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ORIGINAL ARTICLE

The WRKY70 transcription factor of *Arabidopsis* influences both the plant senescence and defense signaling pathways

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Abstract Regulatory proteins play critical roles in controlling the kinetics of various cellular processes during the entire life span of an organism. Leaf senescence, an integral part of the plant developmental program, is fine-tuned by a complex transcriptional regulatory network ensuring a successful switch to the terminal life phase. To expand our understanding on how transcriptional control coordinates leaf senescence, we characterized *AtWRKY70*, a gene encoding a WRKY transcription factor that functions as a negative regulator of developmental senescence. To gain insight into the interplay of senescence and plant defense signaling pathways, we employed a collection of mutants, allowing us to specifically define the role of *AtWRKY70* in the salicylic acid-mediated signaling cascades and to further dissect the cross-talk of signal transduction pathways during the onset of senescence in *Arabidopsis thaliana*. Our results provide strong evidence that *At*WRKY70 influences plant senescence and defense signaling pathways. These studies could form the basis for further unraveling of these two complex interlinked regulatory networks.

Keywords *Atwrky70* mutants · Dark-induced senescence · Salicylic acid · Signaling crosstalk

Abbreviations

ET	Ethylene
JA	Jasmonic acid
SA	Salicylic acid

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Introduction

Leaf senescence, constituting the terminal stage of leaf development, is a type of programmed cell death (PCD) and is manifested by a loss of chlorophyll that occurs during chloroplast disassembly together with other catabolic events such as protein, lipid and nucleic acids degradation (Quirino et al. 2000; Gepstein 2004). Leaf senescence is regulated by both intrinsic and environmental signals, with a number of phytohormones and growth regulators such as cytokinins, abscisic acid, salicylic acid, jasmonates, ethylene, nitric oxide and brassinosteroids acting in a precisely coordinated manner (Gepstein 2004). Although considered to be a highly entropic process, the onset, progression and completion of leaf senescence requires extensive and orchestrated transcriptional reprogramming, de novo protein synthesis as well as post-translational regulation (Guo and Gan 2005). Transcriptome analyses in *Arabidopsis thaliana* revealed that among 2,491 genes expressed during developmentally controlled senescence, approximately 800 genes are specifically up-regulated

(Buchanan-Wollaston et al. 2003; Guo and Gan 2005). Despite a remarkable difference in the induced gene expression patterns observed between developmentally induced and dark-induced senescence, approximately 500 genes representing various functional categories, are common to the two forms of triggered leaf senescence (Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006). Fifty-nine genes encoding transcription factors from 20 different gene families were shown to be induced both in developmental- and dark-induced senescence (Buchanan-Wollaston et al. 2005). The two largest groups of senescence-related transcription factors are the NAC and WRKY superfamilies. In *Arabidopsis*, the NAC domain protein family consists of 109 members, many of which are implicated in different developmental and stress response processes, and more than one-fifth of these genes are specifically induced during developmentally triggered senescence (Buchanan-Wollaston et al. 2005; Olsen et al. 2005). *AtNAP*, coding for a NAC family transcription factor, is highly expressed in senescing leaves and loss-of-function mutants of this gene display significantly delayed leaf senescence (Guo et al. 2004; Guo and Gan 2006).

*At*WRKY proteins, comprising 74 members, constitute the second largest group of transcription factors in the senescence transcriptome (Guo et al. 2004). WRKY proteins contain one or two domains of a conserved peptide stretch of about 60 amino acids, designated the WRKY domain, encompassing a Zn-finger motif (Ülker and Somssich 2004). The WRKY domain shows high binding affinity to a distinct *cis*-acting DNA element termed the W Box (T/CTGACC/T), although altered binding preferences have also been observed (Ülker and Somssich 2004). Specific WRKY family members show altered expression in various tissues and organs as well as in response to a wide range of pathogens, pathogen-mimicking stimuli and different abiotic stresses suggesting a role of WRKY factors both in plant defense and plant developmental programs (Ülker and Somssich 2004). *AtWRKY6* was shown to be strongly up-regulated during the progression of leaf senescence and also by treatments with salicylic acid (SA), jasmonic acid (JA) and ethylene (ET; Robatzek and Somssich 2001). Analyses of *At*WRKY6 target genes identified *Senescence-Induced Receptor Kinase* (*SIRK* = *FRK1*) encoding a signaling component also identified in the *FLS2*-dependent defense pathway (Ülker and Somssich 2004). Overexpression, RNAi or knock-out lines of another gene, *AtWRKY53*, showed accelerated and delayed senescence phenotypes, respectively (Miao et al. 2004). However, the potential functions of the majority of the leaf senescence-associated *AtWRKY* transcription factors remain to be elucidated.

Here we report the functional analysis of *AtWRKY70* exhibiting high expression levels in senescing leaves. The developmental- and dark-induced leaf senescence phenotypes of two independent *Atwrky70* T-DNA insertion lines were analyzed as was the role of *AtWRKY70* on SA- and JA/ET-dependent defense-related genes during leaf senescence. In addition, the expression of *AtWRKY70* was studied in transgenic plants and mutants defective in SA- and JA/ET-mediated signal transduction pathways.

Materials and methods

Plant material and growth conditions

Arabidopsis seeds were sown in plastic pots (diameters 8 × 8 × 8.5 cm) with Minitray soil (Gebr. Patzer, Sinntal-Jossa, Germany), incubated for 3 days at 2–5°C, subsequently transferred to a phytochamber (Heraeus Voetsch, Balingen, Germany) and grown under 12 h light/12 h dark photoperiod and PFD of 80 mmol photons m^{-2} s^{-1} at 22°C and 60% relative humidity. The mutants *cpr*5 (N3770), *npr*1 (N3726), *jar*1 (N8072), *ein*2 (N3071), *ctr*1 (N8057) were obtained from the Nottingham *Arabidopsis* Stock Centre. The mutant *aos* (knock-out line #1,180 from the T. Jack T-DNA lines collection, Columbia−6 background) was supplied by Ken Feldmann (Ceres Inc., Malibu, CA, USA), *ndr*1, *edr*1, and transgenic line expressing *NahG* gene were obtained from Paul Schulze-Lefert (Max-Planck Institute for Plant Breeding Research, Cologne, Germany), and *pad*4 was kindly provided by Jane Parker (Max-Planck Institute for Plant Breeding Research, Cologne, Germany). All plants were derived from the Columbia-0 ecotype unless mentioned. The *Atwrky70-1* insertion mutant SALK_025198 was obtained from the *Arabidopsis* Resource Center whereas *Atwrky70-2* corresponds to GABI-Kat line 752F08 (Rosso et al. 2003).

Construction of plasmids and generation of transgenic lines

DNA sequences of 1.9 kb *AtWRKY70* upstream regulatory region, and the first 87 bp of exon I were amplified from Columbia-0 genomic DNA using primers *AtWRKY70-Pr-F*: 5′ (*GWF*) *tacacaaccactaaactttacggc*3′ and *AtWRKY70-Pr-R*: 5′ (*GWR*) *cgaggagttgctgaagctg agttgt*3′, and cloned into *pDONR*207 Gateway® vector (Invitrogen). The Entry clone was recombined into the Gateway®-compatible binary expression vector

pJawohl11 (a derivative of pBIN19) to generate a translational promoter-GUS fusion construct. To generate $P_{AtWRKY70}$*::GUS* transgenic lines, *Arabidopsis* Col-0 plants were transformed via *Agrobacterium tumefaciens* strain GV3101 carrying the *pMP90RK* plasmid (Koncz and Schell 1986) and the appropriate construct using the inflorescence dipping method of Clough and Bent (1998). The histochemical assay to monitor for GUS activity in $P_{AtWRKY70}$*::GUS* transgenic plants was carried out according to the method described by Jefferson et al. (1987).

