment, 5-week-old plants with a low expression level of *WRKY53* were chosen, and for JA treatment, 7-week-old plants with a high expression level of *WRKY53* were analyzed. *WRKY53* expression was not effected in *npr1-1* plants treated with SA and *jar1-1* plants treated with JA, but repression was abolished in *coi1-1* treated with JA. Expression of *ESR* is not affected in *npr1-1* treated with SA but completely blocked in the *jar1-1* mutant and constitutively high in *coil-1* (Figure 2B). These results demonstrate that ESR is a target of the *JAR1*-mediated and *COI1*-mediated JA signaling pathways and is downstream of *JAR1* and *COI1*. SA signaling on *ESR* and *WRKY53* expression is not mediated by *NPR1*. JA signaling on *WRKY53* expression is mediated by *COI1* but independent of *JAR1*.

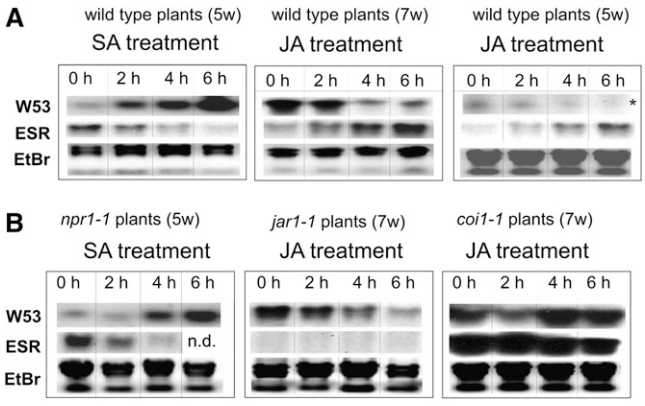

Figure 2. RNA Gel Blot Analyses of the Wild Type and SA or JA Signaling Mutants after JA or SA Treatment.

(A) Wild-type plants.

(B) *npr1-1*, *jar1-1*, or *coi1-1* mutant plants of different ages were treated with JA or SA and were harvested at different time points (0 to 6 h) after treatment as indicated above the lanes. Hybridization probes are indicated at the left.

5w, 5-week-old plants; 7w, 7-week-old plants; EtBr, ethidium bromide staining of total RNA; *, very long exposure time of the x-ray film; n.d., not determined.

In silico analyses of the *ESR* sequence revealed some similarities to the ESP family. ESPs are rather small labile proteins that do not have any enzymatic activity per se. However, they are considered to be a necessary myrosinase cofactor to drive the myrosinase-catalyzed reaction toward the production of cyanoepithioalkanes, starting from glucosinolates containing a double terminal bond. The presence of EPS during the hydrolysis of alkenyl glucosinolates leads to the formation of epithionitriles instead of isothiocyanates by transfer of the sulfur atom from the basic glucosinolate backbone to the terminal alkene residue of the side chain. These hydrolysis products have been found to be most active against herbivores and pathogens (Eckardt, 2001; Lambrix et al., 2001; Tierens et al., 2001; Grupp and Abel, 2006). de Torres Zabala et al. (2005) could show that ESR belongs to the EPS family. To test whether WRKY53 has an influence on the ESR function as epithiospecifier to protect plants against bacterial and fungal pathogens, $35S_{pro}$:*ESR* overexpressing (ESR-OE) and ESR T-DNA insertion lines (ESR-KO) and $35S_{pro}$:*WRKY53* overexpressing (W53-OE) and WRKY53 T-DNA insertion lines (W53-KO) were analyzed for their pathogen responses. Both lines were infected by the bacterial pathogen *Pseudomonas syringae* DC 3000 and the fungal pathogen *Alternaria brassicicola*, respectively. In ESR-OE plants, the growth rate of bacteria in a defined leaf region decreased significantly compared with wild-type plants, whereas in ESR-KO plants, the growth rate of the bacteria increased (Figure 3A). Fungal infection was followed by visible damage of the infected leaves that can be categorized according to damage degrees. The infected leaves of the ESR-OE line looked less damaged compared with wild-type plants, whereas the infected leaves of the ESR-KO line were severely damaged (Figure 3B). By contrast, neither bacterial nor fungal infection revealed any significant difference among wild-type plants, W53-OE, and W53-KO (Figures 3A and 3B). GC-MS analyses of different isothiocyanates or nitriles revealed that by overexpressing *ESR*, the glucosinolate hydrolysis is driven toward the formation of nitrile compounds since much more of 5-methylthio-pentyl-nitrile and 3-indole-acetonitrile can be detected in ESR-OE. Similar effects have already been shown by de Torres Zabala et al. (2005). By contrast, much more isothiocyanates can be detected in ESR-KO plants (Figure 4B). This indicates that ESR can function as epithiospecifier and modifies the profile of the glucosinolate derivatives that might be more effective against the tested pathogens. In accordance with the infection experiments, only slight differences in these compounds can de observed in W53-OE and W53-KO plants. In this case, overexpression led to a reduction of nitrile compounds

