Early Senescence and Cell Death in Arabidopsis *saul1* Mutants Involves the *PAD4*-Dependent Salicylic Acid Pathway[1][W][OA]

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Age-dependent leaf senescence and cell death in Arabidopsis (*Arabidopsis thaliana*) requires activation of the transcription factor ORESARA1 (ORE1) and is not initiated prior to a leaf age of 28 d. Here, we investigate the conditional execution of events that regulate early senescence and cell death in *senescence-associated ubiquitin ligase1* (*saul1*) mutants, deficient in the PLANT U-BOX-ARMADILLO E3 ubiquitin ligase SAUL1. In *saul1* mutants challenged with low light, the switch of age-dependent cell death was turned on prematurely, as indicated by the accumulation of *ORE1* transcripts, induction of the senescence marker gene *SENESCENCE-ASSOCIATED GENE12*, and cell death. However, *ORE1* accumulation by itself was not sufficient to cause *saul1* phenotypes, as demonstrated by double mutant analysis. Exposure of *saul1* mutants to low light for only 24 h did not result in visible symptoms of senescence; however, the senescence-promoting transcription factor genes *WRKY53*, *WRKY6*, and *NAC-LIKE ACTIVATED BY AP3/PI* were up-regulated, indicating that senescence in *saul1* seedlings was already initiated. To resolve the time course of gene expression, microarray experiments were performed at narrow intervals. Differential expression of the genes involved in salicylic acid and defense mechanisms were the earliest events detected, suggesting a central role for salicylic acid in *saul1* senescence and cell death. The salicylic acid content increased in low-light-treated *saul1* mutants, and application of exogenous salicylic acid was indeed sufficient to trigger *saul1* senescence in permissive light conditions. Double mutant analyses showed that *PHYTOALEXIN DEFICIENT4* (*PAD4*) but not *NONEXPRESSER OF PR GENES1* (*NPR1*) is essential for *saul1* phenotypes. Our results indicate that *saul1* senescence depends on the PAD4-dependent salicylic acid pathway but does not require NPR1 signaling.

Plant senescence is an age-dependent phenomenon that closely correlates with cell death. Leaf senescence is developmentally well defined to guarantee the recycling of resources from senescing leaves into young leaves or seeds, thus optimizing the growth and reproductive capacity of plants. This process becomes visible as yellowing of leaves due to chlorophyll degradation. Senescence is genetically associated with aging and involves the regulated expression of senescence-associated genes (*SAG*s; Yoshida, 2003; Lim et al., 2007). Microarray studies on adult Arabidopsis (*Arabidopsis thaliana*) plants indicated large changes in gene expression patterns during age-dependent senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Wagstaff et al., 2009). In Arabidopsis, a feed-forward regulatory switch of age-dependent cell death during senescence has recently been demonstrated (Kim et al., 2009). The NAC transcription factor ORESARA1 (ORE1; also named ANAC092) triggers aging-associated cell death in leaves that are at least 28 d old. In young leaves, *ORE1* accumulation is prevented by *miRNA164* (*miR164*), which targets *ORE1* mRNA for cleavage. This negative regulation of *ORE1* expression by *miR164* is released during aging through the down-regulation of *miR164* (Kim et al., 2009).

In addition to ORE1, other components of the transcription factor network regulating gene expression during senescence have been identified. High expression of the *WRKY6* transcription factor gene leads to leaf necrosis (Robatzek and Somssich, 2002). It has been shown that WRKY6 activates the promoter of

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SENESCENCE-INDUCED RECEPTOR KINASE1 encoding senescence-induced receptor kinase, which is specifically induced in senescent leaves. Premature senescence has been observed in *WRKY53*-overexpressing plants, whereas *wrky53* mutants showed delayed senescence (Miao et al., 2004). Similarly, overexpression of the *NAC-LIKE ACTIVATED BY AP3/PI* (*AtNAP*)/*ANAC029* gene encoding a NAC family transcription factor results in premature senescence (Guo and Gan, 2006). In contrast, senescence was delayed in two independent *atnap* mutant alleles. The NAC transcription factor ORE1 SISTER1/ANAC059 is also a positive regulator of senescence (Balazadeh et al., 2011). The hierarchy within the network of transcription factors during senescence is still not known.

Besides regulation at the level of transcription, protein turnover represents a highly important molecular mechanism for control of the onset and progression of senescence and cell death. On the one hand, bulk protein turnover during senescence occurs in autophagic vesicles involving ubiquitin-like conjugation pathways (Thompson and Vierstra, 2005; Bassham, 2007; Phillips et al., 2008; Yoshimoto et al., 2009). On the other hand, selective protein turnover is mediated by the ubiquitin/proteasome pathway (Sullivan et al., 2003; Smalle and Vierstra, 2004). The F-box protein ORE9 has been suggested to be a positive regulator of leaf senescence, because mutants lacking *ORE9* expression are delayed in senescence (Woo et al., 2001). Generally, F-box proteins are part of SCF-type E3 complexes with ubiquitin ligase activity and recruit target substrates to such complexes (Lechner et al., 2006). It has recently been shown that ORE9 indeed interacts with the core SCF subunits ARABIDOPSIS SKP1 HOMOLOGUE1 and CULLIN1 in planta (Stirnberg et al., 2007). The arginyl-tRNA:protein arginyltransferase ATE1, which is a component of the N-end rule pathway within the ubiquitin-dependent proteolytic system, also positively regulates senescence. Knockout of the *ATE1* gene in *delayed senescence1* mutant plants resulted in delayed leaf senescence (Yoshida et al., 2002). In contrast to ORE9 and ATE1, the RING-type ubiquitin ligase NITROGEN LIMITATION ADAPTATION (NLA) and the PLANT U-BOX (PUB)-ARMADILLO (ARM) E3 ubiquitin ligase SENESCENCE-ASSOCIATED UBIQUITIN LIGASE1 (SAUL1; At1g20780) are negative regulators of plant senescence (Peng et al., 2007; Raab et al., 2009). A mutation in NLA results in early senescence when growing the mutants in nitrogen-limiting conditions. The ubiquitin ligase activity of NLA has indirectly been demonstrated through interaction with the Arabidopsis ubiquitin conjugase AtUBC8 (Peng et al., 2007). Similarly, *saul1* mutant plants lacking any expression of *SAUL1* show early senescence in low-light conditions. Senescence can be prematurely induced in *saul1* seedlings by transfer to low light. Thus, *saul1* mutants have been established as a low-light-inducible and age-independent model system for senescence (Raab et al., 2009).