Full length *AtWRKY70* cDNA (sequence deposited in Genbank under the accession number AF421157) was generated by RT-PCR starting from RNA derived from floral tissue of Columbia-0 ecotype and amplification of the cDNA with the primers *AtWRKY70-F*: 5′(*GWF*) *taatggatactaataaagcaaaaaagc*3′ and *AtWRKY70-R*: 5′ (*GWR*) *cagatagattcgaacatga actgaag*3′. The cDNA was cloned into the *pDONR201* Gateway® vector (Invitrogen). The *AtWRKY70-GFP* expression construct was obtained by recombining the *pENTR201 AtWRKY70* clone into the Gateway®-compatible binary vector *pAM-Kan 2x35S::GW-GFP*, a pPAM (GenBank accession AY027531) derivative.

The universal Gateway–compatible extensions for the BP recombination reactions (between an *att*B-flanked PCR product and a donor vector containing *att*P sites to create an entry clone) were: *GWF* (*att*B1) 5′*ggggacaagtttgtacaaaaaagcaggctta*3′, *GWR* (*att*B2) 5′*gg ggaccactttgtacaagaaagctgggtc*3′.

DNA sequences were determined by the DNA core facility (ADIS) of Max-Planck Institute for Plant Breeding Research on Abi Prism 377, 3100 or 3730 sequencers (Applied Biosystems, Weiterstadt, Germany) using BigDye-terminator v3.1 chemistry. Premixed reagents were from Applied Biosystems.

Subcellular localization studies

Peels of onion (*Allium cepa* L.) epidermal cell layers were placed inside up on Murashige and Skoog (MS) plates. The expression construct *pAM-Kan 2x35S::AtWRKY70-GFP* and control construct *pAM-Kan 2x35S::GFP-GFP* (0.6 mg of DNA) were introduced into onion cells using a pneumatic particle gun (Biorad). The conditions for bombardments were: vacuum of 28 in. Hg, helium pressure of 1,100 or 1,300 psi, and 6 cm of target distance using gold microcarriers. After bombardment, the peels were incubated on the MS plates for 24 h at 22°C. Samples were transferred to glass slides and observed under a fluorescence microscope (Leica MZ12 with Mercury HBO 50 W/Ac lamp and FITC filter).

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg of fresh, healthy *Arabidopsis* leaf tissue. The tissue was flash-frozen and ground in liquid nitrogen. Total RNA was extracted with 1 ml RNAwiz extraction reagent (Ambion, Huntingdon, Cambridgeshire, UK) following the supplier's protocol. DNase I treatment was performed using the DNA-*free* reagent (Ambion) for 20 min at 37°C, and reaction composition was as suggested by the producer. First strand cDNA was synthesized using 2.5 μg of RNA, SuperScript II RNase (Invitrogen) and Oligo dT_{19} RT (Ambion) following manufacturers' instructions.

RT-PCR, RNA-blot analyses and DNA labeling

For RT-PCR analyses, 2 μl of the first strand cDNA was used as template and reactions were standardized using primers specific to *Arabidopsis tubulin* β-subunit (At5g44340) (F: 5′*acgtatcgatgtctatttcaacga*3′ and R: 5′*at atcgtagagagcctcattgtcc*3′). Subsequent PCRs were performed as indicated in the figures for various numbers of cycles under the following conditions: 94°C for 15 s, 60°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min using the following primer sets: *PR1* (At2g14610), F: 5′*tcgtctttgtagctcttgtaggtg*3′ and R: 5′*ttcattagtatggcttctcgttca*3′; *PR2* (At3g57260), F: 5′*ccaccaatgttgatgattcttct*3′ and R: 5′*atactcatccctgaaccttc ctt*3′; *PR3* (At3g12500), F: 5′*tcgaattttgatttcacactgttc*3′ and R: 5′*ctgtagtttcatgggaagtctggc*3′; *PDF1.2* (At5g44420), F: 5′*taatcatcatggctaagtttgctt*3′ and R: 5′*atacacacgatttagcac caaaga*3′; *COR1* (At1g19670), F: 5′*gttacattcttgtagccccac ag*3′ and R: 5′*tttctttgcccattcttacacat*3′; *SEN1* (At4g35770), F: 5′*ccactgcttttaacacaacatca*3′ and R: 5′*agcagtgagaagat cagttgagg*3′; *SEN2* (At1g20620), F: 5′*ccagtctccaacaacatctc ttc*3′ and R: 5′*atatccctcatggtttcaggact*3′; *SEN4* (At4g30270), F: 5′*ttcttcttcacgactcttctcgt*3′ and R: 5′*gtggaaacctcctatggtc agta*3′; *SRG1* (At1g17020), F: 5′*gttgagaaactcgacttcgc*3′ and R: 5′*gatcttagctacgctctgcac*3′; *SRG2* (At3g60140), F: 5′*gacagctgcctcggcgtttcag*3′ and R: 5′*gttaccttgatcataaccccgc*3′, *SAG29* (At5g13170), F: 5′*gatcaatcaccatttcctcgc*3′ and R: 5′*acgaatcccaccacgtttgg*3′; *SAG12* (At5G45890), F: 5′*gta ccaaaacgtttcttctggtg*3′ and R: 5′*ccatgaattcttgatgatccaat*3′; SAG24 (At1g26910), F: 5′*ggaaagccataccctaaatcaag*3′ and R: 5′*aggatatcaactccacatcgaca*3′; *CAB* (At3g27690), F: 5′*gtcaagtctactcctcagagc*3′ and R: 5′*cttccccaagagggaccacc tc*3′; *pectate lyase* (At5G63180), F: 5′*cgtctttgcccgtttccgacc*3′ and R: 5′*attgcatcaattaacccgtcg*3′; *EXGT* (At4G37800), F: 5′*acttgttcggccgtgtgagc*3′ and R: 5′*caatcatcggcttcccataacg*3′; *GST* (At5G17220), F: 5′*tctctctacttcaataaatctccacc*3′ and R: 5′gagtttatgagaattgggcaattag3′; *SIRK* (At2g19190), F: 5′*cat cgattttattcacaagctttgc*3′ and R: 5′*ttctgaactcactatacgcggtgtc*3′; *RPS17* (At1g79850), F: 5′*ctctctctaagcccaattccttc*3′ and

R: 5′*tcagattgaaaacacactcatgg*3′; *UBP8* (At5g22030), F: 5′*taccagaggaggagactttattgg*3′ and R: 5′*ttcaacatttcagcttct atcgag*3′.

Northern blotting and radioactive labeling of the probe was performed using standard molecular procedures and random prime labeling. The *At*WRKY70 cDNA probe was PCR-amplified using the *WK70-F* and *WK70-R* primers. Wherever possible, the primers used for RT-PCR were chosen such that DNA contaminations of the cDNA samples would yield fragments of a larger size due to the presence of intron sequences.

Dark-induced leaf senescence

Leaves or entire aerial parts of 2-week-old *Arabidopsis* plants were excised (seedlings were excised by cutting the hypocotyl approximately 5 mm below the cotyledons) and placed into microtiter plates filled with sterile water allowing good water contact of leaf pedicels and hypocotyls of seedling. The plates were kept in darkness at 22°C for 3–4 days. For intact plant senescence experiments, 2-week-old soil grown plants were transferred from the growth room to a container covered with a black box and placed in a dark room for 5–8 days.

Results

AtWRKY70 is up-regulated during developmentally induced leaf senescence

We took advantage of publicly available microarray data to gain information on the temporal and spatial expression patterns of *AtWRKY70* (At3g56400) in specific organs at various developmental stages during the entire life cycle of *Arabidopsis* (Zimmermann et al. 2004; Schmid et al. 2005). Expression of *AtWRKY70* was not detected in the embryo, in meristematic tissue, in roots, flower buds, petals, carpels, stamen, pollen grains or siliques. However, *AtWRKY70* was constitutively expressed during all leaf development stages with highest signal intensity found in senescing leaves. Additionally, *AtWRKY70* was also found to be expressed in floral abscission zones and flower sepals.