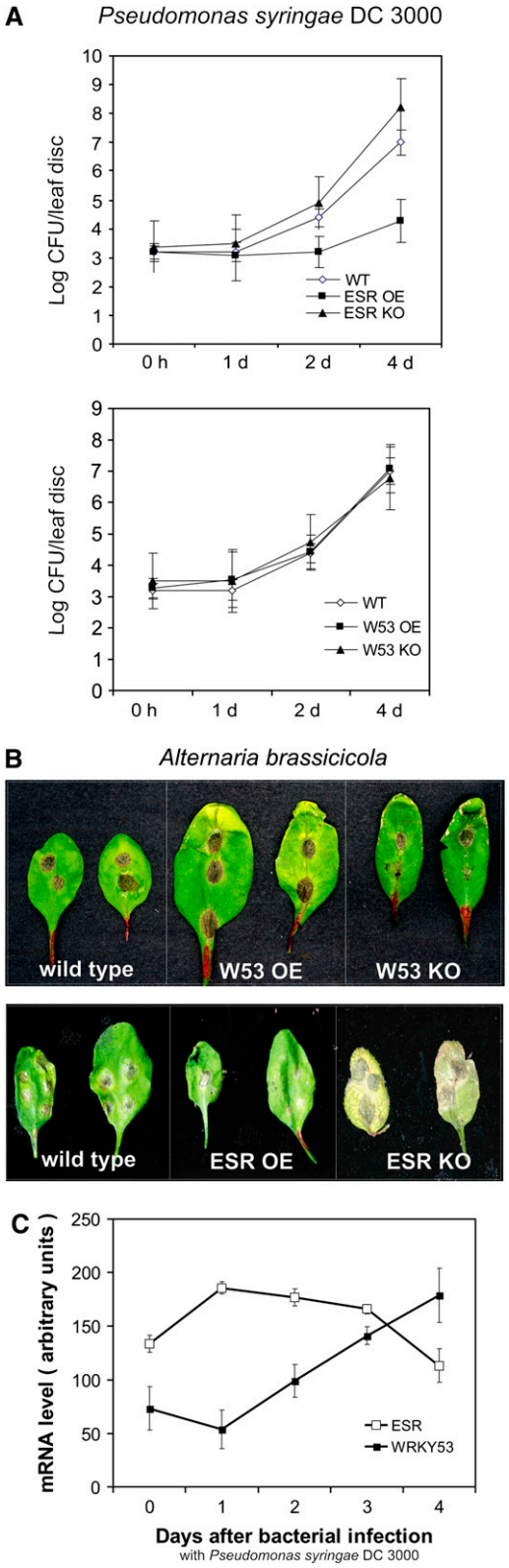

Figure 3. Pathogen Infection of Wild-Type and Transgenic Plants.

(A) W53-OE, W53-KO, ESR-OE, ESR-KO, or wild-type plants were infected with *P. syringae* DC 3000, and the number of colony-forming units (CFU) per leaf disk was determined after 1, 2, or 4 d on agar plates. Leaf discs of six infected plants were pooled for this experiment. Error bars indicate SE.

(B) The same lines were also infected with *A. brassicicola*, and the damage of the leaves was observed phenotypically.

(C) Quantification of RNA gel blot analyses of *WRKY53* and *ESR* after infection as expression units relative to rRNA. Error bars indicate SE of three experiments.

in comparison to wild-type plants, whereas knockout of the gene led to a slight increase of nitriles (Figure 4B).

ESR Function in Leaf Senescence

Since WRKY53 is most likely an important transcriptional regulator in leaf senescence, we analyzed ESR-OE and ESR-KO plants for their senescence phenotype. Comparing the corresponding leaves of 7-week-old plants among ESR-OE, ESR-KO, and wild-type plants, senescence was delayed in ESR-OE leaves, whereas in ESR-KO, senescence was strongly accelerated (Figure 5A). By contrast, W53-OE showed accelerated and W53-KO showed delayed senescence (Figure 5A; Miao et al., 2004). This indicates an antagonistic function of WRKY53 and ESR. A second ESR T-DNA insertion line (SALK010349) showed the same phenotype; however, it was not as pronounced. Therefore, we complemented the mutant line exhibiting the strong phenotype with the ESR gene under the control of its own promoter to assure that the mutant phenotype was really due to the insertion of the T-DNA in the ESR gene. In these plants, the phenotype of the wild-type plants was completely restored (Figure 5B). Overexpression of ESR in the W53-OE background also restored the wild-type phenotype, whereas overexpression of ESR in the W53-KO plant had no additive effect, and the same phenotype as for the W53-KO plants could be observed (Figure 5B). This is an indication that the ESR effect on leaf senescence is transmitted by WRKY53.

Negative Feedback Loop between *WRKY53* and *ESR*

To characterize the interplay between WRKY53 and ESR more precisely, RNA gel blot or RT-PCR analyses were performed using transgenic lines with altered gene expression of either *WRKY53* or *ESR*. Modulation of *WRKY53* transcript levels by constitutive overexpression decreased expression level of the *ESR* gene compared with the wild type. Several W-boxes can be detected in the ESR promoter region, and it was shown before that WRKY53 can also act as transcriptional repressor. By contrast, suppression of *WRKY53* expression in the W53-KO line led to an increase in *ESR* expression. Moreover, higher *ESR* transcript levels in ESR-OE lines decreased the expression of *WRKY53*, and suppression of *ESR* transcripts in ESR-KO lines increased the expression of *WRKY53* (Figure 6A), indicating that WRKY53 can regulate *ESR* expression in a negative feedback loop and vice versa. Transformation of increasing amounts of a $35S_{pro}$:*ESR* construct into $WRKY53_{pro}$:*GUS* protoplasts confirmed that the *WRKY53* promoter-driven expression of a