In plant and animal cells, the ubiquitin/proteasome pathway has critical functions for cell survival and repair (Vernace et al., 2007; Vierstra, 2009). It is the major proteolytic pathway that mediates regulated protein degradation. The age-dependent decline of this process leads to the accumulation of aberrant proteins and has been correlated with certain human diseases (Grune et al., 2004; Hyun et al., 2004). It has also been suggested that the inhibition of proteasome function induces morphological symptoms of plant programmed cell death (Kim et al., 2003). Generally, plant programmed cell death occurs during normal development, for example, embryo formation and leaf senescence, but also during the hypersensitive response following pathogen attack to trigger cell death around infection sites and restrict pathogen spread. In the pathogen response, regulatory functions have been assigned to components of the ubiquitin/proteasome pathway, including many PUB proteins (Vierstra, 2009; Yee and Goring, 2009). Most of these belong to the subfamily of PUB-ARM proteins that contain ARM repeats, named after the *Drosophila* segment polarity gene *armadillo*, in addition to the U-box (Nüsslein-Volhard and Wieschaus, 1980; Riggleman et al., 1989). PUB-ARM proteins carry distinct numbers of ARM repeats where each repeat consists of three α-helices, yielding a conserved three-dimensional structure that is implicated to function as a protein interaction domain (Peifer et al., 1994; Huber et al., 1997; Coates, 2003). Tobacco (*Nicotiana tabacum*) CMPG1 and Avr9/Cf-9 Rapidly Elicited276, rice (*Oryza sativa*) SPOTTED LEAF11, and Arabidopsis AtPUB17, AtPUB22, AtPUB23, and AtPUB24 are PUB-ARM proteins and have been implicated in cell death control during pathogen responses (Zeng et al., 2004; González-Lamothe et al., 2006; Trujillo et al., 2008). The PUB-ARM protein SAUL1 has a role in the regulation of senescence and is localized to the plasma membrane (Drechsel et al., 2011). This localization may render SAUL1 a regulatory component that, by modifying signaling components through ubiquitination at the plasma membrane, is positioned very high in the hierarchy of senescence and cell death control.

To further define the function of SAUL1 during the onset and progression of senescence and cell death, we asked whether the feed-forward regulatory switch of age-dependent cell death during senescence involving *ORE1* induction was turned on in very young *saul1* seedlings. In addition, we aimed to reveal the time course of gene expression changes in *saul1* mutants after transfer to low light on a genomic scale to resolve the underlying signaling events. We show that, indeed, *ORE1* expression and cell death are rapidly induced in *saul1* seedlings after transfer to low light. However, additional knockout of *ORE1/ANAC092* in *saul1-1/anac092-1* double mutants was not sufficient to suppress *saul1* phenotypes. Microarray analyses identified the timing of responses in *saul1* cell death and senescence and indicated that salicylic acid (SA) pathways may play a central role in *saul1* senescence.

Abolishing SA pathways in *saul1-1/pad4* but not in *saul1-1/nonexpresser of PR genes1* (*npr1*) double mutants was sufficient to suppress *saul1* phenotypes. Our results suggested that specifically the PHYTOALEXIN DEFICIENT4 (PAD4)-mediated SA pathway is important for *saul1* senescence.

RESULTS

The Switch for Age-Dependent Cell Death Is Prematurely Turned on in *saul1* Seedlings

Recent mutant analyses in Arabidopsis showed that mutant plants, which do not express *SAUL1* encoding an E3 ubiquitin ligase of the PUB protein family, exhibit early senescence (Raab et al., 2009). When growing in low light (photon flux density less than 30 μmol m^{-2} s^{-1}), *saul1* mutants show premature aging of leaves younger than 10 d (Fig. 1A). In permissive light conditions (photon flux density greater than 60 μmol m^{-2} s^{-1}), *saul1* seedlings are indistinguishable from the wild type, but senescence can be induced rapidly at any developmental stage by transfer to low light. Expression of the *SAG12* gene encoding a Cys protease is strictly associated with senescent tissues, and *SAG12* expression is used as a highly reliable marker of age-dependent senescence (Weaver et al., 1998; Noh and Amasino, 1999). Here, we show that at the molecular level senescence in *saul1* mutants becomes manifested in *SAG12* expression that is normally absent from young seedlings (Fig. 1B). This implies a premature turning on of a regulatory switch in *saul1* mutants leading to *SAG12* expression that is suppressed by SAUL1 in wild-type plants.