To substantiate and to extend on the expression data, we generated *AtWRKY70* promoter GUS reporter lines ($P_{AtWRKY70}$::*GUS*) expressing a translational fusion construct comprising 1.9 kb of *AtWRKY70* upstream regulatory region, the first 87 bp of *AtWRKY70* exon I and the GUS reporter gene. Tissue-specific *AtWRKY70* expression at different developmental stages was determined in 3-day-old etiolated seedlings, 15-day-old light-grown seedlings, 30-day-old plants, and in leaves and inflorescences from soil-grown 42-day-old mature plants. Consistent with the microarray data, GUS activity staining revealed a continuous increase in GUS activity staining during progression of leaf development. The *AtWRKY70* gene was expressed at high levels throughout most stages of leaf growth but increases gradually up to the point of leaf senescence (Fig. 1a, panel 1). Moreover, GUS activity staining was also detected in various floral organs such as stigmatic papillae and the flower abscission zone (Fig. 1a, panels 2–5). However, at later stages of flower development, GUS activity staining was no longer detected in stigmatic papillae, whereas it remained persistent in the abscission zone (Fig. 1a, panel 2). Thus, *AtWRKY70* expression patterns suggest a possible function during senescence of leaves and flowers but also for a need of *At*WRKY70 at earlier stages of leaf development.

*At*WRKY70 targets to the nucleus

The *At*WRKY70 protein is predicted with high probability to be targeted to the nucleus (PENCE PA-SUB, 100%; PSORT, 98%). To test this prediction, we transiently transfected an expression construct, *2x35S*::*At*WRKY70-GFP, encoding a full-length *At*WRKY70 protein fused to GFP into onion (*Allium cepa* L.) epidermal cells. As a control, a construct expressing the GFP reporter (*2x35S*::GFP) was included. Microscopic analysis revealed that at all time points tested (6, 10, and 24 h post transfection) *At*WRKY70-GFP was solely visualized in the nucleus confirming that *At*WRKY70, like other WRKY family members, resides almost exclusively within the nucleus (Fig. 1b). In contrast, *2x35S*::GFP expression resulted in detection of the protein throughout the cells.

Analysis of two *Atwrky70* T-DNA insertion lines

To gain deeper insight into the function of *AtWRKY70 in planta*, we obtained two independent insertion mutants, *Atwrky70-1* (SALK_025198) and *Atwrky70-2* (GABI_752F08), with T-DNAs located within the first intron and third exon of the *AtWRKY70* gene, respectively (Fig. 2a). In both cases, the presence of the T-DNA was confirmed by PCR amplification of specific bands using primer pairs derived from the *AtWRKY70* gene and the left border of the T-DNA. Homozygous *Atwrky70* knock out lines were isolated based on the absence of *AtWRKY70* gene specific PCR products (Fig. 2b). Moreover, both homozygous *Atwrky70* insertion lines showed no detectable *AtWRKY70* transcript

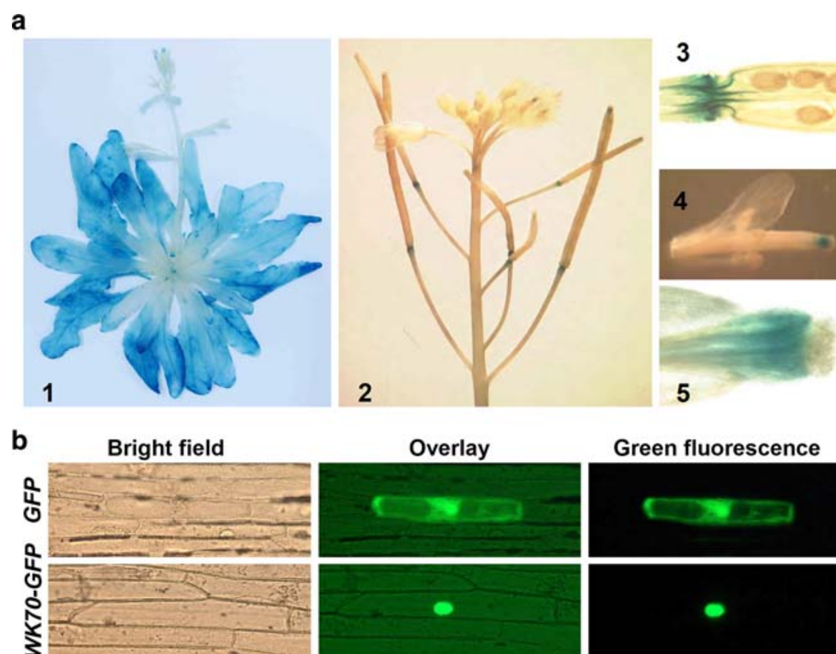

Fig. 1 **a** Expression analysis of a representative $P_{AtWRKY70}$*::GUS* transgenic plant. *Part 1* GUS-activity staining in a 42-day-old plant; *part 2* GUS-activity staining in total inflorescences; *part 3* close-up view of GUS-activity staining detected within a floral organ abscission zone; *parts 4* and *5* GUS-activity staining observed in the residual stigmatic papillae (close-up in e) at later stages of flower development. **b** Nuclear localization of *At*WRKY70. Transient expression of *2x35S::AtWRKY70-GFP* in onion epidermal cells. Bright field images of the respective onion epidermal cells are shown to the *left*. *At*WRKY70-GFP is visible solely in the nucleus (*lower right*), whereas the control GFP-GFP protein is present throughout the entire cell (*top right*). *Middle column* shows bright field image overlays with those obtained using a GFP filter

(Fig. 2c) and thus very likely represent complete loss-of-function mutants. During the entire period of development, the two *Atwrky70* knock-out mutants were slightly reduced in size compared to wild-type plants (Fig. 2d, and data not shown).

Loss of *AtWRKY70* function promotes both developmentally and dark-induced leaf senescence

We investigated the role of *AtWRKY70* in leaf senescence by analyzing both the developmentally induced and dark-induced senescence programs. The *Atwrky70* insertion lines and wild-type plants were grown under standard greenhouse or phytochamber conditions to monitor natural senescence. Under the tested conditions, the *Atwrky70* loss-of-function mutants showed markedly earlier senescence compared to wild-type plants at 6–7 weeks post germination. The wild-type plants displayed significant accumulation of red anthocyanin pigments but moderate leaf yellowing; both considered the hallmarks of the early leaf senescence stages. On the contrary, *Atwrky70* mutant plants exhibited severe yellowing of leaves, resulting from intensified degradation of chlorophyll, which is indicative of an advanced leaf senescence phase (Fig. 3a). However, bolting and flowering times were nearly identical between the *Atwrky70* mutants and wild-type plants revealing that the earlier appearance of leaf yellowing in *Atwrky70* mutants plants was not the consequence of a generally accelerated ontogenesis, but rather occurred due to an earlier onset of the developmentally induced senescence program.

We next examined dark-induced senescence, a procedure that is commonly used to artificially trigger senescence (Weaver and Amasino 2001). Detached leaves or entire aerial parts of 2-week-old soil grown plants of the two independent *Atwrky70* insertion lines and wild-type plants were subjected to darkness for 3–4 days. Within this time period, leaves of the *Atwrky70* mutants showed severe yellowing and cell death while leaves of wild-type plants only became pale green (Fig. 3b). Additionally, we also examined dark-induced leaf senescence of intact plants and observed that leaves of both *Atwrky70* mutants senesced more rapidly than wild-type leaves when kept in the dark for 5–6 days (data not shown) again pointing towards a function of *AtWRKY70* in leaf senescence.