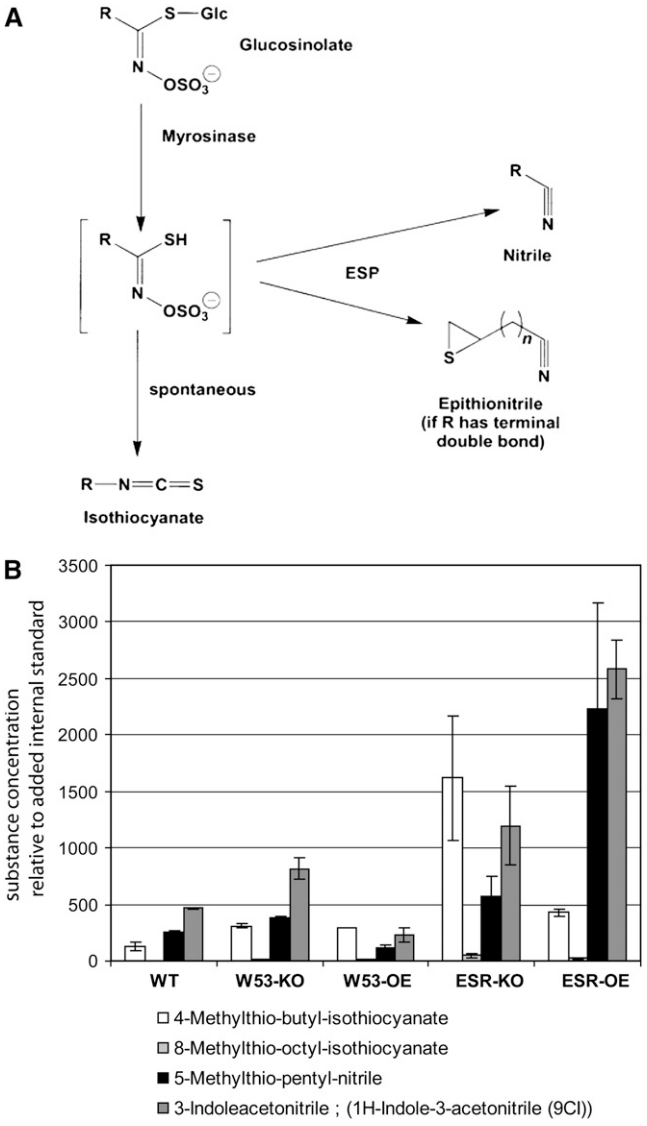

Figure 4. Functional Analyses of ESR and WRKY in Nitrile/Isothiocyanate Composition.

(A) Conversion of glucosinolates to isothiocyantes is catalyzed by the enzyme myrosinase. In the presence of epithiospecifiers as cofactors of myrosinase, the glucosinolates are converted to nitriles or epithionitriles.
(B) GC-MS analyses of W53-OE, W53-KO, ESR-OE, ESR-KO, or wild-type plants. The concentration of two isothiocyanates and two nitriles was determined as relative values compared with the added standards. Mean value of six independent experiments are shown. Error bars indicate SE.

reporter gene was reduced in the presence of ESR (Figure 6B). In vitro analyses by electrophoretic mobility shift assay (EMSA) revealed that in the presence of increasing amounts of ESR protein, the DNA binding activity of WRKY53 was inhibited (Figure 6C). A time course of gene expression after pathogen infection revealed that *ESR* expression reached the highest expression rate already on the first day after inoculation with the pathogens, whereas *WRKY53* expression is slightly reduced. By contrast, *WRKY53* showed higher expression levels 4 d after infection when *ESR* expression is reduced again, also indicating an antagonistic regulation of both genes (Figure 3C). In the ESR-OE plants, which show higher pathogen resistance, an elevated expression of the defensin gene *PDF1.2* can be detected, whereas the expression of *NPR1* appears to be almost unaffected (Figure 6A)

DISCUSSION

Massive changes in the transcriptome can be observed during leaf senescence accompanied by the activation of many transcription factors. Different members of the WRKY transcription factor family have been shown to be induced during leaf senescence (Eulgem et al., 2000), and *WRKY53* was shown to play an important role during this process in *Arabidopsis* (Hinderhofer and Zentgraf, 2001; Miao et al., 2004). However, the role of individual WRKY factors during leaf senescence is still unclear. The involvement of WRKY factors in plant defense is also well documented; some of these factors have been shown to confer disease resistance (Deslandes et al., 2002), trigger expression of defense-related genes in systemic acquired resistance (Eulgem et al., 1999; Maleck et al., 2000; Robatzek and Somssich, 2002), and control *Arabidopsis* innate immunity activated by bacterial flagellin (Asai et al., 2002). Dong et al. (2003) showed that 49 of 72 *WRKY* genes in *Arabidopsis* were differentially regulated in response to exogenous SA or infection by a bacterial pathogen. *WRKY53* was strongly induced by SA but only slightly induced by pathogen challenge (Dong et al., 2003), indicating that WRKY53 is not involved in pathogen defense and SA induction might be part of other signaling processes. Here, we showed that neither overexpression nor knockout of the *WRKY53* gene had an influence on the resistance against bacterial or fungal pathogens like *P. syringae* or *A. brassicicola*, supporting that SA induction of WRKY53 is not involved in pathogen defense but is most likely involved in regulation of leaf senescence. However, SA forms a network of synergistic and antagonistic interactions with other signaling molecules, such as JA and ethylene (Glazebrook, 2001; Kunkel and Brooks, 2002; Spoel et al., 2003), and *WRKY70* was shown to be one node of interaction between SA- and JA-induced gene expression (Li et al., 2004). Microarray analyses of *WRKY53* overexpression and knockout plants revealed that *WRKY70* expression is influenced by WRKY53 and vice versa (U. Zentgraf and T. Laun, unpublished data; Li et al., 2004). In addition, several enzymes involved in JA biosynthesis showed senescence-enhanced expression, and JA levels were shown to increase during senescence. Increased JA levels could induce senescence-associated genes and premature senescence symptoms in *Arabidopsis* (Nam, 1997; He et al., 2002; Buchanan-Wollaston et al., 2003). Since *WRKY53* is expressed only at an early time point of leaf senescence, increasing JA levels during progression of leaf senescence might be involved in the shutoff of *WRKY53* expression after onset of senescence (Hinderhofer and Zentgraf, 2001). Here, we showed that senescence-specific *WRKY53* might also act as a node of convergence of SA and JA signaling in two different ways (illustrated in Figure 7): (1) *WRKY53* expression is antagonistically regulated by JA and SA,

