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SAUR49 Can Positively Regulate Leaf Senescence by Suppressing SSPP in *Arabidopsis*

Running Head: SAUR49 can suppress SSPP during leaf senescence

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SAUR49 Can Positively Regulate Leaf Senescence by Suppressing SSPP in *Arabidopsis*

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List of author contributions: N.N.W. conceived and designed the study, supervised the experiments, and compiled and finalized the article. Z.W., J.Z., and Y.C. performed the experiments. N.N.W., Z.W., Y.M., and D.W. analyzed the data. Z.W., Y.M., and N.N.W drafted and revised the manuscript. All authors read and approved the final manuscript.

Abbreviations: DEX, dexamethasone; SAUR, small auxin-up RNA; SARK, senescence-associated receptor-like kinase; SSPP, senescence suppressed protein phosphatase; GUS, β-glucuronidase; Y2H, yeast two-hybrid; WT, wild type.

Abstract

The involvement of small auxin-up RNA proteins (SAURs) in leaf senescence has been more and more acknowledged, but the detailed mechanisms remain unclear. In the present study, we performed yeast two-hybrid assays (Y2Hs) and identified SAUR49 as an interactor of SSPP, which is a PP2C protein phosphatase that negatively regulates Arabidopsis leaf senescence by suppressing the leucine-rich repeat receptor-like protein kinase (LRR-RLK) SARK, as reported previously by our group. The interaction between SAUR49 and SSPP was further confirmed *in planta*. Functional characterization revealed that SAUR49 is a positive regulator of leaf senescence. The accumulation level of SAUR49 protein increased during natural leaf senescence in *Arabidopsis*. The transcript level of *SAUR49* was upregulated during *SARK*-induced premature leaf senescence but downregulated during *SSPP*-mediated delayed leaf senescence. Overexpression of *SAUR49* significantly accelerated both natural and dark-induced leaf senescence in *Arabidopsis*. More importantly, *SAUR49* overexpression completely reversed *SSPP*-induced delayed leaf senescence. In addition, overexpression of *SAUR49* reversed the decreased plasma membrane (PM) H^+-ATPase activity mediated by *SSPP*. Taken together, the results showed that *SAUR49* functions in accelerating the leaf senescence process via the activation of *SARK*-mediated leaf senescence signalling by suppressing SSPP. We further identified four other SSPP-interacting SAURs, SAUR30, SAUR39, SAUR41 and SAUR72, that may act redundantly with SAUR49 in regulating leaf senescence. All these observations indicated that certain members of the SAUR family may serve as an important hub that integrates various hormonal and environmental signals with senescence signals in *Arabidopsis*.

Keywords: SAUR49, SARK, SSPP, leaf senescence, auxin, H^+-ATPase activity, *Arabidopsis*

Introduction

Leaf senescence is a highly regulated developmental process accompanied by macromolecule breakdown as well as the remobilization of nutrients from senescing leaf cells into seeds, storage organs, or actively growing tissues (Gan and Amasino 1997, Lim et al. 2007). Leaf senescence occurs in an age-dependent manner but also in response to various internal signals and environmental cues. Among the internal signals are phytohormones, which play various roles in the senescence process. For instance, ethylene, abscisic acid (ABA), salicylic acid (SA), jasmonates (JAs), and strigolactone (SL) can promote leaf senescence, whereas gibberellic acids (GAs), and cytokinins suppress leaf senescence (Schippers et al. 2015, Ueda and Kusaba 2015). However, unlike other plant hormones, the detailed functions of auxin in leaf senescence remain controversial. Several previous studies involving the exogenous application of indole-acetic acid (IAA) indicated that auxin negatively regulates leaf senescence (Noh and Amasino 1999, Kim et al. 2011). However, recent emerging evidence has suggested that auxin positively regulates leaf senescence (Xu et al. 2011; Hou et al. 2013).

Small auxin-up RNA (*SAUR*) genes belong to one of the three early auxin-responsive gene families and can rapidly respond to auxin applications without *de novo* protein synthesis (Abel et al. 1994). *SAUR*s comprise 81 family members (including two pseudogenes) in *Arabidopsis* (Hagen and Guilfoyle 2002), most of which lack introns and encode small (10-20 kD) proteins. SAURs exhibit widespread subcellular localization; they occur in the nucleus (Knauss et al. 2003, Park et al. 2007), cytosol (Kant et al. 2009, Kong et al. 2013, Qiu et al. 2013, Xu et al. 2017) and plasma membrane (PM) (Spartz et al. 2012, Chae et al. 2012). SAUR proteins are highly unstable and appear to be subject to ubiquitin-mediated proteolysis. Several *SAUR*s have been reported to be positive regulators of leaf senescence. For instance, compared with wild-type (WT) plants, rice plants overexpressing *SAUR39* exhibited an earlier start of senescence in mature leaves and a greater number of senescing leaves (Kant et al. 2009). Overexpression of *SAUR36* led to early leaf senescence, while the T-DNA insertion mutant *saur36* displayed delayed leaf senescence in

Arabidopsis (Hou et al. 2013). Likewise, the leaves of *SAUR10* overexpression lines also senesced earlier than did the WT leaves (Bemer et al. 2017). Our group recently demonstrated that overexpression of *SAUR72* also led to precocious leaf senescence in *Arabidopsis* (Zhou et al. 2018). However, the detailed mechanism underlying SAUR-regulated leaf senescence has not yet been reported.

In addition to being involved in leaf senescence, *SAUR*s also participate in many other important physiological processes in responses to different hormonal and environmental signals. For instance, overexpression of several *SAUR*s in *Arabidopsis* resulted in pleiotropic phenotypes such as increased hypocotyl length, increased lateral root development, expanded petals and stamen filaments and twisted inflorescence stems (Spartz et al. 2012, Chae et al. 2012, Kong et al. 2013, Li et al. 2015). Most of these processes are associated with increased cell expansion and auxin efflux. Additional studies demonstrated that *SAUR*s promote cell expansion by inhibiting PP2C-D family protein phosphatases, especially the PM-localized PP2C-D proteins (D2, D5, D6), thereby activating PM H^+-ATPase (Spartz et al. 2014, Ren et al. 2018). However, *SAUR* members also act as negative regulators of auxin synthesis and transport. Overexpression of *OsSAUR39* and *OsSAUR45* resulted in reduced free IAA levels and reduced polar auxin transport in rice. The expression levels of five *OsYUCCA* genes involved in auxin biosynthesis and seven *OsPIN* genes encoding auxin efflux carriers were downregulated in *OsSAUR45*-overexpressing rice. In addition, exogenous auxin applications could largely rescue the phenotypes of *OsSAUR39*- and *OsSAUR45*-overexpressing rice plants (Kant et al. 2009, Xu et al. 2017).