Leaf senescence is accompanied by decreased expression of genes related to photosynthesis and protein synthesis and increased expression of senescence-associated genes (SAGs), with different leaf

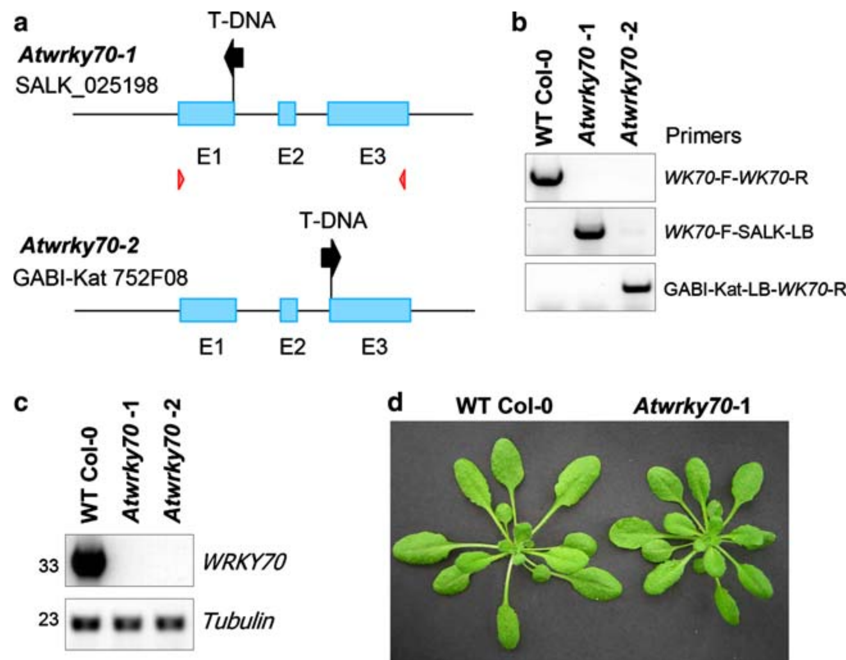

Fig. 2 Identification and characterization of homozygous *Atwrky70* T-DNA insertion alleles. **a** Schematic representation of the T-DNA insertion sites within the *AtWRKY70* locus. The three exons (marked *E1*, *E2* and *E3*) are highlighted in *blue*. Positions of the T-DNA insertions are marked by *black arrows* with the arrowheads pointing in each case towards the left border site within the T-DNA. **b** Verification of homozygous *Atwrky70* mutant lines. Gene-specific and respective T-DNA-specific primers were used to detect the presence of the wild-type and mutant *AtWRKY70* alleles in genomic DNA from representative *Atwrky70-1* and *Atwrky70-2* homozygous plants, and from wild-type control. Origin of the genomic DNA is shown at the *top* whereas the primer combinations used for PCR analysis are given to the right. **c** The *AtWRKY70* T-DNA insertion lines appear to be complete loss-of-function mutants. RT-PCR was performed with *AtWRKY70* gene-specific primers and the respective DNA templates as indicated above each lane. *AtWRKY70* transcript could not be detected in 30-day-old plants homozygous for the T-DNA insertions but was clearly detected in wild-type Col-0 control plants. Expression of *Tubulin* was used as a loading control. Numbers of RT-PCR cycles performed are indicated to the left. **d** *AtWRKY70* insertion lines are somewhat smaller than wild type plants. Shown is a comparison between WT and *Atwrky70-1*

senescence stages classified based on the expression patterns of specific marker genes (Oh et al. 1997; Yoshida et al. 2001). To determine the effect of *At*WRKY70 on senescence signaling, we measured transcript levels of several senescence-associated genes in the *Atwrky70* mutant and wild-type plants using semi quantitative RT-PCR. These included genes related to oxidative stress such as *SRG1* (oxidoreductase), *GST* (glutathione S-transferase) and *SEN2* (catalase 3), genes related to cell wall degradation, breakdown of macromolecules and transport to other plant parts such as *SRG2* (=*DIN2*; *dark-inducible gene 2*, glycosyl hydrolase), pectate lyase, *EXGT* (xyloglucan:xyloglucosyl transferase), and *SEN4* (=*MERI-5*, endo-xyloglucan transferase), genes related to protein synthesis and degradation such as *SAG12* (senescence-specific cysteine proteinase), *RPS17* (chloroplast ribosomal protein S17) and *SAG24* (60S ribosomal protein L10), genes related to the progression of photosynthesis such as *CAB* (chlorophyll *a/b* binding protein), genes related to signaling such as *SIRK* (senescence-induced receptor-like kinase) and genes with unknown functions but shown to be associated with senescence such as *SAG29* (nodulin MtN3 family protein) and *SEN1*, an early indicator of natural and dark induced senescence.

Initially, we examined the expression of senescence-associated genes assumed to be altered in 40-days-old plants showing early signs of senescence. At this stage, plants have bolted and some of the older rosette leaves showed yellowing on their edges. The *Atwrky70-1* mutant plants had more of such yellowing leaves. As expected, the expression of *CAB*, *pectate lyase*, and *EXGT* were down-regulated and the expression of *GST*, *SRG1*, *SRG2* and *SIRK* were up-regulated in the *Atwrky70-1* mutant compared to the wild-type plants (Fig. 4a). The expression of *SAG29* was slightly lower in *Atwrky70-1* than in wild-type plants.

To determine if *At*WRKY70 plays a role in the initiation of senescence we monitored the expression of several genes, including some early senescence-associated marker genes, in 30-days-old *Atwrky70-1 and Atwrky70-2* mutant and wild-type plants showing no visible signs of senescence. To exclude any bias in the selection of individual leaves for sampling and avoid

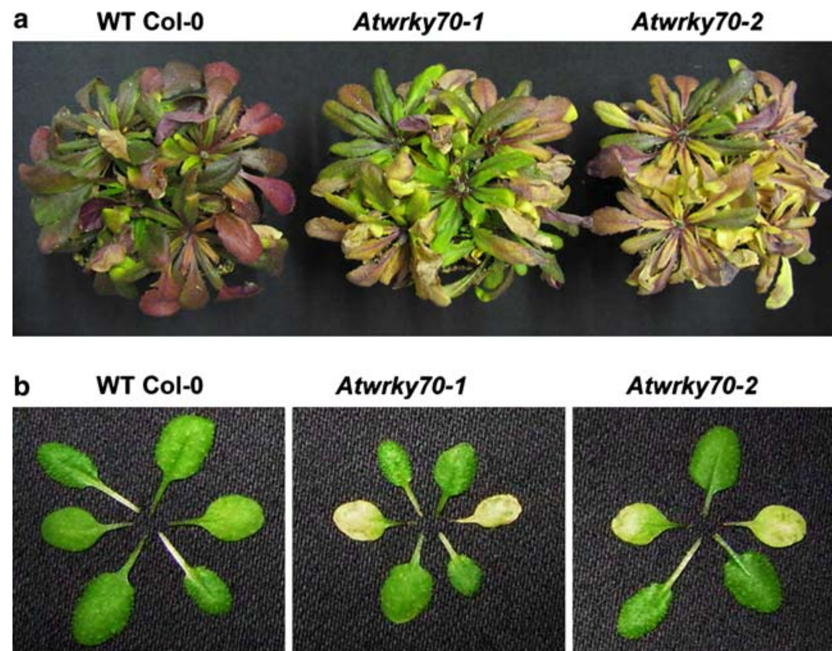

Fig. 3 Senescence phenotype of *Atwrky70* mutant plants. **a** Progression of natural senescence in *Atwrky70* mutants and wild-type plants grown under controlled conditions. The number of yellow and necrotic leaves is higher in 7-week-old *Atwrky70-1* and *Atwrky70-2* mutant plants compared to wild-type due to accelerated developmental senescence. Inflorescenses were removed for clarity of the senescence phenotype. **b** Dark-induced senescence phenotype of *Atwrky70* mutant plants. Aerial parts of 2-weeks-old wild type and *Atwrky70* mutants plants grown under long day condtions in the phytochamber were excised by cutting their hypocotyls 5 mm below their cotyledons. The excised plantlets were transferred to microtiter wells containing water and kept under dark conditions for 3, 4 days. For clarity, the leaves were dissected from the seedlings before being photographed

expression differences resulting from wounding we chose to harvest the entire aerial parts of the plant. No differences in the expression of some late senescence-associated genes (*pectate lyase*, *EXGT*, *SAG12*, and *SAG24*) were observed between wild-type plants and the *Atwrky70-1* mutant (Fig. 4b), but in *AtWRKY70-2* the expression of *SAG12* was lower and that of *pectate lyase* was somewhat higher. Since expression of the *pectate lyase* gene is downregulated and that of *SAG12* is upregulated during progression of senescence (compare with Fig. 4a), one possible explanation for this observed difference was that the physiological states of the two mutants at sampling time were not completely identical. Alternatively or in addition, the two loss-of-function alleles may differ somewhat in their rates of leaf developmental progressions (Fig. 4b). The expression of *SAG29* was again slightly lower in the *Atwrky70* mutants. On the other hand, higher transcript levels of *SEN1*, *SEN2* and *SRG1* were observed in the leaves of the *Atwrky70* mutants compared to leaves of wild-type plants (Fig. 4b). No differences in expression were detected for *SEN4*. Together these data show that loss-of-*AtWRKY70* function influences the expression levels of certain senescence-associated markers genes even at early stages of the leaf senescence program.