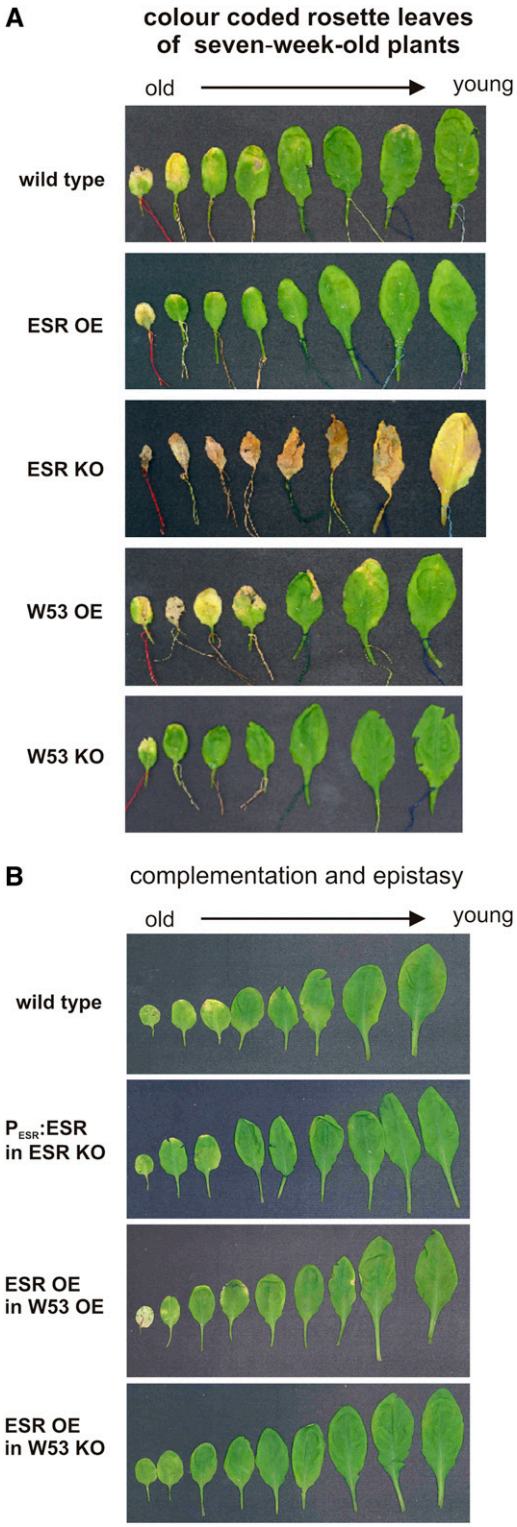

Figure 5. Senescence-Related Phenotype.

(A) The phenotype of the leaves of 7-week-old plants that were arranged according to their age with the help of a specific color code is presented. W53-OE, W53-KO, ESR-OE, ESR-KO, or wild-type plants.

(B) A complementation of the mutant phenotype was achieved by the transformation of ESR under the control of its own promoter into the ESR-KO line (P_{ESR}:*ESR* in ESR-KO). Epistatic analyses of *WRKY53* and *ERS* function was performed by overexpressing *ESR* in the W53-KO (ESR-OE in W53-KO) and in the W53-OE line (ESR-OE in W53-OE).

and (2) WRKY53 action in the nucleus might be affected on the protein level by the interaction with the JA-inducible protein ESR.

WRKY53 Expression Is Antagonistically Regulated by JA and SA

WRKY53 expression is induced by SA independent of *NPR1*. In SA signaling, nuclear localization of NPR1 is essential for SA-mediated defense gene expression but not required for the suppression of JA signaling. NPR1 modulates the crosstalk between SA and JA through a novel function in the cytoplasm (Spoel et al., 2003). However, *WRKY53* induction and *ESR* repression by SA are independent of *NPR1*. In the *jar1* mutant, WRKY53 expression could be reduced in the same way as in the wild-type plant, indicating that JAR1 is not involved in this signaling. By contrast, ESR induction was completely abolished, clearly indicating the involvement of JAR1 in the ESR expression. By contrast, in the *coi1* mutant, WRKY53 expression is increased compared with wild-type plants of the same age, and JA treatment was no longer able to reduce this expression, suggesting an inhibiting function of COI1 on WRKY53 expression also in the 7-week-old wild-type plants. However, the constitutively high expression of ESR in the *coi1* mutant was totally unexpected and cannot be explained by a simple model. This might also reflect compensatory effects of the pleiotropic effects in the *coi1* mutant. The F-box protein COI1 appears to be involved in the expression of both genes, whereas JAR1, which most likely converts JA into JA-Ile (Staswick and Tiryak, 2004), only influences the expression of *ESR*, indicating that *ESR* and *WRKY53* are influenced by different branches of the JA signaling pathway. It has recently been proposed that COI1 can mediate the removal of transcription factors tagged by JA-dependent phosphorylation from the promoters of their target genes (Turner et al., 2002). It has been shown that WRKY53 can be phosphorylated directly by a mitogen-activated protein kinase kinase kinase (MEKK1), but whether this phosphorylation is JA dependent still has to be elucidated (Y. Miao and U. Zentgraf, unpublished data). Moreover, JA activates the coordinate expression of genes involved in the antioxidative metabolism, and this gene activation is abolished in the JA-deficient *opr3* mutant after ozone treatment (Sasaki-Sekimoto et al., 2005). In a different report, MeJA application increased hydrogen peroxide levels in *Arabidopsis* leaves most likely by a direct activation of the superoxide dismutase enzyme (Maksymiec and Krupa, 2002). However, *WRKY53* expression is induced by hydrogen peroxide, and hydrogen peroxide levels increase during onset of leaf senescence (Miao et al., 2004; Zimmermann et al., 2006), but is reduced by JA and induced by SA, indicating that a more complex regulation is involved in WRKY53 expression. Recently, Mur et al. (2006) showed that synergistic and antagonistic interaction between SA