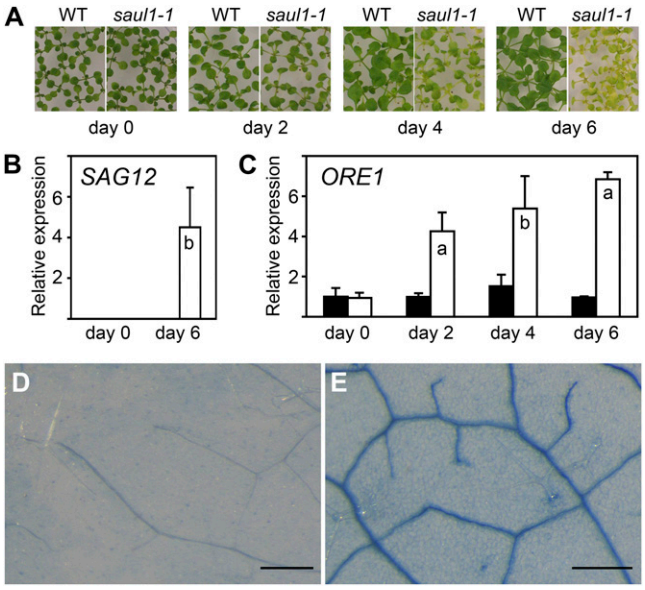

Figure 1. Premature turning on of the switch for age-dependent cell death in *saul1* seedlings. A, Senescence phenotype of *saul1-1* mutants. Wild-type (WT) and *saul1-1* plants were grown for 12 d at 60 μmol m^{-2} s^{-1} photon flux density followed by growth for 2, 4, and 6 d at 20 μmol m^{-2} s^{-1} photon flux density. Yellowing of leaves appears as a visible symptom of senescence. B, *SAG12* transcript level in young *saul1-1* seedlings. Bars show relative expression of the senescence marker gene *SAG12* in wild-type (black bars) and *saul1-1* (white bars) plants at the indicated time points (mean ± SD; $n = 3$) as determined by qPCR. Note that *SAG12* transcripts are absent from wild-type seedlings and *saul1-1* mutant seedlings without the senescence phenotype. C, Increased *ORE1* transcript levels in *saul1* seedlings. Bars show relative expression determined by qPCR of the senescence regulatory gene *ORE1* in growth kinetics as in A in wild-type (black bars) and *saul1-1* (white bars) seedlings at the indicated time points (mean ± SD; $n = 3$). D and E, Premature cell death in *saul1* mutants. Trypan blue staining indicated dead or dying cells in *saul1-1* mutant (E) but not in wild-type (D) seedlings. Assayed leaves ($n = 16$) were less than 20 d old, and identical leaves and leaf areas were chosen for comparison. Bars = 250 μm.

To identify the status of the feed-forward regulatory switch of age-dependent cell death during senescence (Kim et al., 2009) in *saul1* mutants after transfer to low light, we tested for *ORE1* expression through real-time reverse transcription-PCR (qPCR) experiments and for symptoms of cell death. Indeed, *ORE1* transcript levels were up-regulated in *saul1-1* leaves with leaf ages below 10 d counted from the time of leaf emergence (Fig. 1C). Accumulation of *ORE1* transcripts was followed by cell death in *saul1* mutants, as expected and indicated by the presence of trypan blue-stained cells (Fig. 1, D and E; Salt et al., 2011). These findings suggest that the feed-forward regulatory switch of age-dependent cell death has prematurely been turned on in *saul1* mutant seedlings.

High Salinity Severely Affects the Growth and Development of *saul1* Mutants

A common abiotic stress that may cause senescence and cell death in plants is high salinity. Next to its role in age-dependent cell death, ORE1/ANAC092 has been shown to mediate salt-induced senescence and cell death, too. Compared with the wild type, *anac092-1* mutants were more tolerant to salt (Huh et al., 2002; Balazadeh et al., 2010). To study whether SAUL1 is also involved in the salt response, wild-type and *saul1-1* mutant seedlings were grown in permissive light conditions for 5 d, transferred to agar plates containing 0 or 150 mM NaCl, and kept in permissive light. Whereas *saul1-1* mutants were indistinguishable from wild-type plants in the absence of salt, *saul1-1* mutants but not wild-type plants showed leaf yellowing as visible symptoms of senescence in the presence of NaCl (Fig. 2A). The expression of selected genes that are known to be induced by salt or regulated by ORE1 (Balazadeh et al., 2010), namely *PATHOGENESIS-RELATED GENE1* (*PR1*), *SAG13*, *ENHANCED DISEASE SUSCEPTIBILITY16* (*EDS16*), *At5g39520*, and *At2g15830*, also increased upon NaCl treatment in *saul1-1* in comparison with wild-type plants (Fig. 2B). These data suggested that SAUL1 is a regulatory factor integrating not

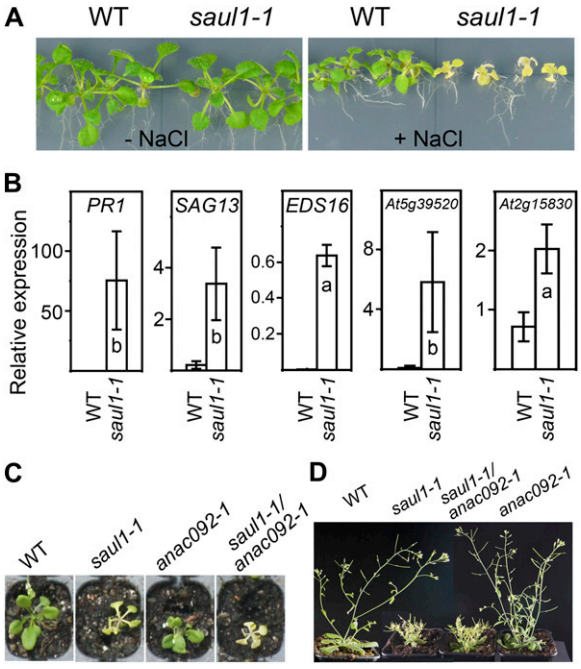

Figure 2. Hypersensitivity of *saul1* mutants to high salt concentrations. A, Wild-type (WT) and *saul1-1* mutant seedlings were grown in permissive light conditions (80 μmol m^{-2} s^{-1}) for 5 d and then transferred to 0 or 100 mM NaCl to investigate the effect of salt on growth and development in permissive light conditions. In contrast to the wild type, *saul1-1* mutant seedlings showed leaf yellowing on high salt. B, Relative expression levels were determined by qPCR to demonstrate salt induction of *PR1*, *SAG13*, *EDS16*, *At5g39520*, and *At2g15830* in *saul1-1* seedlings. Values represent means ± SD from three independent experiments. C, Leaf yellowing was monitored in wild-type, *saul1-1*, *anac092-1*, and *saul1-1/anac092-1* seedlings that were grown at 100 μmol m^{-2} s^{-1} on soil. D, Growth defects in adult plants were observed in *saul1-1* and *saul1-1/anac092-1* but not in *anac092-1* plants.

only low-light-induced, but also salt-stress-triggered, senescence and cell death.

The Absence of *ORE1/ANAC092* Is Not Sufficient to Suppress *saul1* Phenotypes

We were aiming to test whether the knockout of *ORE1/ANAC092* may suppress *saul1* senescence and cell death, because this important switch during age-dependent cell death was turned on in low-light-treated *saul1* mutants (Fig. 1). By crossing *saul1-1* mutants to *anac092-1* mutants, *saul1-1/anac092-1* double knockout mutants were generated and studied with respect to the occurrence of leaf yellowing. In growth conditions that led to *saul1-1* senescence and cell death, *saul1-1/anac092-1* double mutants also showed yellowing of leaves, indicating that additional knockout of *ORE1/ANAC092* did not suppress *saul1* senescence and cell death phenotypes (Fig. 2C). The growth defect of *saul1-1* mutants at later developmental stages (Raab et al., 2009) was also not suppressed in these double mutants (Fig. 2D).