Our group previously reported that the leucine-rich repeat receptor-like protein kinase (LRR-RLK) SARK positively regulates leaf senescence (Xu et al. 2011); moreover, the protein phosphatase SSPP negatively regulates leaf senescence by directly interacting and dephosphorylating the cytoplasmic domain of AtSARK (Xiao et al. 2015). To elucidate the molecular mechanism of *SARK*/*SSPP*-mediated leaf senescence signalling in *Arabidopsis*, we performed a yeast two-hybrid (Y2H) assay against a normalized *Arabidopsis* cDNA library in which SSPP was used as bait. A

putative candidate was obtained containing the complete open reading frame of SAUR49. Here, we demonstrate that SAUR49 can interact with SSPP both *in vitro* and *in planta*. Overexpression of *SAUR49* significantly accelerated leaf senescence and increased PM H^+-ATPase activity in *Arabidopsis*. Moreover, overexpression of *SAUR49* completely reversed the *SSPP*-induced delayed leaf senescence and cell expansion-related phenotypes. Taken together, our results suggest that SAUR49 positively regulates leaf senescence by inhibiting SSPP and thus indirectly activates the function of SARK. Besides SAUR49, four other senescence-upregulated SAURs were also found to interact with SSPP and may function redundantly in this process. Since the *SAUR* family genes respond to a wide variety of upstream signals, the interactions between SSPP and these SAURs may serve as an important cross-talk hub that integrates various hormonal and environmental stimuli with SARK-mediated leaf senescence signalling.

Results

SAUR49 interacts with SSPP

Using Y2H assays, we identified SAUR49 as protein that putatively interacts with SSPP. To confirm the interaction between SSPP and SAUR49 *in vitro*, a protein pull-down assay was carried out. In this assay, 6xHis-tagged SSPP (His-SSPP) and glutathione S-transferase (GST)-tagged SAUR49 (GST-SAUR49) fusion proteins were expressed separately. As shown in Fig. 1a, GST-SAUR49 bound to His-SSPP, while the GST negative control did not, suggesting that SAUR49 physically interacts with SSPP *in vitro*. We also tested this interaction in yeast using a Y2H assay. It was shown that the yeast cells co-expressing SAUR49-AD and SSPP-BD could grow on the quadruple dropout medium (SD/-Leu-Trp-His-Ade), the same as the yeast cells harbouring pGADT7-T and pGBKT7-53 which served as the positive control (Fig. 1b), further suggesting a physical interaction between SAUR49 and SSPP.

We further examined the subcellular localization of SAUR49 by transforming *Arabidopsis* mesophyll cell protoplasts with *35S:SAUR49-eYFP* plasmids. The results showed that SAUR49 was localized to the cytoplasm (Fig. 1c), which was the same for SSPP. To determine whether these two proteins could interact in plant cells, we carried out a bimolecular fluorescence complementation (BiFC) assay. A yellow fluorescent protein (YFP) fluorescence signal was observed in Agrobacterium-infiltrated tobacco leaves that expressed both SAUR49-nYFP and SSPP-cYFP fusion proteins. However, no fluorescence signal was observed in the leaf samples infiltrated with Agrobacterium that harboured one gene construct in combination with an empty vector that expressed only the split YFP counterpart (Fig. 1d). These results indicated that SAUR49 could interact with SSPP *in planta*.

SAUR49 protein accumulates during leaf senescence

The expression of *SAUR*s is regulated at multiple levels (Hong and Gray 2015). *SAUR* transcripts were shown to be unstable due to their high turnover rates (Chae et al. 2012). To investigate the role of *SAUR49* in natural leaf senescence, we generated both *SAUR49:GUS* and *SAUR49:SAUR49-GUS* transgenic *Arabidopsis* plants to

examine *SAUR49* promoter activity and protein accumulation during leaf senescence by histochemical β-glucuronidase (GUS) staining. The results revealed stronger GUS activity in mature and early senescent leaves but lower GUS activity in late senescent leaves in 28-day-old *SAUR49:GUS* plants (Fig. 2a, top), suggesting that the promoter activity of *SAUR49* decreased during late senescence. Consistently, quantitative RT-PCR analysis showed that the accumulation level of *SAUR49* transcript gradually declined as the sixth leaves of wild-type *Arabidopsis* developed from 14 to 32 days after emergence (DAE) (Fig. 2a, bottom). However, the blue colouring of the 28-day-old *SAUR49:SAUR49-GUS* plants did not decrease in the old leaves when compared to that in the mature leaves (Fig. 2b, top). Furthermore, anti-GUS western blots in which anti-actin served as a loading control confirmed that SAUR49 accumulation gradually increased during leaf senescence (Fig. 2b, bottom). These results suggested that SAUR49 may undergo posttranscriptional modification and gradually accumulated as the plants aged.

A CHX-chase assay was used to investigate whether the accumulation of SAUR49 protein is regulated in an age-dependent manner. The sixth true leaves of *35S:SAUR49-Flag* transgenic *Arabidopsis* plants at both young and early senescent stages were treated with cycloheximide (CHX) and harvested at four different time points. As shown in Figure 2c, the accumulation of SAUR49-Flag protein nearly disappeared within 1 hour in CHX-treated young leaves. However, it was still clearly detectable even after 2 hours of CHX treatment in early senescent leaves. These results indicated that the stability of SAUR49 protein was increased by aging signals.