AtWRKY70 represses both SA and JA/ET mediated defense marker genes expression

The regulatory network governing leaf senescence has substantial crosstalk with plant defense signaling pathways (Grbic and Bleecker 1995; Morris et al. 2000; He et al. 2002). In *Arabidopsis*, at least three genetically distinguishable defense pathways are characterized: the SA-, JA- and ET-mediated resistance pathways (Glazebrook 2005). The levels of these signaling molecules increase during senescence and can modulate the expression of specific downstream genes (Morris et al. 2000; Buchanan-Wollaston et al. 2005). SA also induces the expression of pathogenesis-related (*PR*) genes encoding the acidic isoforms of proteins such as *PR1*, *PR2*, *PR3*, and *PR5* as well the transcriptional regulator *NPR1* (*non-expressor of PR1*; Ward et al. 1991). Moreover, JA/ET activate the expression of *plant defensin1.2* (*PDF1.2*), and *coronatine resistant 1* (*COR1*; Benedetti et al. 1998; Glazebrook 2005). To determine the activation state of the SA, JA and ET pathways in the *Atwrky70* mutants during senescence, the steady-state transcript levels of *NPR1*, *PR1*, *PR2*, *PR3*, *PR5*, *COR1*, and *PDF1.2* were evaluated and compared to that of wild-type plants 30 days post germination. Basal expression levels of the SA-responsive

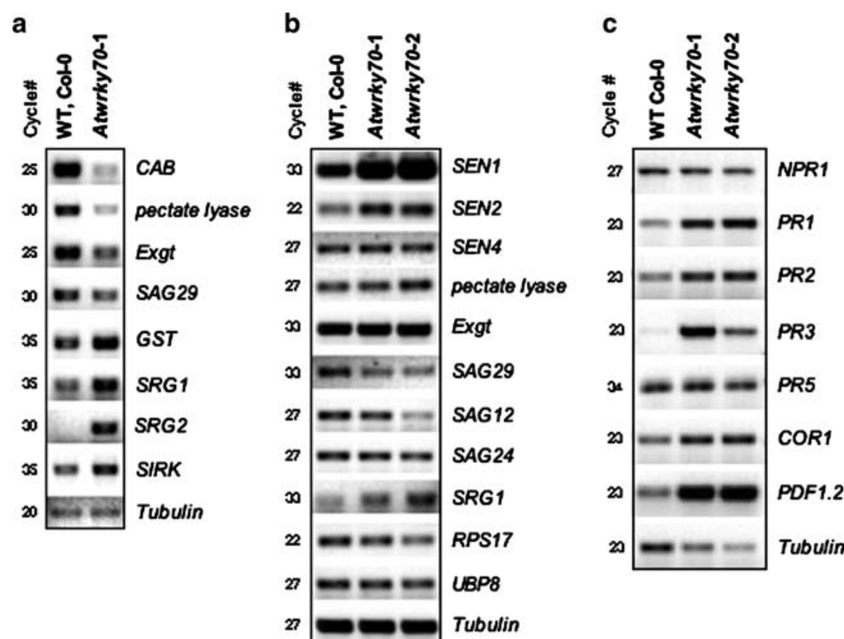

Fig. 4 **a** Early senescence symptoms in the *Atwrky70-1* mutant correlate with transcriptional up-and down-regulation of genes associated with senescence. Semi-quantitative RT-PCR analyses of 40-days-old Col-0 wild-type and *Atwrky70-1* mutant plants using senescence-related marker genes (indicated to the *right*). Number of RT-PCR cycles are given to the *left*. Ten plants were sampled by punching out 10 mm diameter leaf disks from the seventh oldest leaf, counting from the first true leaf, of each plant grown in the phytochamber. RNA was isolated from the pooled samples. *CAB* chlorophyll *a*, *b* binding protein coding gene, *EXGT* xyloglucan:xyloglucosyl transferase, *SAG29* senescence-associated gene 29, *GST* glutathione S-transferase gene, *SRG1* senescence-related gene 1, *SRG2* senescence-related gene 2 (=DIN2), *SIRK* senescence-induced receptor-like kinase. **b** Expression of genes associated with early senescence are elevated in *Atwrky70* mutant plants. RT-PCR analyses on 30-day-old Col-0 wild-type and *Atwrky70* mutant plants using senescence-related marker genes (indicated to the *right*). cDNA templates used in each reaction are labeled above each *lane*. Number of RT-PCR cycles are given to the *left*. *SEN1* senescence-associated gene 1, *SEN2* senescence-associated gene 2, *SEN4* senescence-associated gene 4, *EXGT* xyloglucan:xyloglucosyl transferase, *SAG29* senescence-associated gene 29, *SAG12* senescence-associated gene 12, *SAG24* senescence-associated gene 24, *SRG1* senescence-related gene 1, *RPS17* chloroplast ribosomal protein coding gene, *S17*, *UBP8* ubiquitin-specific protease coding gene 8. **c** The basal expression levels of some pathogen-responsive marker genes are elevated in *Atwrky70* mutant plants. Transcript levels of *NPR1*, *PR1*, *PR2*, *PR3*, *PR5*, *COR1* and *PDF1.2* in wild-type, *Atwrky70-1* and *Atwrky70-2* plants were detected by RT-PCR. *NPR1* non expressor of PR1, *PR1* pathogenesis related 1, *PR2* pathogenesis related 2, *PR3* pathogenesis related 3, *PR5* pathogenesis related 5, *COR1* coronatine resistant 1, *PDF1.2* plant defensin1.2. The number of RT-PCR cycles performed are given to the *left*. The expression levels of *tubulin* (At5g44340) and *UBP8* (At5g22030) ubiquitin-specific protease eight were used as loading controls for **b** and **c**. RNA derived from entire aerial plant organs was isolated from pooled samples of six plants each grown in a phytochamber

PR1, and *PR2* genes, the JA/ET-responsive defense marker genes *COR1* and *PDF1.2*, as well as that of *PR3* which responds to both stimuli, were significantly elevated in the *Atwrky70* mutant plants when compared to wild-type (Fig. 4c). In contrast to this, no major changes were observed in the expression of *NPR1* and *PR5*. The accelerated senescence of the *Atwrky70* mutants may in part be the consequence of deregulated expression of some defense genes resulting in an elevated stressed-state of such plants.

To gain additional insight into the nature of the signals that are common to plant defense and the *Atwrky70*-mediated early senescence phenotype, we studied the expression patterns of *AtWRKY70* in different mutants and transgenic plant lines affected in known host defense signal transduction pathways. The SA hyper-accumulating mutant *cpr5* (*constitutive PR gene expressor 5*) also showed early senescence in older leaves (Yoshida et al. 2002b). *NahG* transgenic plants failed to accumulate SA, whereas *pad4* (*phytoalexin-deficient 4*) and *npr1* (*non-expressor of PR 1*) were defective in SA signaling. All of these SA mutants exhibited a delayed senescence phenotype (Morris et al. 2000). It is known that JA application induces premature senescence and that endogenous jasmonate levels increase fourfold to fivefold during plant senescence (He et al. 2002). The *aos* (*allene oxide synthase*) and *jar1* (*jasmonic acid resistant 1*) mutants are impaired in JA biosynthesis or its activation, respectively (Park et al. 2002; Glazebrook 2005). The ET-dependent pathway mutant *ein2* (*ethylene insensitive 2*) also shows an early senescence phenotype (Oh et al.