Regulatory Events in *saul1* Seedlings Precede the Occurrence of a Visible Phenotype and the Induction of *ORE1* Expression

To investigate whether senescence in *saul1* seedlings was initiated at the molecular level in the absence of visible symptoms and before the induction of *ORE1* expression, plants were grown in permissive light conditions for 14 d and then transferred to low light. One day after transfer, *saul1-1* seedlings still looked like the wild type (Fig. 3A). At this time, *ORE1* expression was identical in *saul1-1* mutant and wild-type plants (Fig. 3B). We tested for the expression of other well-known senescence regulatory genes encoding transcriptional regulators of the NAC and WRKY transcription factor families. Interestingly, the *WRKY53*, *WRKY6*, and *AtNAP* transcript levels were significantly increased in *saul1-1* seedlings already 24 h after transfer to low light (Fig. 3B). This line of evidence demonstrates that changes in the regulatory network of transcription took place even before the occurrence of visible symptoms and before the increase of *ORE1* expression. These observations implied that important regulatory events at the molecular level were established in the first 24 h after transfer to low light. In wild-type plants, the plasma membrane-localized ubiquitin ligase SAUL1 functions to prevent these expression changes. This prompted us to use a genomics approach to resolve the time course of

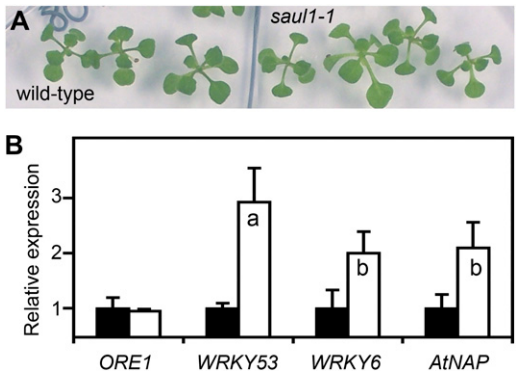

Figure 3. Regulatory events at the molecular level in the absence of visible symptoms of senescence. A, Wild-type and *saul1-1* mutant seedlings were grown for 14 d in permissive light (60 μmol m^{-2} s^{-1}) followed by growth for 24 h in low-light conditions (20 μmol m^{-2} s^{-1}). Note that wild-type and *saul1-1* mutant seedlings were indistinguishable. B, Induction of transcription factor gene expression. Relative expression levels for *ORE1*, *WRKY53*, *WRKY6*, and *AtNAP* were determined by qPCR from three independent experiments. Means ± SD ($n = 3$) are shown for wild-type (black bars) and *saul1-1* mutant (white bars) seedlings. Whereas *ORE1* transcript levels were indistinguishable in wild-type and *saul1-1* seedlings, the expression of *WRKY53*, *WRKY6*, and *AtNAP* was induced significantly.

gene expression changes that result in the onset and progression of *saul1* senescence and cell death.

Microarray Analyses Reveal Different Levels of Response in Low-Light-Induced *saul1* Senescence and Cell Death

To study gene expression changes, *saul1-1* mutant and wild-type seedlings were grown at permissive light (75 μmol m^{-2} s^{-1}) for 11 d and then challenged with low light (20 μmol m^{-2} s^{-1}) for 6, 24, or 48 h. Differential expression profiles of genes in *saul1-1* compared with wild-type seedlings were determined by using Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays. Microarray hybridizations, data acquisition, and bioinformatics analysis of the microarray experiments were performed as described (Deeken et al., 2006; Duy et al., 2007) with adaptations (see "Materials and Methods"). For each time-point, T_0 (low-light challenge: time = 0 h, just before transfer to low light), T_6 (6 h), T_{24} (24 h), and T_{48} (48 h), the fold changes and adjusted *P* values for genes differentially expressed were calculated from three replicate *saul1* mutant samples versus three replicate wild-type samples. On the basis of fold changes ($\log_2$) greater than 0.585 or ($\log_2$) less than -0.585 that met the significance criterion of adjusted $P < 0.01$, we found 24 up-regulated genes and one down-regulated gene at T_0, 224 up-regulated and 134 down-regulated genes at T_6, 2,031 up-regulated and 1,994 down-regulated genes at T_{24}, and 2,737 up-regulated and 3,281 down-regulated genes at T_{48} (Supplemental Tables S1 and S2).

Previously, we determined the low-light-induced accumulation of abscisic acid (ABA) in *saul1* mutant plants (Raab et al., 2009). Therefore, we checked for the regulation of ABA-dependent genes in low-light-grown *saul1* mutants. Frequently used ABA marker genes were not regulated before T_{24} (ABI1 [At4g26080] and ATHB7 [At2g46680]) or T_{48} (ATHB12 [At3g61890] and RD26 [At4g27410]), suggesting that the ABA regulation of gene expression was not initiated at T_6 (Supplemental Table S1). To confirm this suggestion, we screened the differential expression of genes that we previously identified to be responsive to ABA (Hoth et al., 2002) for fold changes ($\log_2$) greater than 0.379 or less than -0.379 meeting the significance criterion of an adjusted $P < 0.05$ in our microarray experiments. Of the 598 up- and 617 down-regulated ABA-responsive genes that were also present on the array, 31.4% and 37.3% were also up- and down-regulated in low-light-challenged *saul1* plants, respectively (Supplemental Table S3). However, induction or repression did not appear until 24 h or even 48 h after transfer to low light (with very few exceptions), indicating that ABA control (like ORE1 regulation) of gene expression was not the earliest event.

To elucidate the events leading to *saul1* cell death and senescence, we scanned the genes that were differentially expressed at T_6 already. Strikingly, among the 224 up-regulated genes at T_6, we found many genes that have previously been related to pathogen defense and the response to SA in Arabidopsis, including *PR1*, *PR5*, *EDS1*, *EDS16*, and *PAD4* (Supplemental Table S1). Therefore, we compared the genes that were differentially expressed at T_6 with gene expression changes upon pathogen or SA treatment. Of the 224 induced genes, 66% were also up-regulated in response to powdery mildew, indicating substantial overlap with biotic stress responses (Supplemental Table S4; Nishimura et al., 2003). Almost one-quarter of the 224 genes (22%) were also induced upon SA treatment (Supplemental Table S4; Blanco et al., 2009). Plant defense involves SA-mediated inhibition of auxin signaling by repressing 21 genes related to auxin signal transduction (Wang et al., 2007). With two exceptions, these genes were repressed at T_{24} in *saul1* mutants (Supplemental Table S5). These data pointed to a participation of defense mechanisms and SA in the response of *saul1* mutants to low light.