Our group previously generated dexamethasone (DEX)-inducible *GVG:SARK* transgenic *Arabidopsis* and *GVG:GUS* transformation control plants (Xu et al. 2011). The induced overexpression of *AtSARK* led to early leaf senescence. We also obtained *SSPP*-overexpressing *Arabidopsis* (*SSPPox*) plants, which experienced delayed leaf senescence by suppressing *SARK*-mediated senescence signal transduction (Xiao et al. 2015). To analyse whether *SAUR49* is involved in *SARK*-*SSPP*-mediated leaf senescence, we measured the relative transcript levels of *SAUR49* in the fifth and sixth leaves of either DEX-treated 21-day-old *GVG:SARK* or *SSPP*-overexpressing

Arabidopsis at developmental stage 5.10 (Boyes et al. 2001) by quantitative RT-PCR using *TIP41-like* as an internal control. The results showed that the transcript levels of *SAUR49* were comparatively low in the leaves of mock- and DEX-treated *GVG:GUS* control plants as well as in the leaves of mock-treated *GVG:AtSARK* plants. However, the expression of *SAUR49* was significantly upregulated in the leaves of DEX-treated *GVG:AtSARK* transgenic plants (Fig. 2d, left). Compared with that in the wild-type control (WT), the relative expression level of *SAUR49* decreased markedly in the leaves of *SSPPox* plants (Fig. 2d, right). In the loss-of-function mutant of *SSPP*, *sspp-1*, the accumulation level of *SAUR49* transcripts showed no significant difference with that in the WT control during natural leaf senescence, however, it was significantly increased in the dark-induced leaf senescence (Supplementary Fig. S1).

Ectopic expression of *SAUR49* causes premature leaf senescence

To further investigate the biological functions of *SAUR49*, we examined the phenotypes of five independent lines of *35S:SAUR49-Flag* transgenic *Arabidopsis* (*SAUR49ox*). Compared with the WT control, all transgenic lines displayed premature leaf senescence (Fig. 3a). To gain a better view of the function of *SAUR49* in leaf senescence, the rosette leaves of two typical lines, line 8 and line 15, were compared with their corresponding WT controls at the same developmental stage. As shown in Fig. 3b, the *SAUR49ox* transgenic plants presented clearly accelerated leaf senescence. While the leaves of the WT plants remained green, the first eight leaves of line 8 and the first five leaves of line 15 had already began to turn yellow (Fig. 3b). In addition to their early senescence, the rosette leaves of *SAUR49ox* also became elongated and narrow. In addition, the growth of inflorescence axis in *SAUR49ox* was slowed and appeared twisted (Supplementary Fig. S2a). Compared with those of the WT flowers, the calyxes of the transgenic flower organs were over-expanded, and the petals failed to open completely (Supplementary Fig. S2b).

The role of *SAUR49* in dark-induced senescence was also investigated. The sixth leaves of 21-day-old *SAUR49ox* and its WT control were detached and incubated for three days in darkness. As shown in Fig. 3c, loss of chlorophyll content was

accelerated in *SAUR49ox,* suggesting that over-expression of *SAUR49* promoted dark-induced leaf senescence.

To characterize the senescence phenotypes at the molecular level, we measured the transcript levels of several photosynthesis-related or senescence-related genes present in the fifth and sixth leaves of four-week-old *SAUR49ox* plants. As expected, quantitative RT-PCR analysis revealed that, compared with those in the leaves of WT *Arabidopsis* plants, the transcript levels of photosynthesis-related genes such as *GTR1* (Mccormac et al. 2001) and *RUBISCO LARGE SUBUNIT* (*RbcL*) (Krebbers et al. 1988) in the leaves of *SAUR49ox* plants decreased substantially, while the transcript levels of *ACCELERATED CELL DEATH1* (*ACD1*), which encodes the chlorophyll breakdown enzyme pheide a-oxygenase (Pružinská et al. 2003), increased (Fig. 4a). Consistently, compared with those of the WT plants, the transcript levels of the widely used senescence marker gene *SAG12* (Gan and Amasino 1995); the PP2C family protein phosphatase-encoding gene *SAG113* (Zhang et al. 2012); and four critical senescence-related transcription factors, including *NAC1* (Kim et al. 2009), *WRKY6* (Robatzek and Somssich 2002), *WRKY53* (*Miao* et al. *2004*), and *AtNAP* (Guo and Gan 2006), in the leaves of the *SAUR49ox* plants all markedly increased (Fig. 4b). All these results indicated that *SAUR49* positively regulates senescence in *Arabidopsis*.

Since SAUR49 interacted with SSPP and was involved in SARK-induced leaf senescence, the expression levels of *SSPP* and *SARK* in the fifth and sixth leaves of four-week-old *SAUR49ox* plants were also compared with those of WT plants. The results showed that the levels of *SARK* transcript significantly increased in the *SAUR49ox* leaves, while the levels of *SSPP* transcript markedly decreased (Fig. 4c), implying that overexpression of *SAUR49* could promote *SARK*-mediated leaf senescence signal transduction.

Overexpression of *SAUR49* suppresses the function of SSPP in delaying leaf senescence

To explore the functional relationship of *SAUR49* and *SSPP* in the regulation of leaf

senescence, we generated *SAUR49ox/SSPPox* hybrid *Arabidopsis* plants by crossing *SAUR49ox* plants with *SSPPox* plants. The overexpression of exogenous *SAUR49* and *SSPP* was confirmed by semi-quantitative RT-PCR (Fig. 5a, bottom). While *SAUR49ox* displayed early leaf senescence and *SSPPox* displayed delayed leaf senescence, the newly formed hybrid *Arabidopsis* exhibited accelerated leaf senescence, which was the same as that of *SAUR49ox* (Fig. 5a, b). This finding suggests that overexpression of *SAUR49* reversed the delayed leaf senescence induced by *SSPP*. In addition, the delayed bolting and flowering time of *SSPPox* were also reversed by *SAUR49* overexpression under the long-day photoperiod (Fig. 5c).

Consistently, when the expression levels of genes related to chlorophyll metabolism and chloroplast function such as *ACD1* were downregulated and those of *RbcL* and *RbcS* were upregulated in the fifth and sixth leaves of *SSPPox* at stage 5.10, the expression levels of those same genes were up- and downregulated instead, respectively, in the hybrid *Arabidopsis* at the same developmental stage (Fig. 5d). In addition, the transcript levels of several senescence-associated marker genes, such as *SAG113*, *AtNAP* and *WRKY6*, were all upregulated in *SAUR49ox/SSPPox* and did not show significant differences from those in *SAUR49ox*, representing expression patterns that were opposite to those observed in *SSPPox* (Fig. 5d).