1997). The basal *AtWRKY70* transcript levels were unaltered in the *aos*, *jar1* and *ein2* mutants indicating that both JA- and ET-dependent signaling are not required for *AtWRKY70* expression during developmental senescence. Contrastingly, *AtWRKY70* transcript levels were slightly reduced in the *cpr5* mutant, more strongly reduced in *npr1* and *pad4* and completely abolished in *NahG* plants compared to wild-type at 40 days post germination (Fig. 5a). Thus, functional SA signaling pathway is required for *AtWRKY70* expression during leaf senescence. Since overexpression or antisense suppression of *AtWRKY70* caused no major alterations in endogenous levels of free SA, JA and ET in *Arabidopsis* leaves (Li et al. 2004), the *Atwrky70*-mediated early senescence phenotype observed here was not a consequence of changes in the balance of these hormones.

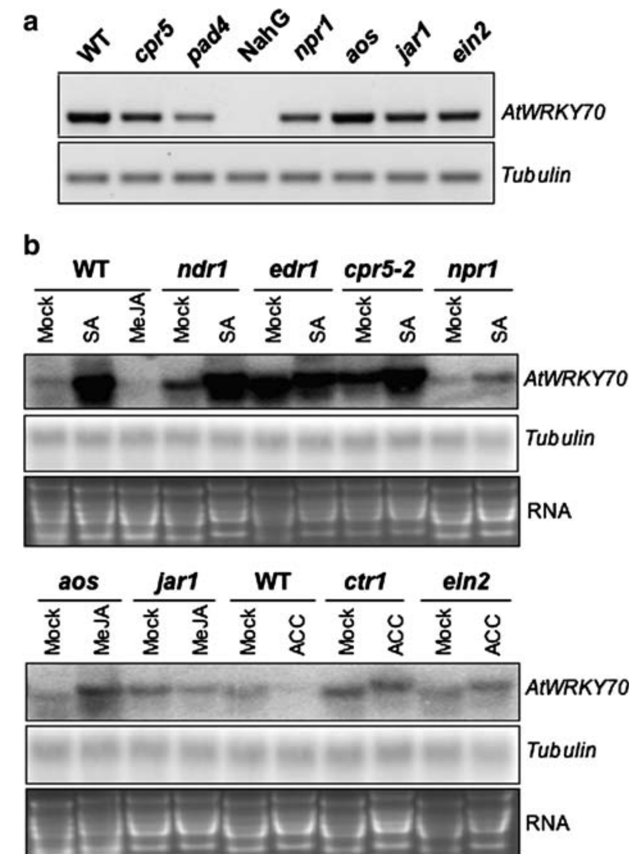

Fig. 5 **a** Endogenous *AtWRKY70* transcript levels in various defense signaling mutants. Comparative basal *AtWRKY70* transcript levels observed in the indicated *Arabidopsis* mutants impaired in different defense signaling pathways. *cpr5*, *pad4*, *NahG* and *npr1* define the SA dependent pathway, *aos* and *jar1* the JA-dependent pathway and *ctr1* and *ein2* the ET-dependent pathway. PCR on genomic DNA showed no DNA contamination (data not shown). *Tubulin* was used as loading control. **b** Expression of *AtWRKY70* in various defense signaling mutants upon different treatments. RNA samples were prepared from leaves of 22-day-old wild-type or mutant plants as indicated. After electrophoretic separation on the gel and blotting onto nylon membranes, the blot was probed with an *AtWRKY70* specific DNA fragment. A *tubulin* probe and ethidium bromide stained ribosomal RNA bands were used to monitor equal RNA loading of each *lane*. Wild-type and mutant plants are indicated at the *top* and *underlined*, and the respective treatments are given for each *lane*. *SA* salicylic acid, *MeJA* methyl jasmonate, *ACC* 1-aminocyclopropane-1-carboxylic acid

We next analyzed *AtWRKY70* transcript levels in 22-days-old mutant plants impaired in components of various defense signaling pathways upon specific treatments. Moreover, we expanded the array of mutants by including *ndr1* (*non-race-specific disease resistance 1*), compromised in the activation of CC-NB-LRR class of resistance proteins, *edr1* (*enhanced disease resistance 1*), conferring elevated resistance to powdery mildew disease and *ctr1* (*constitutive triple response1*) exhibiting constitutive activation of the ethylene signaling pathway (Kieber et al. 1993; Century et al. 1995; Frye et al. 2001). The mutants *ndr1*, *edr1*, *cpr5* and *npr1* were treated with SA, *aos* and *jar1* were treated with methyl jasmonate (MeJA) and *ctr1* and *ein2* were treated with the natural precursor of ET 1-aminocyclopropane-1-carboxylic acid (ACC). The endogenous basal and stimulus-dependent transcript levels of *AtWRKY70* were not significantly affected by a 4 h treatment with MeJA or ACC either in wild-type plants or in the mutants impaired in JA/ET signaling strongly suggesting that the JA/ET signaling transduction pathways are not required for *AtWRKY70* expression (Fig. 5b). Note that the slight reduction of *AtWRKY70* transcript levels in ACC-treated wild-type plants was not consistently reproducible. In contrast to MeJA or ACC treatments, *AtWRKY70* displayed a strong increase in expression upon treatment with SA. Basal expression of *AtWRKY70* was elevated in *ndr1* and *cpr5* mutants, but could be induced to higher levels upon SA application supporting the hypothesis that SA signaling positively affects *AtWRKY70* expression (Fig. 5b). In contrast, high constitutive levels of *AtWRKY70* transcript were observed in the *edr1* mutant with no significant changes detected upon SA treatment. The reduction in *AtWRKY70* transcript levels in the *npr1* mutant before or after SA treatment (Fig. 5b) is consistent with previous reports (Li et al. 2004). Collectively, our results support the role of *AtWRKY70* as a senescence-associated gene and indicate a functional requirement of SA for its normal expression.

Discussion

Over the past decade our knowledge on the molecular events occurring during leaf senescence has expanded

significantly (Buchanan-Wollaston et al. 2003). Moreover, global expression data revealed a common subset of genes, many encoding transcription factors, that are expressed both during developmentally and artificially induced senescence (Lin and Wu 2004; Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006). These findings were key steps forward in identifying potential transcriptional regulators modulating signal transduction pathways during the onset and progression of senescence. Our data on *AtWRKY70* adds additional evidence that this individual transcription factor plays an important regulatory role in both of these processes and possibly also in the abscission of floral organs.

Leaf senescence can also be induced by several biotic and abiotic stresses (Buchanan-Wollaston et al. 2003) for which global transcript profiling data is available (Zimmermann et al. 2005). Heat, darkness and UV-B radiation are potent inducers of premature senescence that cause loss of chlorophyll and an increase in lipid damage, mimicking natural senescence and resulting in the regulation of senescence-associated genes (John et al. 2001; Swidzinski et al. 2002). *AtWRKY70* is strongly down-regulated by these treatments which is consistent with *Atwrky70* mutants' early senescence phenotypes. In contrast, cycloheximide (CHX), an inhibitor of RNA-directed protein synthesis and senescence progression (Bieleski and Reid 1992) strongly induces *AtWRKY70* expression levels. This strong activation of *AtWRKY70* in the presence of CHX also indicates that this gene is very likely under negative regulatory control by a factor with a short half-life.