The PAD4-Dependent SA Pathway Is Required for *saul1* Senescence and Cell Death

To further substantiate the role of SA in *saul1* mutant phenotypes, we measured SA and SA glucoside contents in the wild type and *saul1-1* mutants at 6, 12, 24, and 48 h after transfer to low light. Whereas no increase was observed in wild-type seedlings, SA and SA glucoside (and thus total SA) contents increased considerably in *saul1-1* mutant seedlings at 24 and 48 h (Fig. 4A). However, increased contents could be detected already at 12 h (Fig. 4A, inset). In the next step, we studied the growth of mutant seedlings in the presence of SA in permissive light conditions. As expected from our gene expression data, *saul1-1* mutant seedlings were indeed more sensitive to SA treatment than wild-type seedlings and showed yellowing of leaves (Fig. 4B). We monitored the expression of SA-responsive genes in the presence of SA in *saul1-1* mutants compared with wild-type seedlings. Indeed, SA induction of *EDS16* and *PAD4* expression was much stronger in *saul1-1* plants (Fig. 4C). The expression of *AAO3* was also slightly changed. Taken together, our data suggested that SA signaling is an important event in low-light-induced *saul1* cell death and senescence. To test this hypothesis, we crossed *saul1-1* to *pad4* plants that are defective in SA responses and isolated *saul1-1/pad4* double mutants. In contrast to *saul1-1* mutant seedlings showing growth arrest and yellowing of leaves in low light, these *saul1-1/pad4* plants were indistinguishable from wild-type plants (Fig. 5A). We confirmed this also at the molecular level by analyzing *AAO3*, *WRKY6*, and *EDS16* expression. Whereas transcript levels of all three genes were highly increased in *saul1-1* mutants, their expression resembled wild-type levels in *saul1-1/pad4* double mutants (Fig. 5B). The increase of SA and SA glucoside that was observed in low-light-treated *saul1-1* mutants was also absent in *saul1-1/pad4* double mutants (Fig. 4A).

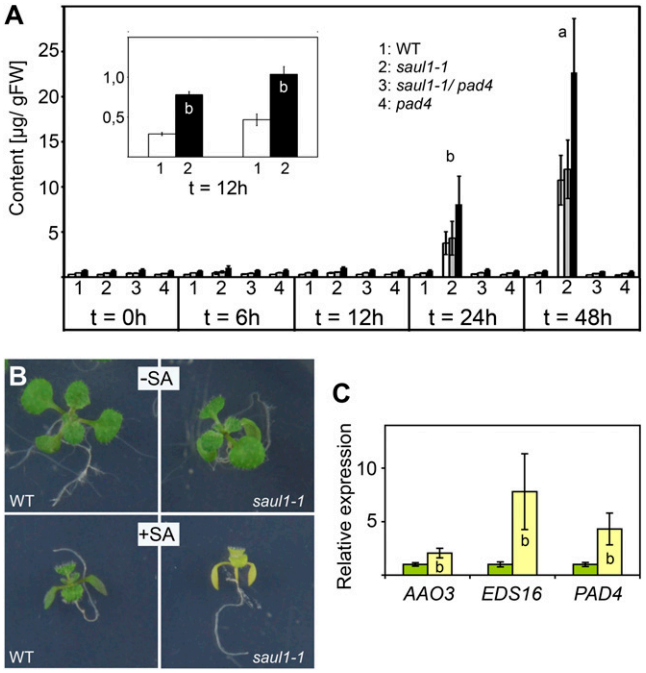

Figure 4. Determination of SA content and SA-induced *saul1* senescence. A, SA (white bars), SA glucoside (gray bars), and total SA (black bars) contents at the indicated times are shown for wild-type (WT), *saul1-1*, *saul1-1/pad4*, and *pad4* plants. Means ± SE ($n = 6$) are shown. Significant differences in SA content and total SA already at 12 h were only detected in *saul1-1* mutants (inset). Significance differences were determined by Student's *t* test analysis (a, $P < 0.01$; b, $P < 0.05$). FW, Fresh weight. B, When growing in the absence of SA in permissive light conditions, wild-type (top left) and *saul1-1* mutant (top right) seedlings developed indistinguishably. SA treatment resulted in growth arrest of both wild-type (bottom left) and *saul1-1* (bottom right) seedlings, but only *saul1-1* mutants showed symptoms of senescence, namely yellowing of leaves. C, Relative expression levels for *AAO3*, *EDS16*, and *PAD4* in the presence of SA were determined by qPCR. In comparison with the wild type, the expression of these genes was significantly increased in *saul1-1* mutants. Means ± SD ($n = 3$) are shown for wild-type (green bars) and *saul1-1* (yellow bars) plants.

Disruption of the *PAD4*-dependent SA pathway was thus sufficient to suppress the low-light-induced *saul1* phenotype. In contrast, disruption of the major SA signaling component NPR1 or of EDS5, which is required for SA biosynthesis, in *saul1-1/npr1-1* or *saul1-1/eds5-1* double mutants, respectively, did not suppress *saul1* phenotypes (Supplemental Fig. S1, A and B). These data suggested that *saul1* senescence specifically requires the PAD4-dependent SA pathway.

DISCUSSION

In this study, we demonstrate that *saul1* mutants lacking expression of the E3 ubiquitin ligase gene *SAUL1* appear to misjudge their developmental age and turn on a regulatory switch of age-dependent cell death in the young seedling stage. In wild-type Arabidopsis, this age-dependent switch involving *ORE1*, *miR164*, and *EIN2* ensures that aging triggers cell death in leaves (Kim et al., 2009). During leaf development, down-regulation of *miR164* allows for *ORE1* accumulation. This age-dependent down-regulation requires *ETHYLENE INSENSITIVE2*, encoding a putative transport protein in the endoplasmic reticulum membrane, which is involved in ethylene signaling and leaf senescence (Oh et al., 1997; Alonso et al., 1999; Bisson et al., 2009). A critical developmental turning point occurs between leaf ages of 26 and 28 d. At this time, *ORE1* accumulation apparently becomes sufficient to induce *SAG12* expression and cell death (Kim et al., 2009). In *saul1* seedlings challenged with low light, the turning point leading to cell death is reached at leaf ages below 10 d. Accordingly, *ORE1* accumulation, *SAG12* expression that is normally strictly associated with age-dependent senescence, and cell death can be detected prematurely (Fig. 1). In wild-type plants, SAUL1 function is thus crucial to prevent cell death under low-light conditions. However, through genetic analyses, we could show that disruption of *ORE1/ANAC092* in *saul1-1/anac092-1* double mutants did not result in the suppression of *saul1* phenotypes. Apparently, enhanced *ORE1* expression by itself is not sufficient to cause senescence and cell death in *saul1* plants.