Overexpression of *SAUR49* reverses the reduced PM H^+-ATPase activity mediated by SSPP

Previous studies have demonstrated that PP2C-D proteins function as repressors of PM H^+-ATPases by promoting the dephosphorylation of their C-terminal autoinhibitory domain, thus inhibiting hypocotyl growth via an acid-growth mechanism (Hager 2003, Spartz et al. 2014, Ren et al. 2018). The *SSPPox* plants in the current study indeed exhibited reduced hypocotyl elongation phenotypes (Supplementary Fig. S3). However, the *SAUR49ox/SSPPox Arabidopsis* hybrid had elongated hypocotyl which was similar to that of *SAUR49ox* (Supplementary Fig. S3). In addition, the *SSPPox* plants consistently displayed phenotypes with decreased proton motive force, including insensitivity to Li^+ in root growth inhibition assays

(Fig. 6a) and decreased medium acidification, as indicated by bromocresol purple (Fig. 6b). By contrast, the *SAUR49ox* plants displayed phenotypes with increased PM H^+-ATPase activity, such as hypersensitivity to Li^+ and increased medium acidification. The same results were observed for the *SAUR49ox/SSPPox* hybrid *Arabidopsis* plants (Fig. 6a, b). These findings indicated that the insensitivity to LiCl and decreased medium acidification of the *SSPPox* plants were also reversed by overexpression of *SAUR49*. Taken together, these results revealed that *SAUR49* overexpression reversed the reduced PM H^+-ATPase activity mediated by SSPP.

Identification of four SAURs that may act redundantly with SAUR49 in promoting leaf senescence

To evaluate the role of SAUR49 in the regulation of leaf senescence, we also generated *saur49* null mutant alleles using the clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-Associated 9 (Cas9) system, in which the target sequence is located within a 39-57 bp region in the *SAUR49* genomic DNA. Two *saur49* gene-editing mutant alleles were obtained: designated *saur49-c2* and *saur49-c7*. Sequencing analysis revealed that the homozygous *saur49-c2* and *saur49-c7* mutants had 11 and 17 base deletions, respectively, which resulted in premature translation terminations at positions 22 and 24 of their amino acid sequence, respectively (Supplementary Fig. S4a). We examined the senescence-related phenotypes of the two mutants but observed no significant differences between those phenotypes and that of the WT plants (Supplementary Fig. S4b). This finding is most likely due to the functional redundancy of the multiple *SAUR* members.

There are 79 *SAUR* family members in *Arabidopsis*. In addition to *SAUR49*, nine other *SAUR* genes were found to be up-regulated during leaf senescence based on *Arabidopsis* eFP browser prediction and further confirmation by qPCR (Fig. 7a). Yeast-two-hybrid assays were used subsequently to examine the interactions between these nine SAUR proteins and SSPP. As shown in Fig. 7b, four SAURs, namely SAUR30, 39, 41 and 72, could interact with SSPP, implying that they may act

redundantly with SAUR49 during leaf senescence.

Discussion

SAUR49 is a positive regulator of leaf senescence

Our results demonstrated that *SAUR49* proteins gradually accumulated in an age-dependent manner, although the transcriptional expression of *SAUR49* slightly decreased during late senescence (Fig. 2a, b). Moreover, the expression of *SAUR49* increased during *SARK*-induced leaf senescence but was inhibited during *SSPP*-mediated delayed leaf senescence (Fig. 2d). Furthermore, transgenic plants overexpressing *SAUR49* exhibited accelerated natural and dark-induced leaf senescence (Fig. 3, 4). These results suggest that SAUR49 positively regulates leaf senescence.

Our previous study revealed that the expression of *SARK* was detected in mature leaves and gradually increased with increasing leaf age (Xiao et al. 2015). The transcript level of *SAUR49* was elevated in *SARK*-overexpressing *Arabidopsis* at 24 hours after DEX treatment before the onset of a visible senescence phenotype (Fig. 2d). Consistently, histochemical GUS staining of *SAUR49:GUS* transgenic *Arabidopsis* revealed a similar expression pattern of *SAUR49* with *SARK* in mature and early senescent leaves. These observations suggest an indirect up-regulation of *SAUR49* by *SARK*, probably through a yet-unknown mechanism with specific transcription factor(s) involved. Further investigations including identification and functional characterization of these transcription factors are desirable.

The opposite accumulation patterns of *SAUR49* transcripts and proteins at the late stage of leaf senescence implied that *SAUR49* may be also regulated at the posttranslational level. The SAUR proteins were highly unstable and subject to degradation by the ubiquitin-26S proteasome pathway (Knauss et al. 2003, Chae et al. 2012, Spartz et al. 2012). In our study, SAUR49 seemed to be stabilized in an age-dependent manner by posttranslational modification (Fig. 2c). The detailed mechanism, however, still needs to be elucidated.

SAUR49 positively regulates leaf senescence by suppressing SSPP

SAUR49 was originally identified in our Y2H assay as a protein that interacts with the negative senescence regulator SSPP. The interaction between SSPP and SAUR49 was further confirmed by Y2H assays and a protein pull-down experiment, as well as *in planta* bimolecular fluorescence complementation (BiFC) assay that further demonstrated the interaction in the cytoplasm (Fig. 1). Phenotypic analysis revealed that overexpression of *SAUR49* could reverse the delayed leaf senescence phenotype caused by *SSPP* (Fig. 5). In line with these observations, the expression levels of several *SAG*s and photosynthesis-related genes in the fifth and sixth rosette leaves of *SAUR49ox/SSPPox* were up- and downregulated, respectively, and their expression levels did not significantly differ with those in the leaves of *SAUR49ox* (Fig. 5d). This finding indicates that the expression pattern of these marker genes was opposite to that observed in the leaves of *SSPPox*.