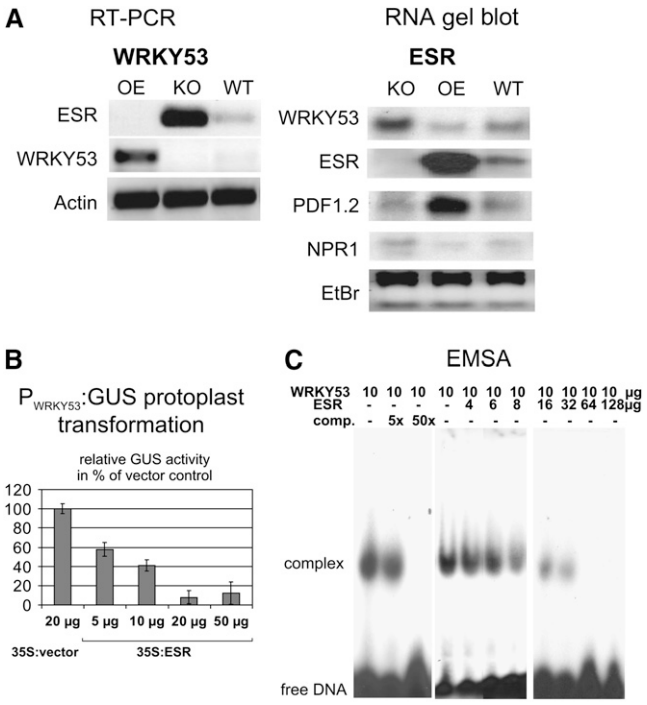

Figure 6. Negative Feedback Loop between *WRKY53* and *ESR*.

(A) RT-PCR or RNA gel blot analyses of W53-OE, W53-KO, ESR-OE, ESR KO, or wild-type plants. *ACTIN2* was used as reference for equal amounts of cDNA in the RT-PCR, whereas ethidium bromide staining (EtBr) was used as loading control in RNA gel blot analyses.

(B) Protoplasts prepared from WRKY53$_{pro}$:*GUS* transgenic plants were transformed with different amounts of 35S$_{pro}$:*ESR* (35S:ESR) or the empty vector (35S:vector) and analyzed for GUS activity. Mean values of five experiments are shown; error bars indicate SE.

(C) EMSA of [γ-^{32}P]ATP-labeled DNA fragment containing a triple W-box with 10 µg of crude protein extract of an *E. coli* M15 strain expressing recombinant *WRKY53* (Miao et al., 2004). Addition of nonlabeled DNA fragments as competitor DNA or 0 to 128 µg of crude protein extract of an *E. coli* BL21 strain expressing recombinant *ESR* is indicated above the lane.

and JA are highly concentration dependent. Consulting the Genevestigator database (https://www.genevestigator.ethz.ch), *WRKY53* and *ESR* are expressed at the same time point during development, and both mRNAs can be found in leaf tissue, indicating that the negative crosstalk on the protein level can actually occur in planta. However, there are some discrepancies in *WRKY53* and *ESR* expression patterns in response to SA and JA and in different mutants. According to Genevestigator data, *WRKY53* expression is induced by SA and reduced by MeJA; by contrast, *ESR* expression is not altered after treatment with both hormones despite the fact that The Arabidopsis Information Resource database assigns a response to JA to the ESR gene. However, the treatment and growing conditions were different in these experiments. *WRKY53* expression is not altered in the *coi1* mutant compared with the wild type, and ESR expression appears to be downregulated. Therefore, the role of COI1 in this signaling is still an unanswered question.

WRKY53 Interacts in the Nucleus with the JA-Inducible Protein ESR on the Protein Level

The interacting protein partner of WRKY53 appears to have a dual function: one in pathogen defense and one in senescence regulation, most likely depending on its localization in the cell. Overexpression or knockout of the *ESR* gene clearly showed a senescence-retarding or -accelerating phenotype, respectively. Overexpression or knockout of the *WRKY53* gene had an antagonistic effect. Epistatic analyses confirmed that ESR function in senescence is most likely mediated trough WRKY53 since overexpression of ESR in W53-KO plants had no additive effect, whereas overexpression of ESR in W53-OE restored the wild-type situation. In the mutant *coi1*, high levels of ESR also could be detected, but *coi1* plants did not show a retarded senescence phenotype under the conditions we used for our experiments. However, in contrast with the ESR-OE plants showing only low levels of *WRKY53* transcripts, *WRKY53* expression levels are also very high in the *coi1* mutant. Thus, provided that transcript levels also reflect protein levels, both protein amounts would be elevated in the *coi1* mutant plants, indicating that ESR might act on senescence through its interaction with WRKY53 in the nucleus.

Pathogen challenge revealed that ESR also confers resistance to fungal and bacterial pathogens, whereas WRKY53 had no effect on pathogen resistance. JA signaling is important for resistance to the fungus *A. brassicicola*, whereas SA signaling is

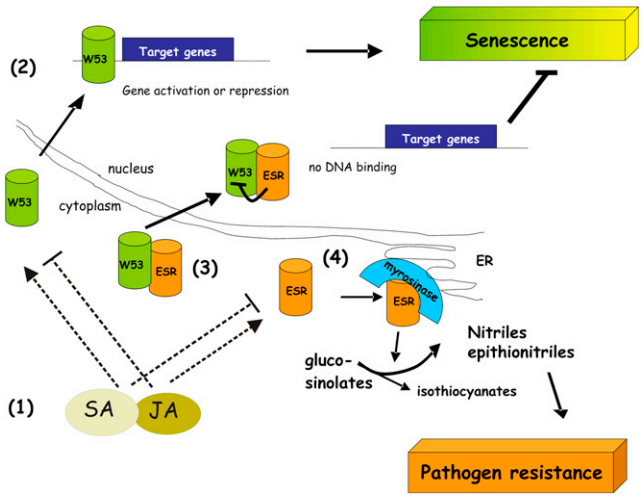

Figure 7. Model of the Regulatory Network of WRKY53 and ESR under the Control of SA and JA.