Obviously, our data raise an important question: is the onset of senescence and cell death in *saul1-1* seedlings established at the molecular level before symptoms such as growth arrest and yellowing of leaves are visible? *ORE1* accumulation in *saul1* seedlings was observed after 2 d in low-light conditions, but *ORE1*

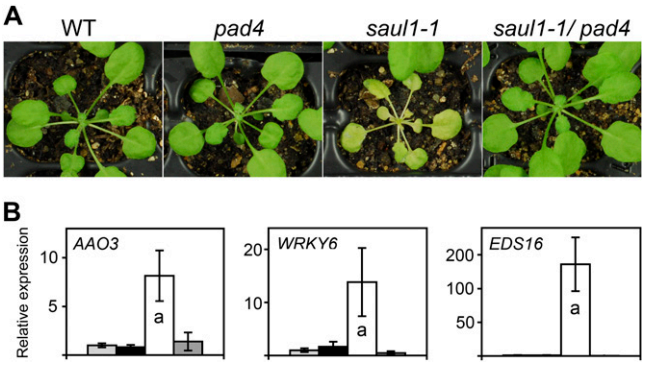

Figure 5. Rescue of the *saul1* senescence and molecular phenotypes in *saul1/pad4* double mutants. A, The phenotypes of wild-type (WT), *saul1-1* and *pad4* single mutant, and *saul1-1/pad4* double mutant plants, which were grown for 4 weeks in permissive light, were studied 3 d after transfer to low light. The *saul1* mutants but not the *saul1/pad4* double mutants exhibited yellowing of leaves and reduced growth. B, Induction of gene expression of senescence- and SA-related genes. Relative expression levels for *AAO3*, *WRKY6*, and *EDS16* were determined by qPCR from three independent experiments. Means ± SD ($n = 3$) are shown for wild-type (light gray bars), *pad4* (black bars), *saul1-1* (white bars), and *saul1-1/pad4* double mutant (dark gray bars) seedlings. Induction of all three genes was only seen in *saul1-1* seedlings. This induction was prevented in *saul1-1/pad4* double mutants.

transcript levels in *saul1* mutants were still indistinguishable from those in wild-type plants after 1 d of low-light treatment. In contrast, *WRKY53*, *WRKY6*, and *AtNAP* transcript levels were already significantly increased after 1 d (Fig. 3). Expression of these transcription factor genes has previously been shown to induce plant senescence (Robatzek and Somssich, 2002; Miao et al., 2004; Guo and Gan, 2006). These data suggest that *WRKY53*, *WRKY6*, and *AtNAP* are activated prior to *ORE1* in *saul1* mutants. It will be necessary to find out the hierarchy in the regulatory network of transcription during low-light-induced senescence and cell death in *saul1* mutants compared with age-dependent processes in wild-type plants.

The microarray analyses indicated that SA-dependent signaling represents the first detectable regulatory event in low-light-challenged *saul1-1* mutant seedlings. The SA biosynthetic genes *EDS16* and *EDS5* and the SA signaling components *PAD4*, *EDS1*, and *WRKY70* that are crucial for SA signaling were present at higher levels already at T_6. Various presumed defense effector genes were also activated, including *PR1*, *PR2*, and *PR5* (Supplemental Table S1; Glazebrook, 2005). In line with the predominant association of SA with resistance to biotrophic pathogens, we not only found an overlap of gene expression changes in *saul1* mutants with SA-triggered expression changes but also a high degree of overlap with expression changes upon treatment with powdery mildew (Nishimura et al., 2003; Wang et al., 2007). Full activation of SA and defense marker genes requires the signaling component NPR1 and involves transcriptional regulators such as TGACG MOTIF-BINDING FACTOR5 (TGA5) and TGA6 (Kim and Delaney, 2002; Kesarwani et al., 2007; Trujillo and Shirasu, 2010). The *NPR1* gene and the genes encoding the transcriptional regulators TGA5 and TGA6 were indeed increased with a small delay at T_{24} (Supplemental Table S1). At T_6, many genes with functions in the control of host defense against pathogens and of cell death, which is not essentially required for defense gene expression, were also regulated (Supplemental Table S1). It has been shown previously that SA causes the repression of auxin genes (Wang et al., 2007). This repression, which is thought to be an important aspect of SA-dependent defense responses, was observed in low-light-grown *saul1* mutants at T_{24}/T_{48} and may partly be responsible for the growth defects of *saul1* seedlings in low light (Supplemental Table S5). At this point in time, and thus also following SA action, transcript levels of ABA-regulated genes were changed (Supplemental Table S3). At the gene expression level, an overlap between pathogenicity responses and ABA application has previously been described (de Torres-Zabala et al., 2007). In addition, genetic and molecular analyses of *constitutive expressor of PR genes22* (*cpr22*) mutants showed that elevated SA content resulted in alterations of ABA signaling (Mosher et al., 2010).