Transgenic *Arabidopsis* plants overexpressing *SAUR49* also exhibited several growth-related phenotypes phenotypes, such as elongated and narrow rosette leaves, and twisted inflorescence axes (Supplementary Fig. S2). Interestingly, the *SAUR49ox/SSPPox Arabidopsis* hybrid exhibited a phenotype that was similar to that of *SAUR49ox* but opposite to that of *SSPPox* (Figure5, Supplementary Fig. S3). In addition, similar to *SAUR49ox*, *SAUR49ox/SSPPox* exhibited elongated hypocotyl and over-expanded calyxes with increased PM H^+-ATPase activity, such as hypersensitivity to LiCl and increased medium acidification (Fig. 6). Together, these results suggest that SAUR49 functions as a repressor of SSPP during leaf senescence and cell expansion process.

The interaction between SSPP and certain members of SAUR family is an important cross-talk hub that integrates multiple hormonal and environmental signals with SARK-mediated leaf senescence signalling

In addition to SAUR49, four other senescence-upregulated SAUR family members, namely SAUR30, 39, 41 and 72, were also found to interact with SSPP and may function redundantly in regulating leaf senescence (Fig. 7). It is noteworthy that the

expression levels of *SAUR39* and *SAUR72* during leaf senescence were both relatively low (Fig. 7a). Consistently, as we previously reported, the premature leaf senescence in *SAUR72ox* was much weaker than those in *SAUR49ox* (Zhou et al. 2018). We therefore infer that the role of *SAUR39* and *SAUR72* in leaf senescence might be limited. By contrast, the expression levels of *SAUR30* and *SAUR41* during leaf senescence are much higher (Fig. 7a). These two *SAUR* members, together with *SAUR49,* may play more important roles in leaf senescence regulation. The other five senescence-upregulated SAUR members, including SAUR36, could not interact with SSPP (Fig. 7b), implying that they may regulate leaf senescence independently from SARK-SSPP mediated signalling pathway.

SAURs can respond very dramatically to a wide variety of upstream signals, including many hormones and environmental conditions, which are translated into gene expression via transcription factors involved in these pathways, such as PIFs, ARFs and BZRs (Ren and Gray 2015, Stortenbeker and Bemer 2019). Among the five *SAUR* genes identified in this study, only *SAUR41* and *SAUR72* are responsive to auxin (Kong et al. 2013, Ren and Gray 2015). *SAUR49* was found to be induced by gibberellin (Bai et al. 2012) while suppressed by jasmonate (Nemhauser et al. 2006). *SAUR30* was induced by both ABA and brassinosteroids (Nemhauser et al. 2006, Wang et al. 2012). All these suggest that the involvement of *SAURs* in the leaf senescence pathway may enable the plant to respond very dynamically to different hormonal signals and integrate them at the level of SAUR-SSPP to either induce or repress senescence. Similarly, abiotic stimuli such as the light conditions, temperature and drought can also influence *SAUR* expression (Stortenbeker and Bemer 2019). For example, *SAUR30, SAUR41, SAUR49* and *SAUR72* are responsive to dark-to-light transitions (Sun et al. 2016). *SAUR41* and *SAUR72* were found to be involved in salt stress tolerance (Qiu et al. 2019). Therefore, different environmental factors may also be integrated at the level of SAUR-SSPP to have an impact on leaf senescence.

Based on the results of the present study and on previous findings by our group (Xu et al. 2011, Xiao et al. 2015), we proposed a working model describing how *SAURs*, *SSPP*, and *SARK* regulate leaf senescence (Figure 8). The protein kinase

SARK is an important positive regulator of leaf senescence. By contrast, the protein phosphatase SSPP interacts with and dephosphorylates the cytosolic domain of SARK and works as a brake of the SARK-mediated leaf senescence process. SSPP is highly expressed in young leaves, while the expression of SARK is very limited. A high expression of SSPP inhibits the activity of SARK, thereby preventing young leaves from early senescence and maintaining normal leaf development and function. In senescing leaves, the expression of SSPP is reduced, but the expression of SARK is significantly increased. The expression of SAUR49 and other SSPP-interacting SAURs also increases, probably as a result of developmental and environmental signals, and influenced by the accumulation of SARK. In addition, SAUR49 protein abundance appears to be further enhanced by increased stabilization in the senescing leaves. The highly abundant SAURs then interacts with SSPP to repress its phosphatase activity, thereby increasing the SARK phosphorylation level and promoting leaf senescence. Because *SAUR49* is upregulated in *SARK* overexpression lines and *vice versa*, there may be a positive feedback loop, which would lead to an accelerated progression of leaf senescence. Our work made back-to-back support to the speculation in a recent review that SAURs may regulate leaf senescence by suppressing SSPP (Stortenbeker and Bemer 2019). And, we demonstrated that the above-mentioned five SAUR members especially SAUR49 participated in this process. It is also worthy to note that SSPP belongs to PP2C-D family which consists of nine members (Ren et al. 2018). It is therefore likely that inhibition of SSPP may only represent one of the possible mechanisms underlying the accelerated leaf senescence induced by SAUR49. To verify the authenticity of the working model proposed, effects of *SAUR49* overexpression in loss-of-function mutants of *SARK* and *SSPP* need to be further addressed.

It is worthy to note that we focused only on the senescence up-regulated SAUR members in this work. It's likely that there are other SSPP-interacting SAUR proteins that are not generally up-regulated in aging leaves, but specifically induced in response to environmental factors, such as extensive light or drought, thereby contributing to the dynamic switch to leaf senescence. A more thorough and extensive

investigation of this gene family is needed.

Future perspectives:

Recently, Gou's group demonstrated that SARK can function as either a co-receptor of CLV1, CLV2/CRN and RPK2 to regulate shoot apical meristem homeostasis (Hu et al. 2018) ， or co-receptor of BAM1/2 and RPK2 to regulate early anther development through a CLV-independent pathway (Cui et al. 2018). Besides, Anne et al (2018) recently suggested that SARK was required for full sensing of root-active CLE peptides in the early developing protophloem independently of CLV2-CRN. In addition, PP2C-D phosphatases were recently reported to be involved in the negative regulation of auxin-mediated cell expansion (Ren et al. 2018). Together, these results suggest that SARK and SSPP are essential components involved in different developmental processes. However, whether SARK and SSPP still work in concert, whether other key components are jointly involved in these processes, and whether auxin is still involved remain unclear. The proliferation and differentiation of stem cells maintained by the CLAVATA (CLV)-WUSCHEL (WUS) negative-feedback loop are crucial for continuous organ initiation and outgrowth. Whether these developmental signals exert effects on plant organ senescence or overall ageing requires further exploration. Moreover, how *SARK* integrates environmental cues, endogenous developmental cues and leaf senescence signals will also be an exciting topic for future research.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 was used in this study. *Arabidopsis* seeds were surface sterilized in 10% (v/v) sodium hypochlorite for 2 min, washed 10 times with sterilized water, germinated, and then grown on vertical plates (one-half-strength Murashige and Skoog [MS] medium that contained 0.8% [w/v] agar [pH 5.7] and 1% [w/v] sucrose and that was supplemented with or without antibiotics and chemical reagents) at 20°C ± 1°C with cycles of 16 h of light and 8 h of darkness under 100 to 150 μmol m^{-2} s^{-1} light intensity. Seven-day-old seedlings were then transplanted into soil and grown under the same conditions for additional experiments and seed production.