(1) *WRKY53* and *ESR* expression is antagonistically regulated by SA and JA. (2) WRKY53 protein can enter the nucleus and can bind to W-boxes in its target promoters and either activate or repress its target genes, resulting in the onset of leaf senescence. (3) WRKY53 can interact with ESR in the cytoplasm, and ESR can be shifted to the nucleus in this complex. There, ESR can inhibit binding activity of the WRKY53 protein to its target genes, leading to the inhibition of the senescence process. (4) If the ESR protein stays in the cytoplasm, it can act as a cofactor of the membrane-associated myrosinase to drive the hydrolysis of glucosinolates into the direction of nitriles and epithionitriles, which are most likely part of an enhanced resistance against *P. syringae* and *A. brassicicola*.

not required since NahG plants are as resistant as wild-type plants (Thomma et al., 1998). By contrast, a complex network integrating SA and JA signaling is involved in the response to the bacterial pathogen *P. syringae* (Glazebrook et al., 2003). GC-MS analyses demonstrated that ESR could function as an epithiospecifier that does not have enzymatic activity per se but rather acts as a cofactor of myrosinase to drive the conversion of glucosinolates into nitriles (de Torres Zabala et al., 2005). Plants with increased *ESR* expression clearly showed higher amounts of nitriles, whereas ESR-KO plants revealed higher levels of isothiocyanates. In plants with altered expression of *WRKY53*, the nitrile contents were only slightly modulated. Moreover, ESR is located in the cytoplasm in the absence of WRKY53 where it can function as a cofactor of myrosinase. To our knowledge, a nuclear localization has not been reported to date for any member of the epithiospecifiers nor for myrosinase. Myrosinase is localized either in specific cells (myrosin cells) in vacuoles or to a low extent in normal cells in the cytoplasm with a tendency to associate with membranes of the tonoplast, plasmalemma, endoplasmic reticulum, or mitochondria (Bones and Rossiter, 1996; Lambrix et al., 2001). Higher amounts of nitriles produced in the ESR-OE plants appear to confer higher resistance to *P. syringae* and *A. brassicicola* infection than high levels of isothiocyantes produced in ESR-KO plants. Lambrix et al. (2001) showed that different ecotypes of *Arabidopsis* accumulate different hydrolysis products and that different hydrolysis products had different effects on herbivore feeding. In this case, isothiocyanates were stronger feeding inhibitors for a generalist lepidoteran herbivore, the cabbage looper *Trichoplusia ni*, than were nitriles. In addition, in the ESR-OE line, the defensin *PDF1.2* is more highly expressed than in the wild-type plants probably also participating in the higher pathogen resistance. The expression of the *NPR1* gene, which acts as a regulator for pathogenesis-related gene expression (Cao et al., 1997), was not affected by *ESR* overexpression.

In the presence of the WRKY53 protein, ESR is partly directed to the nucleus. EMSA experiments revealed that ESR inhibited DNA binding of WRKY53 in vitro. Several senescence-associated genes (SAGs) and many transcription factors, including other members of the WRKY family, were shown to be direct target genes of WRKY53 (Miao et al., 2004). Therefore, the nuclear levels of ESR might have an indirect influence on expression levels of SAGs and transcription factors via WRKY53. ESR also had an influence on the expression of the *WRKY53* gene itself. However, if this was a direct effect, *WRKY53* expression should increase in ESR-OE since high levels of ESR would abolish WRKY53 binding to its own promoter and WRKY53 was shown to negatively regulate its own expression (Miao et al., 2004). It is most likely that ESR also has an indirect effect on *WRKY53* expression by affecting the expression of target genes of WRKY53, including the other WRKY factors since WRKY factors influence the transcription of each other rather in a regulatory network than in linear signal transduction pathways (Robatzek and Somssich, 2002; Li et al., 2004; Miao et al., 2004). Whether ESR can also interact with other WRKY factors still has to be elucidated.

There are some rare examples in nature for such unusual protein–protein interactions of transcription factors. In vertebrates and *Drosophila*, the Armadillo/β-catenin has a dual function: it acts as a component of the cadherin-based cell adhesion system, and β-catenin regulates gene expression by direct interaction with transcription factors, such as T cell factors of the wnt/wingless signaling pathway. Deregulation of wnt signaling is associated with cancer formation in humans (Städeli et al., 2006). In *Arabidopsis*, Lesions Simulating Disease Resistance1 (LSD1) acts as a cytoplasmic retention protein for the bZip10 transcription factor, which regulates cell death after infection. In this case, the transcription factor is kept in the cytoplasm by the interaction with this protein and is prevented from its function as transcription factor. However, it is still unclear whether LSD1 has an additional function in the cytoplasm (Kaminaka et al., 2006). In conclusion, WRKY53 and ESR mediate a negative crosstalk between pathogen resistance and senescence influenced by the JA/SA equilibrium, and according to the phenotypes, ESR appears to have a greater impact on WRKY53 function in senescence than WRKY53 on ESR function in pathogen resistance.

METHODS

Plant Material

Seeds of *Arabidopsis thaliana*, ecotype Columbia (Col), were grown in a climatic chamber at 22°C with 16 h of illumination under low light conditions (60 μmol s^{-1} m^{-2}). Under these conditions, plants developed flowers within 7 to 8 weeks, and mature seeds could be harvested after 10 to 12 weeks. Five- and seven-week-old plants were used for spraying with 2 mM SA and 80 μM JA.