The importance of SA pathways for *saul1* phenotypes was supported by physiological and genetic analyses. Increased SA content was determined in low-light-grown *saul1-1* mutants, exogenous application of SA was sufficient to trigger *saul1* senescence (Fig. 4), and disruption of *PAD4* in *saul1-1/pad4* double mutants was sufficient to fully suppress *saul1* defects (Fig. 5). However, disruption of *NPR1* and *EDS5* in *saul1-1/npr1-1* and *saul1-1/eds5-1* double mutants did not suppress *saul1* phenotypes, thus pointing to a specific requirement of *PAD4*. The *PAD4* gene encodes a protein with similarity to triacyl glycerol lipases, but PAD4 function could not be experimentally shown yet (Jirage et al., 1999). Initially, *pad4* mutant alleles were identified in a screen for enhanced disease susceptibility (Glazebrook et al., 1996), and additional alleles were found when searching for mutants with reduced *R* gene-mediated resistance to *Peronospora parasitica*-specified resistance (Feys et al., 2001). When infected with the virulent bacterial pathogen *Pseudomonas syringae* pv *maculicola* ES4326, these *pad4* mutants show typical properties, such as lower SA content, reduced expression of *PR1*, and lower content of the phytoalexin camalexin. Application of SA rescues many of the *pad4* mutant phenotypes. Together with the putative lipase EDS1, PAD4 is thought to be part of an amplification loop that generates increased SA levels sufficient for SA signaling and defense (Wiermer et al., 2005). Direct interactions have been detected between PAD4 and EDS1, between EDS1 and SAG101, a well-known positive regulator of senescence, and between all three proteins in a ternary complex (He and Gan, 2002; Rietz et al., 2011; Zhu et al., 2011). It has been shown that PAD4 and EDS1 participate not only in resistance to pathogens but also in the transduction of photooxidative stress signals resulting in cell death and growth inhibition. Cell death and growth inhibition phenotypes of *lsd1*, *acd6-1*, *cpr1*, and *cpr6* mutants require the presence of *PAD4* and *EDS1*, as demonstrated by the rescue of mutant phenotypes in double mutants with *pad4* or *eds1* (Jirage et al., 2001; Rustérucci et al., 2001; Mateo et al., 2004; Ochsenbein et al., 2006; Mühlenbock et al., 2008; Ng et al., 2011). The SA amplification loop is also crucial for cell death and senescence, increased SA content, and gene expression changes in *saul1* mutants challenged by low light (Fig. 5). Such a role of SA in the regulation of gene expression in cell death during the final stage of leaf senescence has previously been suggested from microarray analyses (Morris et al., 2000).

From the presented data, a timing of events for low-light-induced *saul1* senescence and cell death can be deduced. The E3 ubiquitin ligase SAUL1 is associated with the plasma membrane (Drechsel et al., 2011). We hypothesize that target proteins are either modulated by monoubiquitination or subjected to degradation by polyubiquitination through SAUL1 at the plasma membrane. This regulation of target proteins is important to prevent cell death and senescence in response to low light or salt stress. In the absence of SAUL1 in *saul1* mutants, the regulation of genes involved in defense and/or the SA responses was the

first event (T_6) that we resolved after transfer to low light. In line with a role of SA in *saul1* senescence and cell death, SA content increased in *saul1* mutants in low light and application of SA triggered growth arrest and yellowing of leaves in *saul1* mutant seedlings growing in permissive light (Fig. 5). However, SA application did not result in yellowing of leaves but only in growth arrest in wild-type seedlings at this time. This indicated that SA is not sufficient to trigger yellowing of leaves in the presence of functional SAUL1 as easily as in the absence of SAUL1 in *saul1* mutants, and thus that other changes in *saul1* mutants are required for the complete SA response. This may depend on the activation of *PAD4*, because *saul1* phenotypes were suppressed in *saul1-1/pad4-1* double mutants. Following regulation of defense and SA genes, repression of auxin genes and regulation of ABA genes were detected at T_{24}. This might be due to increased SA activity, because SA effects on the expression of auxin and ABA signaling components have been published previously (Wang et al., 2007; Mosher et al., 2010).

We have shown that during senescence and cell death, plasma membrane-associated SAUL1 is important in suppressing prodeath events. In animals, extrinsic signaling leading to programmed cell death has recently been shown to involve ubiquitin-dependent steps at the plasma membrane, too (Jin et al., 2009). Cell death control at the plasma membrane thus represents another facet of ubiquitination. The Arabidopsis RING1 E3 ligase that has a function in pathogen-induced programmed cell death is also associated with the plasma membrane in lipid rafts (Lin et al., 2008). Recently, it has been shown that the pepper (*Capsicum annuum*) plasma membrane-associated *CaRING1* is part of the SA-dependent defense responses (Lee et al., 2011). Regulated ubiquitin/proteasome-dependent processing at membranes has been suggested as a possible mechanism of transcription factor regulation and thus gene expression control (Hoppe et al., 2001). Future research will help to identify in vivo targets that are modified by SAUL1 at the plasma membrane.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Treatments

Prior to sowing on petri dishes containing Murashige and Skoog salts, pH 5.7, 0.05% MES, and 0.8% phytoagar (Duchefa Biochemie), Arabidopsis (*Arabidopsis thaliana*) wild-type and *saul1* mutant seeds were surface sterilized and stratified in the dark at 4°C for 3 d. Plants were grown in long-day conditions (16 h of light, 8 h of dark) at 21°C and photon flux densities of 20 to 100 μmol m^{-2} s^{-1} (as indicated in the text and/or figure legends) for the indicated numbers of days starting with transfer from dark to light after stratification. Photon flux densities were determined using a LI-250A light meter in combination with a Quantum sensor (LI-COR Biosciences). For SA treatment, SA was added to the medium to a final concentration of 0.5 mM. For studying NaCl-dependent gene expression, wild-type and *saul1-1* plants were grown on agar for 8 d and then transferred to a hydroponics system containing 150 mM NaCl or control solution for 2 d prior to harvesting the samples. To provide independent biological replicates, three wild-type samples and three *saul1-1* samples were collected for all treatments, and each sample consisted of five individual seedlings.