For the dark-induced senescence assay, the sixth rosette leaves were detached from three-week-old soil-grown *Arabidopsis*. Detached rosette leaves were incubated on 3 mM MES buffer (pH 5.7) in complete darkness for 3 days and sampled for analysing chlorophyll contents.

Constructs, plant transformation, and crossing

To construct *35S:SAUR49-Flag* and *35S:SAUR49-eYFP* fusion genes, the full-length coding sequence of *SAUR49* without its termination codon was amplified from *Arabidopsis* cDNA by RT-PCR. The primer pairs used for PCR were *SAUR49-35S* and *SAUR49-eYFP*. The DNA fragments were subsequently inserted into pCAMBIA3301 or pCAMBIA3301 binary vectors that contained an eYFP coding sequence to create the recombinant transcription unit.

To generate *saur49* CRISPR/Cas9 genome-editing mutants, equal volumes of 100 μmol/L *SAUR49 guide sequence F* and *SAUR49 guide sequence R* were mixed together, incubated at 95°C for 5 min and then cooled slowly to room temperature, which resulted in a double-stranded gRNA with 4-nt overhangs at both ends. The gRNA was then cloned into a pHEE401E vector (Wang et al. 2015).

To construct the *SAUR49:GUS* fusion gene, an 894-bp DNA fragment containing the upstream genomic sequence of *SAUR49* was amplified via PCR from

Arabidopsis genomic DNA and then cloned into the *Bam*H I and *Nco* I sites of pCAMBIA3301. To construct the *SAUR49:SAUR49-GUS* fusion gene, a DNA fragment containing 894 bp of the upstream genomic sequence of *SAUR49* and the *SAUR49* coding sequence without its termination codon was cloned and inserted into a pCAMBIA3301 binary vector.

To construct *GST-SAUR49* recombinant transcription units, the *SAUR49* coding region was cloned and inserted into a pGEX-6P-1 vector, yielding *GST-SAUR49* recombinant transcription units. To construct the *SAUR49-nYFP* recombinant transcription units, the SAUR49 coding sequence without its termination codon was cloned and inserted into a pSPYNE-35S vector, yielding a *SAUR49-nYFP* construct, and the construction of *SSPP-cYFP* was performed as previously described (Xiao et al. 2015). The *SAUR49* coding sequence was amplified from *Arabidopsis* cDNA via RT-PCR and inserted into a pGADT7 vector to create an *AD-SAUR49* fusion gene recombinant transcription unit. All primers used for generating constructs in the present study are listed in the Table S1.

The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101, which were subsequently transformed into WT Columbia-0 *Arabidopsis* plants using the floral dip method (Clough and Bent 1998). The transformants were screened on one-half-strength MS medium that contained 15 mg L^{-1} glufosinate, and the resistant seedlings were transplanted into soil and verified by genomic PCR followed by semi-quantitative RT-PCR. The PCR primers used to confirm the recombinant transgenes in the transgenic plants are listed in the Table S1. Homozygous T3 plants were used for all experiments.

SSPPox/SAUR49ox plants were obtained by crossing *SAUR49ox* transgenic plants with *35S:SSPP* plants. *DR5:eGFP-GUS/SAUR49ox* plants were obtained by crossing *SAUR49ox* transgenic plants with *DR5:eGFP-GUS* plants. *D Ⅱ:Venus/SSPPox* plants were obtained by crossing *SSPPox* transgenic plants with *D Ⅱ:Venus* plants. Homozygous plants were identified by segregation analysis and PCR-based genotyping among the F3 progeny.

Protein expression and pull-down assays

The resulting *GST-SAUR49* fusion vector was expressed in BL21 *Escherichia coli* cells and purified with glutathione-Sepharose 4 Fast Flow (GE Healthcare, 17513201) in accordance with the manufacturer's instructions. *In vitro* pull-down assays were performed as previously described (Zhang et al. 2013). Briefly, Ni Sepharose 6 Fast Flow beads (GE Healthcare, 17-5318-01) bound with His-SSPP were washed with phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). To each reaction, approximately 30 μg of GST-tagged fusion proteins was added, and the reaction mixtures were incubated for 2 h at 4°C under gentle rotation. After being washed ten times with washing buffer, the interactive proteins were eluted and then boiled for 5 min. The proteins were subsequently separated by 12% (w/v) SDS-PAGE followed by immunodetection with anti-GST antiserum (CST, 2624S) at a 1:5,000 dilution.

Analysis of protein stability

To assess protein levels, the sixth true leaves of *35S:SAUR49-Flag* transgenic *Arabidopsis* at young and early senescent stages were incubated with cycloheximide (100 μM). Samples were harvested at the indicated time points and further immunoblotted with anti-actin antibody or anti-Flag antibody.

Y2H assays

Y2H assays were performed using the Matchmaker GAL4 system (Clontech) as described in the Yeast Protocols Handbook (Clontech). The bait plasmids containing *SSPP* and prey plasmids containing *SAUR49* were sequentially co-transformed into cells of the yeast strain AH109 (Clontech). The presence of both vectors was confirmed by growing the yeast on media that lacked Trp and Leu. Experimental protein-protein interactions were assessed by growth on synthetic dextrose/-Leu-Trp-His-Ade plates. For the control experiments, yeast strains were generated with the pGADT7-T plasmid and either the pGBKT7-53 or pGBKT7-Lam vector for positive and negative controls, respectively. The yeast was transformed with the BD-SSPP plasmids for transactivation activity assays.