The concomitant changes in the transcriptional activities of several genes, especially the up-regulation of genes associated with early senescence such as *SEN1, SEN2* and *SRG1* in the *Atwrky70* mutants, lends further support for a role of *AtWRKY70* in partly regulating critical steps within the process of leaf senescence. *SEN1* is known to be up-regulated in leaves during the initial stages of dark-, ethylene-, or ABA-induced senescence (Oh et al. 1996). *SEN1*, *SEN2*, *SEN4* and *SRG1* contain numerous W box elements, the cognate binding sites for WRKY transcription factors. Thus, *At*WRKY70 may play a role in the regulation of these genes either directly or by activating other *WRKY* genes. In fact, the *SEN1* promoter has eight W box motifs (TGACC/T or C/TTGAC) five of which are extended W box elements (C/TTGACC/T). A similar situation is found in the promoters of *SEN2*, (7 W boxes, 2 of which are extended W box elements), of *SEN4* (6 W box motifs of which 3 are extended W boxes), and of *SRG1* (11 W boxes of which 3 are extended W-box elements). Except for *SEN4*, all of these genes were up-regulated in the *Atwrky70* mutant plants.

Since senescence-associated genes are shown to accumulate in plant tissues not only during various phases of senescence but also during another form of PCD termed the hypersensitive response (HR; an active defense strategy against numerous pathogens), a significant overlap in gene transcription between these two physiological processes likely exists (Maleck et al. 2000; Quirino et al. 2000; Schenk et al. 2005). Indeed, *AtWRKY70* expression is also altered in response to various pathogens including the hemibiotrophic bacterium *Pseudomonas syringae* pv *tomato* and the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Li et al. 2004; Zimmermann et al. 2004; AbuQamar et al. 2006). Overexpression of *AtWRKY70* caused heightened resistance to *P. syringae* and *Erysiphe chichoracearum* and enhanced susceptibility to *A. brassicicola* (Li et al. 2004, 2006). In contrast, loss-of-*AtWRKY70* function resulted in enhanced susceptibility to *E. cichoracearum* and *B. cinerea* (AbuQamar et al. 2006). Moreover, recent studies showed that necrotrophs deliberately trigger HR in the host, which subsequently leads to cell death and necrosis and hence might be a strategy used by these pathogens to initiate colonization of host tissue (Govrin and Levine 2000; La Camera et al. 2005). The fact that *AtWRKY70* expression is regulated both by defense- and senescence-associated signals acting in a coordinated manner indicates that *AtWRKY70* function may be required to integrate signals that link both pathways.

Treatment with SA appears to be a very potent inducer of *AtWRKY70* expression and increases its transcript levels almost 30-fold within 2 h of application (Fig. 5b; Li et al. 2004; Ülker and Somssich 2004; Zimmermann et al. 2005). Recently, SA was proposed to promote the expression of senescence-related genes solely in naturally occurring senescing leaves (Morris et al. 2000; Buchanan-Wollaston et al. 2005; van der Graaff et al. 2006). SA appears to be a signal required for *AtWRKY70* expression in both young and senescing leaves, as no endogenous transcript can be found in SA-deprived *NahG* transgenic plants, and a strong reduction is observed in SA defective signal transduction mutants *eds1*, *pad4* and *npr1* (Fig. 5b; Li et al. 2004; Zimmermann et al. 2005; Bartsch et al. 2006). Conversely, increased basal transcript levels of *AtWRKY70* in SA hyper-accumulating mutants *edr1*, *cpr5* and *acd11* (*accelerated cell death 11*), at an early stage of plant development suggest that SA promotes *AtWRKY70* expression (Fig. 5b; Frye et al. 2001; Brodersen et al. 2002; Yoshida et al. 2002a). The high constitutive levels of *AtWRKY70* transcript in the *edr1*

mutant are intriguing. *EDR1* encodes a Raf-like mitogen-activated protein kinase kinase kinase (MAPKKK) that negatively regulates disease resistance (Frye et al. 2001). *edr1* plants are not altered in their timing of senescence compared to wild-type plants. However, *edr1* mutants display an enhanced ethylene-induced senescence response, a phenotype that can be phenocopied by overexpressing a dominant negative variant of *EDR1* (Frye et al. 2001; Tang and Innes 2002). It is therefore possible that *AtWRKY70* contributes to this enhanced response by regulating/or interacting with an ethylene-dependent component. Endogenous *AtWRKY70* expression kinetics varied in *cpr5* mutant depending upon the developmental stage of the plants. At the early age (e.g., 22 days), *AtWRKY70* expression is induced, while in senescing leaves a slight repression relative to wild-type plants was observed. The biological significance of these findings is currently unknown.

Our data suggest that *At*WRKY70 can act as a repressor of some SA- and JA-response genes such as *PR1*, *PR2*, *PR3*, *COR1* and *PDF1.2*. This is partly in agreement to the data of AbuQamar et al. (2006) who also showed that in *Atwrky70* mutant plants basal expression of the SA-dependent *PR1* is elevated. This finding, however, contradicts results obtained by Li et al. (2004), who found no impairment of *PR1* expression in an *AtWRKY70* antisense line. Unfortunately, comparison of *PR1* expression of this antisense line with the *Atwrky70* mutants recently analyzed by Li et al. (2006) was not reported. Thus, it remains unclear whether this observed difference is due to variations of the experimental set-ups used or due to off-target effects of the silencing construct used, since the antisense line is phenotypically not identical to the *Atwrky70* mutant plants (Li et al. 2006). Still, several defense-related genes are known to be induced during leaf senescence, including *PR1*, *PR2*, *PR3* and *PDF1.2* (Wyatt et al. 1991; Hanfrey et al. 1996; Morris et al. 2000; Quirino et al. 2000; He et al. 2002), in a phenomenon known as Age-Related Resistance (Kus et al. 2002). Expression of *PR* genes during senescence is probably linked to a protective function in aging leaves against invasion by opportunistic pathogens, thereby maintaining viability of the leaf until mobilization is complete and preventing infection of the rest of the plant (Kus et al. 2002).

In contrast to our study, AbuQamar et al. (2006) did not observe elevated basal expression levels of *PDF1.2* in *Atwrky70* mutants although a difference in the temporal expression kinetics of *PDF1.2* following pathogen challenge was seen in such mutants compared to wild-type plants. *Atwrky70* mutants showed a clearly accelerated expression of *PDF1.2* transcript in *Botrytis*-inoculated plants (maximum levels reached at 36 h pi in the mutant compared to 60 h in wild-type plants). Basal expression of *PDF1.2* was not reported for the *AtWRKY70* antisense line but was slightly enhanced in the *Atwrky70-1* mutant (Li et al. 2006) consistent with our observations.

Finally, the increased basal *AtWRKY70* transcript levels in the *coi1* mutant reported recently (Li et al. 2004; AbuQamar et al. 2006) stand partially in disagreement with our findings revealing that *AtWRKY70* expression is not significantly altered in two JA-signaling mutants *aos* and *jar1* (Fig. 5b) and with microarray data showing that *AtWRKY70* expression was not altered in the mid-flowering stage of *coi1* mutant plants (Buchanan-Wollaston et al. 2005). Again, these discrepancies may be due to the experimental differences or alternatively, specific to the *coi1* mutant and thus not a general JA-related effect.

In spite of the biological and practical importance of leaf senescence, elucidation of the genes that control senescence has been complicated by the complexity of signaling pathways that appear to be involved. Due to an apparent functional redundancy within the signaling networks the task of defining the specific contribution of individual pathways to the senescence program is difficult and may explain why forward screens have only identified a very limited number of mutants directly associated with a senescence phenotype (Buchanan-Wollaston et al. 2005). It is for this reason that the analysis of potential transcriptional regulators remains an important area within this field of research. Here we have identified one transcriptional regulator, which signals through the SA-mediated pathway and thereby regulates a subset of genes common to senescence and plant defense. Identifying specific *At*WRKY70 in vivo target genes will be the next step required to gain a more in-depth knowledge of its role within these pathways and to more precisely define specific convergence points. Thus, elucidating the senescence mechanisms at the molecular level will remain a challenge but should yield valuable information not only on the regulation of developmental cell death, but also provide the necessary tools to prudently manipulate the senescence process in crops for increased plant longevity, productivity, stress tolerance, pre- or post-harvest storage and shelf life.