qPCR Analysis of Transcript Levels

qPCR analysis was performed as described (Raab et al., 2006). Samples were standardized to *ACTIN* mRNA levels. Gene expression levels were normally given relative to the expression levels in the wild type (set to 1) at the respective control conditions. Significances of differences were determined by Student's *t* test analysis. The respective columns were labeled with letters a ($P < 0.01$) and b ($P < 0.05$) in the figures. Primers for PCR amplification were 5′-CTTACCATGGAAGGCTAAGATGGG-3′ and 5′-TTCCAATAACCGGCTTCTGTCG-3′ for *ORE1* (At5g39610), 5′-TCCTTACAAAGGCGAAGACGCTAC-3′ and 5′-AACCGGGACATCCTCATAACCTG-3′ for *SAG12* (At5g45890), 5′-CGCATCCTCACCGAGCGTACAAC-3′ and 5′-GGTGTTCTTGTGATCGCCTGC-3′ for *WRKY53* (At4g23810), 5′-GGGAAGAGACTTGGGCGTG-3′ and 5′-GCAACGGATGGTTATGGTTTC-3′ for *WRKY6* (At1g62300), 5′-GCCATTCACAGCGGTTCAAG-3′ and 5′-CAACAAATGAGCCAGCGAAC-3′ for *AtNAP* (At1g69490), 5′-CTCCGCCATGGTCTCTACTCATAC-3′ and 5′-ACTTAGGGAAGAAAGCCGATGATG-3′ for *At2g15830*, 5′-ACGACCATTAGCTTCGTGCTTCC-3′ and 5′-AAATCGCATTGGCCATGTTTCTCC-3′ for *At5g39520*, 5′-GCTTGGCTAGCACAGTTACAGC-3′ and 5′-CACTGCAGACACCTAATTGAGTCC-3′ for *EDS16* (At1g74710), 5′-CGTGCTCATATCCTCTGCTGC-3′ and 5′-CCGTCAACGCAAATGGTCTG-3′ for *SAG13* (At2g29350), 5′-CTCGTAATCTCAGCTCTTATTTG-3′ and 5′-CACTACACTCAAGTTGTTTGGAG-3′ for *PR1* (At2g14610), 5′-GAGCATTTGTTCAAGGCATCGG-3′ and 5′-GCAAAGAGACTTCACCACAGGC-3′ for *AAO3* (At2g27150), and 5′-AGATACGCGAGCACAACGCAAG-3′ and 5′-TTTCTCGCCTCATCCAACCACTC-3′ for *PAD4* (At3g52430).

Trypan Blue Staining

To visualize dying cells, leaves were detached and submerged in lactophenol-trypan blue solution (0.03% trypan blue, 33% [w/v] lactic acid, 33% water-saturated phenol, and 33% glycerol). Samples were incubated at 99°C for 1 min followed by incubation at room temperature for 24 h, washed in chloral hydrate solution (2.5 g mL^{-1}) to reduce background staining, and photographed using a Leica MZLIII stereomicroscope (Leica Microsystems).

Determination of SA and SA Glucoside Contents

Free SA and SA glucoside were extracted and analyzed as described with minor modifications (Voll et al., 2012). Per sample, four to seven seedlings (28–38 mg fresh weight) that were grown for 13 d in 100 μmol m^{-2} s^{-1} followed by low-light challenge (20 μmol m^{-2} s^{-1}) for 0, 6, 12, 24, and 48 h were harvested, supplemented with 250 ng of *o*-anisic acid (Acros Organics, Thermo Fisher Scientific) as an internal standard, and then extracted once with 600 μL of 70% methanol at 65°C for 1 h and once with 600 μL of 90% methanol at 65°C for 1 h. The solvent of the combined extracts was then evaporated with a vacuum concentrator, followed by a precipitation step with 500 μL of 5% (w/v) TCA. Free phenols were partitioned two times against 600 μL of cyclohexane:ethyl acetate (1:1). The combined organic phases were evaporated and resuspended in 400 μL of 20% acetonitrile in 25 mM KH_2PO_4 (pH 2.6). The remaining aqueous phase was acidified with 1 volume of 8 M HCl and incubated at 80°C for 1 h. After partitioning as above, the organic phase was supplemented with 20 μL of water to prevent sublimation of SA, evaporated, and resuspended in 400 μL of 20% acetonitrile in 25 mM KH_2PO_4 (pH 2.6; i.e. the HPLC starting mobile phase). HPLC separation of SA and *o*-anisic acid was performed on a Dionex Summit system (P680, ASI-100, TCC-100, RF-2000) equipped with a Phenomenex Luna Security Guard C18 column (4.0 × 3.0 mm) followed by a 5-μm Luna C18(2) reverse-phase column (250 × 4.6 mm) as described by Voll et al. (2012).

Microarray Analysis, Data Preprocessing, and Differential Gene Expression Analysis

Total RNA for microarray analysis was isolated in two consecutive steps using Trizol reagent (Invitrogen) and the Plant RNeasy extraction kit (Qiagen) from wild-type and *saul1-1* mutant seedlings grown on petri dishes in permissive light (60 μmol m^{-2} s^{-1}) for 11 d and then challenged with low light (20 μmol m^{-2} s^{-1}) for 6, 24, or 48 h. To provide biological replicates, three wild-type and three *saul1-1* mutant samples were harvested for each time point. Each sample consisted of at least 15 individual seedlings. For microarray analysis, 5 μg of RNA was processed and hybridized to Affymetrix GeneChip Arabidopsis ATH1 Genome Arrays using the Affymetrix One-Cycle Labeling and

Control (Target) kit according to the manufacturer's instructions. Raw signal intensity values were computed from the scanned array images using the Affymetrix GeneChip Command Console 3.0. Statistical gene expression analysis was performed analogously to the analysis described by Deeken et al. (2006). Data from gene expression microarrays were analyzed using the statistical software R (R Development Core Team; www.R-project.org) and add-on packages for microarray analysis from Bioconductor (Gentleman et al., 2004). Raw data were normalized with a variance stabilization algorithm (Huber et al., 2002). To summarize individual probes of an Affymetrix probe set, the median polish algorithm from the Robust Multichip Average normalization was used (Irizarry et al., 2003). Differentially expressed genes were assessed with a linear model approach implemented in the R package LIMMA (Smyth, 2004). Lists of differentially expressed genes were corrected for multiple testing with a false discovery rate (Benjamini and Hochberg, 2000), yielding an adjusted *P* value for each gene.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Analysis of *saul1/npr1-1* and *saul1/eds5-1* double mutants.

Supplemental Table S1. Up-regulated genes in low-light-treated *saul1-1* mutant seedlings.

Supplemental Table S2. Down-regulated genes in low-light-treated *saul1-1* mutant seedlings.

Supplemental Table S3. Regulation of ABA-induced genes in low-light-treated *saul1-1* mutant seedlings.

Supplemental Table S4. Regulation of genes up-regulated at T_6 in low-light-treated *saul1-1* mutant seedlings by powdery mildew and SA.

Supplemental Table S5. Regulation of auxin-related genes, which were identified to be modulated by SA, in low-light-treated *saul1-1* mutant seedlings.

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