Protoplast preparation, BiFC assays, and fluorescence microscopy analyses

Protoplasts were prepared and transiently transformed according to previous methods (Yoo et al. 2007). For infiltration of *Nicotiana benthamiana*, the *Agrobacterium tumefaciens* strain GV3101 was infiltrated into the abaxial air space of 2- to 4-week-old plants as described previously. The p19 protein of Tomato bushy stunt virus was used to suppress gene silencing (Voinnet et al. 2003). Co-infiltration of *Agrobacterium tumefaciens* strains containing both the BiFC constructs and the p19 silencing plasmid was carried out at an optical density (600 nm) of 0.6:0.6:0.3. The epidermal cell layers of the *Nicotiana benthamiana* leaves were assayed for fluorescence 2 days after infiltration. The fluorescence signal was visualized with a laser scanning confocal microscope (Zeiss LSM710).

Histochemical GUS staining and determination of chlorophyll content

Histochemical GUS staining was performed as described previously (Liu et al. 2010). Briefly, the staining process was performed at 37 °C for 4 h in a solution containing 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_3Fe[CN]_6$, 0.1% triton X-100, and 1 mM X-Gluc (INALCO). Then samples were washed in 75% ethanol and 25% acetic acid to remove chlorophyll. The chlorophyll content was spectrophotometrically measured as described previously (Arnon 1949). At least three independent samples were examined, all of which produced the typical results reported in this article.

Medium acidification and LiCl root growth inhibition assays

For root acidification assays, 8-day-old seedlings were transplanted to plates of water agar that contained 30 mM Tris and 0.003% (w/v) bromocresol purple (pH 6.0), as described previously (Spartz et al. 2014). Colour changes were scanned 8 h after transplanting. Each treatment was replicated three times. For LiCl root growth inhibition assays, 4-day-old seedlings were transplanted to MS medium that was supplemented with 10 mM LiCl, and the position of the root tips was marked. The root lengths were measured after growing for an additional 4 days.

RNA isolation and RT-PCR of gene expression

RNA extraction, cDNA synthesis, and RT-PCR were performed as described previously (Liu et al. 2010). Real-time RT-PCR was performed using SYBR Green Perfect mix (TaKaRa) on an iQ5 (Bio-Rad) system following the manufacturer's instructions. Triplicate assays were performed, all of which produced the typical results shown here. All primers used for RT-PCR are listed in the Table S1.

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Disclosures

The authors have no conflict of interest to declare.

Accession numbers

Sequence data from this article can be found in The Arabidopsis Information Resource or GenBank/EMBL database under the following accession numbers: *SAUR49* (At4g34750), *SAUR30* (At5g53590), *SAUR39* (At3g43120), *SAUR41* (At1g16510), *SAUR72* (At3g12830), *SSPP* (At5g02760), *AtSARK* (At4g30520), *ACD1* (At3g44880), *RbcL* (AtCg00490.1), *GTR1* (At1g58290), *SAG12* (At5g45890), *AtNAP* (At1g69490), *NAC1* (At1g56010), *SAG113* (At5g59220), *WRKY6* (At1g62300), *WRKY53* (AT4G23810), *RbcS* (At1g67090), and *TIP41-like* (At4g34270).

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Figure legends

Figure 1. SAUR49 physically interacts with SSPP both *in vitro* and *in planta*

(a) *In vitro* pull-down assay demonstrating the direct interaction between SAUR49 and SSPP. GST-SAUR49 or GST proteins were incubated with His-SSPP-bound Ni-NTA beads. Proteins bound to His-SSPP were pelleted, subjected to 12% (w/v) SDS-PAGE and detected by immunoblot analysis using anti-GST antibody (top) and anti-His antibody (bottom). A solid arrow indicates the migration of each protein.

(b) Y2H assay showing the interaction between SAUR49 and SSPP. pGBK-T7/pGAD-SAUR49 and pGBK-SSPP/pGAD-T7 combinations were used as negative controls. The "+" and "-" represent the positive and negative control samples from Clontech.

(c) Subcellular localization of SAUR49 in *Arabidopsis* protoplasts. The GFP-fused SAUR49 driven by the *35S* promoter (*35S:SAUR49-YFP*) was expressed in *Arabidopsis* protoplasts, and YFP fluorescence was detected at 12 h after transformation.

(d) BiFC analysis of the interaction between SAUR49 and SSPP in *Nicotiana benthamiana* leaves. Fluorescence images of *Nicotiana benthamiana* epidermal cells infiltrated with a mixture of *Agrobacterium tumefaciens* strains harbouring constructs that encode the indicated fusion proteins.

Figure 2. SAUR49 accumulates during leaf senescence and is involved in AtSARK-SSPP-mediated leaf senescence

(a) The spatial and temporal expression of *SAUR49* during leaf development. Top, twenty-eight-day-old *SAUR49:GUS* transgenic *Arabidopsis* plants was sampled for histochemical GUS staining. Leaves from the transgenic plants are shown in order of emergence. Plant shown represents the typical results of three independent lines. Bottom, quantitative RT-PCR (qPCR) analyses of *SAUR49* transcript levels in the sixth rosette leaves of wild-type plants harvested at 14, 20, 26 and 32 days after emergence (DAE). *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

(b) Protein accumulation of SAUR49 during leaf development. Top, 28-day-old

SAUR49:SARU49-GUS transgenic *Arabidopsis* plants were sampled for histochemical GUS staining. Leaves from the transgenic plants are shown in order of emergence. Plant shown represents the typical results of three independent lines. Bottom, western blot analysis of SAUR49 protein accumulation in the fifth and sixth leaves of *SAUR49:SARU49-GUS* at four different developmental stages. Actin was used as an internal control. Numbers below the blots indicate the intensity ratios of the SAUR49-GUS band to the β-actin control band in each lane. YL, young leaves with leaf area approximately 50% of mature leaves; ML, fully expanded mature leaves; ES, early-senescent leaves, with <25% yellowing leaf area; LS, late-senescent leaves, with >50% yellowing leaf area.

(c) Stability of SAUR49-Flag protein. Time-course analysis of protein abundance in the sixth true leaves of *35S:SAUR49-Flag* plants at young and early senescent stages after treatment with 100 μM cycloheximide (CHX). Numbers below the blots indicate the intensity ratios of the SAUR49-Flag band to the β-actin control band in each lane.