Transgenic Plants

ESR cDNA fragments that contained the full coding sequence were PCR-amplified with primers that contain *Bam*HI and *Xba*I restriction sites at the ends, cloned into pBluescript K, and sequenced to verify PCR product sequences. Subsequently, the *ESR* coding sequence fragment was excised with *Bam*HI and *Xba*I and ligated to pRT-bar binary vector. This vector was transformed into wild-type plants (Col-0), into the W53-KO line, and into the W53-OE line. The *ESR* knockout line was received from the Nottingham Arabidopsis Stock Centre (NASC) (SALK line 055029), which has a T-DNA-insertion in intron 1. In addition, a second T-DNA insertion line (SALK line 10349) was analyzed for the same phenotype. The W53-KO line was also received from the NASC (SALK line 034157) and has a T-DNA-insertion in exon 2. W53-OE and WRKY53$_{pro}$:*GUS* lines were described previously (Miao et al., 2004). *Arabidopsis* transformation was performed by the vacuum infiltration procedure (Bechtold and Pelletier, 1998). The seeds were collected from the infiltrated plants and selected by spraying with 0.1% Basta in a growth chamber.

RNA Gel Blot Analysis

Total RNA was purified from *Arabidopsis* leaves using the Gentra purescript kit (Biozyme). The total RNA was separated on MOPS-formaldehyde (6.2%) agarose gels (1.0%) and transferred to nylon membranes using 10× SSC as transfer buffer. The membranes were hybridized at 42°C using 5× SSC, 50% formamide, 5× Denhardt's solution, 1% SDS, 100 μg/mL heat-denatured, sheared, nonhomologous DNA as hybridization buffer, and DNA probes that were labeled with [α-^{32}P]dCTP in a random priming reaction. After hybridization, membranes were washed once in 2× SSC at room temperature, twice with 2× SSC and 0.1% SDS at 65°C, and once with 0.2× SSC and 0.1% SDS at 65°C. The membranes

were stripped for 30 min to 3 h in 5 mM Tris, pH 8, 0.2 mM EDTA, 0.05% Na-pyrophosphate, and 0.1× Denhardt's solution at 75 to 85°C until no radioactivity could be detected on the membrane. Rehybridization was performed following the hybridization protocol.

Yeast Two-Hybrid System Screen and Confirmation

The activation domain of the full-length *WRKY53* cDNA was deleted (W290), and the truncated cDNA was cloned into the bait pGBKT7 vector, which harbors the Trp1 selection marker, and was transformed to the yeast strain Y187. The cDNA expression library was prepared from 7-week-old rosette leaves of *Arabidopsis* and cloned into pGADT7-Rec vector containing the GAL4 activation domain and the leu2 selection marker. The yeast strain AH109 was used to express this cDNA library. The two-hybrid screenings and assay were performed via matting protocol as described in Clontech's Matchmaker GAL4 Two-Hybrid System 3 and libraries user manual (Clontech/Biosciences). For the confirmation of the protein–protein interaction, the full-length *ESR* and *WRKY53* cDNAs were cloned into the prey pGADT7 vector, which harbors the leu2 selection marker, and the bait pGBKT7 vector, which contains the Trp1 selection marker. If *WRKY53* was used as bait construct in pGBKT7, the truncated version of *WRKY53* (without activated domain) was inserted. The two-hybrid assays were performed as described in Clontech's Matchmaker GAL4 Two-Hybrid System 3 manual to confirm the interaction.

Bimolecular Fluorescence Complementation Assay

A c-myc–tagged version of the full-length *WRKY53* cDNA and the truncated form of *WRKY53* cDNA were cloned to pGFPn173c vector (created by Binghua Wu, University of Tuebingen) via *Bam*HI/*Xba*I and sequenced. The full-length *ESR* cDNA with HA-tag was cloned to pGFPc155c vector (created by Binghua Wu) via *Bam*HI/*Xba*I and sequenced. All plasmids were transformed to the *Agrobacterium tumefaciens* strain GV3101/pMESR0. Both constructs ($35S_{pro}$*:GFPc155*-HA-*ESR* and $35S_{pro}$*:GFPn173*-c-myc-*WRKY53*) were transformed to *Arabidopsis* plants by vacuum infiltration.

Arabidopsis leaves were transiently transformed by injection of *A. tumefaciens* cells harboring the appropriate plasmids as previously described (Batoko et al., 2000). Protein expression was examined 24 to 48 h after injection. GFP-dependent fluorescence was analyzed 48 h after infection in cells of the lower epidermis using an epifluorescence microscope. Small pieces of the leaves were randomly cut from the infected area and mounted in water for microscopy observations. Images were processed using the Adobe Photoshop software package.

Coimmunoprecipitation

Coimmunoprecipitation was performed as described by the user manual of the Clontech Matchmaker Co-IP kit without using in vitro–translated ^{35}S-Met–labeled proteins but using extracts of *Escherichia coli* strains expressing a His-tagged version of *WRKY53* or a GST-tagged version of *ESR*. The protein extracts of the recombinant *E. coli* strains were incubated together for 1 h at room temperature. The anti-GST antibody was added, and the mixture was incubated for 1 h at room temperature on a rotating wheel. Protein A beads were added to the preincubated proteins and were further incubated for 1 h at room temperature on a rotating wheel. The beads were washed and centrifuged as described in the Clontech manual. Subsequently, the proteins were separated on two identical SDS-PAGEs, and a protein gel blot was performed as described below. One membrane was immunodetected with anti-GST antibodies. The second membrane was immunodetected with a combination of anti-GST and anti-HIS antibodies.