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References

AbuQamar S, Chen X, Dhawan R, Bluhm B, Salmeron J, Lam S, Dietrich RA, Mengiste T (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to *Botrytis* infection. Plant J 48:28–44

Bartsch M, Gobbato E, Bednarek P, Debey S, Schultze JL, Bautor J, Parker JE (2006) Salicylic acid-independent enhanced disease susceptibility1 signaling in *Arabidopsis* immunity and cell death is regulated by the monooxygenase FMO1 and the nudix hydrolase NUDT7. Plant Cell 18:1038–1051

Benedetti CE, Costa CL, Turcinelli SR, Arruda P (1998) Differential expression of a novel gene in response to coronatine, methyl jasmonate, and wounding in the *coi1* mutant of *Arabidopsis*. Plant Physiol 116:1037–1042

Bieleski RL, Reid MS (1992) Physiological changes accompanying senescence in the ephemeral daylily flower. Plant Physiol 98:1042–1049

Brodersen P, Petersen M, Pike HM, Olszak B, Skov S, Ødum N, Jørgensen LB, Brown RE, Mundy J (2002) Knockout of *Arabidopsis* accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. Genes Dev 16:490–502

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D (2003) The molecular analysis of leaf senescence—a genomics approach. Plant Biotechnol J 1:3–22

Buchanan-Wollaston V, Page T, Harrison E, Breeze E, Lim PO, Nam HG, Lin JF, Wu SH, Swidzinski J, Ishizaki K, Leaver CJ (2005) Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J 42:567–585

Century KS, Holub EB, Staskawicz BJ (1995) *NDR1*, a locus of *Arabidopsis thaliana* that is required for disease resistance to both a bacterial and a fungal pathogen. Proc Natl Acad Sci USA 92:6597–6601

Clough SJ, Bent AF (1998) Floral dip:a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743

Frye CA, Tang D, Innes RW (2001) Negative regulation of defense responses in plants by a conserved MAPKK kinase. Proc Natl Acad Sci USA 98:373–378

Gepstein S (2004) Leaf senescence—not just a 'wear and tear' phenomenom. Genome Biol 5:212

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu Rev Phytopathol 43:205–227

Govrin EM, Levine A (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol 10:751–757

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flugge UI, Kunze R (2006) Transcription analysis of *Arabidopsis* membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141:776–792

Grbic V, Bleecker AB (1995) Ethylene regulates the timing of leaf senescence in *Arabidopsis*. Plant J 8:595–602

Guo Y, Gan S (2005) Leaf senescence: signals, execution, and regulation. Curr Top Dev Biol 71:83–112

Guo Y, Gan S (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J 46:601–612

Guo Y, Cai Z, Gan S (2004) Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ 27:521–549

Hanfrey C, Fife M, Buchanan-Wollaston V (1996) Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. Plant Mol Biol 30:597–609

He Y, Fukushiga H, Hildebrand DF, Gan S (2002) Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol 128:876–884

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

John CF, Morris K, Jordan BR, Thomas B, A-H-Mackerness S (2001) Ultraviolet-B exposure leads to up-regulation of senescence-associated genes in *Arabidopsis thaliana*. J Exp Bot 52:1367–1373

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a Raf family of protein kinases. Cell 72:427–441

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

Kus JV, Zaton K, Sarkar R, Cameron RK (2002) Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. Plant Cell 14:479–490

La Camera S, Geoffroy P, Samaha H, Ndiaye A, Rahim G, Legrand M, Heitz T (2005) A pathogen-inducible patatin-like lipid acyl hydrolase facilitates fungal and bacterial host colonization in *Arabidopsis*. Plant J 44:810–825

Li J, Brader G, Palva ET (2004) The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell 16:319–331

Li J, Brader G, Kariola T, Tapio Palva E (2006) WRKY70 modulates the selection of signaling pathways in plant defense. Plant J 46:477–491

Lin J-F, Wu S-H (2004) Molecular events in senescing *Arabidopsis* leaves. Plant J 39:612–628

Maleck K, Levine A, Eulgem T, Morgen A, Schmid J, Lawton K, Dangl JL, Dietrich RA (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. Nat Genet 26:403–410

Miao Y, Laun T, Zimmermann P, Zentgraf U (2004) Targets of the WRKY53 transcription factor and its role during leaf senescence in *Arabidopsis*. Plant Mol Biol 55:853–867

Morris K, A-H-Mackerness S, Page T, John CF, Murphy AM, Carr JP, Buchanan-Wollaston V (2000) Salicylic acid has a role in regulating gene expression during leaf senescence. Plant J 23:677–685

Oh SA, Lee SY, Chung IK, Lee C-H, Nam HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol 30:739–754

Oh SA, Park JH, Lee GI, Paek KH, Park SK, Nam HG (1997) Identification of three genetic loci controlling leaf senescence in *Arabidopsis thaliana*. Plant J 12:527–535

Olsen AN, Ernst HA, Leggio LL, Skriver K (2005) NAC transcription factors: structurally distinct, functionally diverse. Trends Plant Sci 10:79–87

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann K, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in *Arabidopsis* due to a block in jasmonic acid biosynthesis. Plant J 31:1–12

Quirino BF, Noh Y-S, Himelblau E, Amasino RM (2000) Molecular aspects of leaf senescence. Trends Plant Sci 5:278–282

Robatzek S, Somssich IE (2001) A new member of the *Arabidopsis* WRKY transcription factor family, *At*WRKY6, is associated with both senescence- and defense-related processes. Plant J 28:123–133

Rosso MG, Li Y, Strizhov N, Reiss B, Dekker K, Weisshaar B (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol 53:247–259

Schenk PM, Kazan K, Rusu AG, Manners JM, Maclean DJ (2005) The SEN1 gene of *Arabidopsis* is regulated by signals that link plant defence responses and senescence. Plant Physiol Biochem 43:997–1005

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Scholkopf B, Weigel D, Lohmann JU (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet 37:501

Swidzinski JA, Sweetlove LJ, Leaver CJ (2002) A custom microarray analysis of gene expression during programmed cell death in *Arabidopsis thaliana*. Plant J 30:431–446

Tang D, Innes RW (2002) Overexpression of a kinase-deficient form of the *EDR1* genes enhances powdery mildew resistance and ethylene-induced senescence in *Arabidopsis*. Plant J 32:975–983

Ward ER, Uknes SJ, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl-Goy P, Métraux J-P, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085–1094

Weaver LM, Amasino RM (2001) Senescence is induced in individually darkened *Arabidopsis* leaves, but inhibited in whole darkened plants. Plant Physiol 127:876–886

Wyatt S, Pan S, Kuc J (1991) Beta-1,3-Glucanase, chitinase and peroxidase activities in tobacco tissues resistant and susceptible to blue mould as related to flowering, age and sucker development. Physiol Mol Plant Pathol 39:433–440

Yoshida S, Ito M, Nishida I, Watanabe A (2001) Isolation and RNA gel blot analysis of genes that could serve as potential molecular markers for leaf senescence in *Arabidopsis thaliana*. Plant Cell Physiol 42:170–178

Yoshida S, Ito M, Callis J, Nishida I, Watanabe A (2002a) A delayed leaf senescence mutant is defective in arginyl-tRNA:protein arginyl transferase, a component of the N-end rule pathway in *Arabidopsis*. Plant J 32:129–137

Yoshida S, Ito M, Nishida I, Watanabe A (2002b) Identification of a novel gene *HYS1/CPR5* that has a repressive role in the induction of leaf senescence and pathogen-defense responses in *Arabidopsis thaliana*. Plant J 29:427–437

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004) GENEVESTIGATOR. *Arabidopsis* microarray database and analysis toolbox. Plant Physiol 136:2621–2632

Zimmermann P, Hennig L, Gruissem W (2005) Gene-expression analysis and network discovery using Genevestigator. Trends Plant Sci 10:407–409

Ülker B, Somssich IE (2004) WRKY transcription factors: from DNA binding towards biological function. Curr Opin Plant Biol 7:491–498