Protein Gel Blot Analyses

To extract soluble proteins of plant tissue, 200 mg of leaf material were batch-frozen in liquid nitrogen, ground into powder, resuspended in 100 μL of extraction buffer (100 mM Tris, pH 7.2, 10% sucrose, 5 mM $MgCl_2$, 5 mM EGTA, and protease inhibitor), and centrifuged at 15,000*g* for 10 min. The supernatant was used for immunoblot analysis. Proteins were separated on 8% acrylamide gels and transferred to nitrocellulose membranes using standard protocols. Membranes were blocked for 1 h at room temperature in TBS containing 5% (w/v) nonfat dry milk. The membranes were incubated either with anti-HA or anti-c-myc monoclonal antibodies for 1 h. Blots were washed in TBST for 10 min (three times) before incubation with secondary antibodies. The blots were washed again with TBST for 10 min (three times), and secondary antibody conjugates were detected with a chemoluminescent substrate and exposed to x-ray films.

Transient Transformation of *Arabidopsis* Protoplasts

Protoplasts were prepared from leaves of wild-type or transgenic plants as follows. Approximately 20 leaves were cut into 1 × 1-mm pieces and digested with 20 μL enzyme solution (2% cellulase Onozuka R-10/0 and 5% Macerozyme R-10). All subsequent steps were performed as described by Asai et al. (2002). Protoplast prepared from wild-type plants were transformed with $35S_{pro}$*:GFP-WRKY53* or $35S_{pro}$*:GFP-ESR* constructs. In addition, protoplast prepared from W53-KO plants were transformed with the $35S_{pro}$*:GFP-ESR* construct. Protoplasts prepared from $35S_{pro}$*:GFPc155-HA-ESR* plants were transformed with plasmids containing the construct $35S_{pro}$*:GFPn173-cmyc-WRKY53*. After 36 h of incubation, transformed protoplasts were observed using a confocal laser scanning microscope (Leica). The laser settings were the following: 488 nm at 37% of maximal power and 543 nm at 100% of maximal power. The photomultiplier was set using a 500- to 530-nm window to collect the GFP fluorescence. Ethidium bromide (1 μg/μL) was used to stain the nuclei. For each construct, at least eight different independently transformed *Arabidopsis* protoplast batches were analyzed. Protoplasts of the $WRKY53_{pro}$*:GUS* transgenic plants were transformed with different amounts of $35S_{pro}$*:ESR*, and reporter gene expression was measured 24 h after transformation as described previously (Miao et al., 2004).

Analysis of Glucosinolate Hydrolysis Products

Leaf samples (50 mg) of each line (W53-OE, W53-KO, ESR-OE, ESR-KO, and Col-0) were ground with a glass-stirring rod for 15 s in 2.5 mL of water at the bottom of a 4-mL glass tube. The tube was quickly sealed with a septum cap and left to stand for 5 min at room temperature. After the addition of 4 mL of dichloromethane including 5 μg/μL propylisothiocyanate as internal standard through the septum, the tube was vortexed for 10 s and passed through an Extrelut NT3 column of anhydrous sodium sulfate with a plug of glass wool in a Pasteur pipette. The water was filtered and the dichloromethane layer was extracted by adding 2 mL of dichloromethane and then concentrated under nitrogen to 80 μL.

Samples were analyzed by GC-MS and GC flame ionization detection. The analytical conditions were as follows: capillary column, HP5-MS (30 m × 0.25 mm × 0.25 μm); the injector and detector temperatures were 250 and 280°C, respectively; helium was used as carrier gas at a flow of 1 mL/min. The temperature program was as follows: starting temperature, 60°C for 4 min; final temperature 220°C and a rate of 10°C/min as described by Lambrix et al. (2001).

Bacterial and Fungal Infection

The bacterial strain *Pseudomonas syringae* pv *tomato* DC 3000 was kindly provided by Birgit Kemmerling (University of Tuebingen). Bacteria were grown to OD 1.2 and applied in a density of 10^4 cfu/mL with a

needleless syringe in the middle of a leaf half, and leaf discs were cut immediately 1, 2, and 4 d after infection. Before cutting the leaf discs, leaves were washed for 1 min in 70% ethanol and 1 min in water. Subsequently, two leaf discs per leaf were cut with a cork borer and put into 100 μL of 10 mM $MgCl_2$ and were immediately homogenized. Appropriate dilutions were plated on Kings B plates with the corresponding antibiotics plus 50 μg/mL of cycloheximid and incubated for 24 to 36 h at 28°C, and at least two dilutions per sample were counted.

The fungal strain *Alternaria brassicicola* was kindly provided by Birgit Kemmerling (University of Tuebingen). Fungi were applied in a density of 5×10^5 spores/mL with a needless syringe on the leaf. After incubation for 48 h under normal growth conditions, damage was observed on the infected leaves and the leaves were categorized according to their degree of damage.

In Vitro DNA Binding Assays

The EMSA was performed essentially as described by Promega (http://www.promega.com/tbs/tb110). The DNA–protein binding reaction was performed by incubation of 0.02 pmol of [γ-^{32}P]ATP-labeled DNA fragment with 10 μg of crude protein extract of an *E. coli* strain expressing recombinant WRKY53. Different amounts (0 to 128 μg) of crude protein extracts of an *E. coli* strain expressing recombinant ESR and 1 μg of poly(dI-dC) were added in a total volume of 20 μL. The reaction products were analyzed on 5% nondenaturing polyacrylamide gels. In addition, a 5- or 50-fold excess of the nonlabeled DNA fragment was added to show specificity of the DNA–protein interaction. Specificity of the DNA–protein interaction has been shown by Miao et al. (2004).

RT-PCR

Total RNA was isolated from *Arabidopsis* leaves using the Gentra purescript kit, and reverse transcription was performed with the Superscript RNase H-reverse transcriptase (Invitrogen) and gene-specific primers. PCR reaction was performed in triplicate on the same amounts of cDNA. To avoid saturation of the PCR reaction, the cycling was stopped after 20 to 25 cycles.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM118512 for *WRKY53* (At4g23810) and AY074550 for *ESR*/*ESP* (At1g54040).

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