(d) Relative expression levels of *SAUR49* in *GVG:SARK* and *SSPPox* transgenic *Arabidopsis*. Left, 21-day-old *GVG:SARK* plants were treated with 30 μM DEX to induce the overexpression of *AtSARK* or treated with a mock solution as a control. The fifth and sixth leaves were harvested at 24 h after DEX treatment for RNA extraction and subsequent quantitative RT-PCR. Right, relative expression levels of *SAUR49* in *SSPPox* transgenic *Arabidopsis*. Fifth and sixth rosette leaves from developmental stage 5.10 were used to analyse the *SAUR49* transcript accumulation. The expression of *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

Figure 3. Ectopic expression of *SAUR49* causes premature leaf senescence

(a) Five independent transgenic lines of *35S:SAUR49-Flag* (lines 5, 8, 15, 17, and 23) and their WT controls were cultivated under long-day conditions. An image was taken at 28 d. The *SAUR49* transcript levels in the fifth and sixth leaves of the five different lines were determined by semi-quantitative RT-PCR. The *TIP41-like* gene was used as an internal control.

(b) Rosette leaves detached from 25-day-old WT and line 8 and line 15 *of 35S:SAUR49-Flag* are shown in the reverse order of emergence.

(c) Overexpression of *SAUR49* accelerates dark-induced leaf senescence. The sixth leaves of 21-day-old wild-type plants and *35S:SAUR49-Flag* transgenic plants (*SAUR49ox*) were detached and incubated for three days in darkness. Total chlorophyll contents were monitored before and after the dark treatment. Data are mean ± SE from three biological replicates. Asterisks indicate statistically significant difference in student's t-test (**$p < 0.01$).

Figure 4. Overexpression of *SAUR49* alters the transcript levels of several senescence-associated marker genes and the transcript levels of genes involved in

chlorophyll metabolism and chloroplast functions in *Arabidopsis*

(a) Overexpression of *SAUR49* alters the expression levels of genes involved in chlorophyll metabolism and chloroplast function in *Arabidopsis*.

(b) Overexpression of *SAUR49* increased the transcript levels of several senescence-related marker genes in *Arabidopsis*.

(c) Relative expression levels of *SSPP* and *SARK* in WT and *35S:SAUR49-Flag* transgenic plants (*SAUR49ox*). The fifth and sixth leaves of 28-day-old *SAUR49ox* and WT control plants were sampled. The transcript levels of the marker genes were determined by quantitative RT-PCR, with *TIP41-like* used as an internal control. Data are mean ± SE from three biological replicates. Asterisk indicates statistically significant differences in student's t-test (**$p < 0.01$; *$p<0.05$).

Figure 5. Overexpression of *SAUR49* reverses the delayed senescence phenotype of *SSPPox*

(a) Twenty-eight-day-old *35S:SAUR49-Flag* (*SAUR49ox*), *35S:SSPP* (*SSPPox*), and *SSPPox/SAUR49ox* plants and their WT control plants were imaged. The transcript levels of *SAUR49* and *SSPP* in the fifth and sixth rosette leaves of transgenic plant at developmental stage 5.10 were analysed by semi-quantitative RT-PCR. The *TIP41-like* gene was used as an internal control. The experiment was performed three times, each yielding similar results.

(b) The *SAUR49ox*, *SSPPox*, and *SSPPox/SAUR49ox* plants and their WT controls were cultivated under long-day conditions for up to 50 days.

(c) The bolting time and flowering time of *SAUR49ox*, *SSPPox*, and *SSPPox*/*SAUR49ox* plants and their WT controls under long-day conditions. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p< 0.05$).

(d) Overexpression of *SAUR49* effectively reverses the changes in the expression levels of genes involved in chloroplast function and the downregulated expression of senescence-associated marker genes in *SSPPox*. Quantitative RT-PCR was used to determine the expression levels of *SAG113*, *AtNAP*, *WRKY6*, *ACD1*, *RbcL*, and *RbcS* in the fifth and sixth leaves of *SAUR49ox*, *SSPPox*, and *SSPPox/SAUR49ox* plants as well as their control plants at developmental stage 5.10. *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p< 0.05$).

Figure 6. Overexpression of *SAUR49* suppresses the regulation of H^+-ATPases activity by SSPP

(a) Left, 4-day-old *35S:SAUR49-Flag* (*SAUR49ox*), *35S:SSPP* (*SSPPox*), *SSPPox/SAUR49ox* and their WT control seedlings were transferred to media that contained 10 mM LiCl or a mock control solution and grown for 5 days. The blue points indicate root tip positions every day. Right, comparison of relative root length of each transgenic line with and without LiCl treatment every day for a

total of five days.

(b) Medium acidification around the root system. Twelve-day-old seedlings of *SAUR49ox*, *SSPPox*, *SSPPox/SAUR49ox* and their WT controls were transferred to plates containing the pH indicator dye bromocresol purple, and colour changes were recorded after 8 h.

Figure 7. Identification of SAURs that were up-regulated in leaf senescence and interacted with SSPP.

(a) The transcript levels of nine *SAURs* in the sixth leaves of wild-type *Arabidopsis* at four different stages including YL, ML, ES and LS were confirmed by qPCR.

(b) Yeast-two-hybrid assay showing the interactions between the senescence up-regulated SAURs and SSPP. The "+" and "-" represent the positive and negative control samples from Clontech.

Figure 8. Proposed model for the role of certain members of SAUR family in SARK-SSPP-mediated leaf senescence.

As demonstrated in our previous study, SARK positively regulates leaf senescence, while SSPP functions to repress SARK and negatively regulates leaf senescence. In young leaves, SSPP is highly expressed, and it inhibits the activity of SARK and helps maintain leaf longevity. In senescing leaves, the expression of SSPP is reduced, but the expression of SARK is markedly increased. Together with developmental and environmental signals, SARK-mediated leaf senescence signalling promotes the stabilization and accumulation of certain members of the SAUR family (SAUR30, 39, 41, 49 and 72). These SAUR proteins then interact with and suppress SSPP and thus indirectly increase the phosphorylation level of SARK, leading to the progression of leaf senescence.

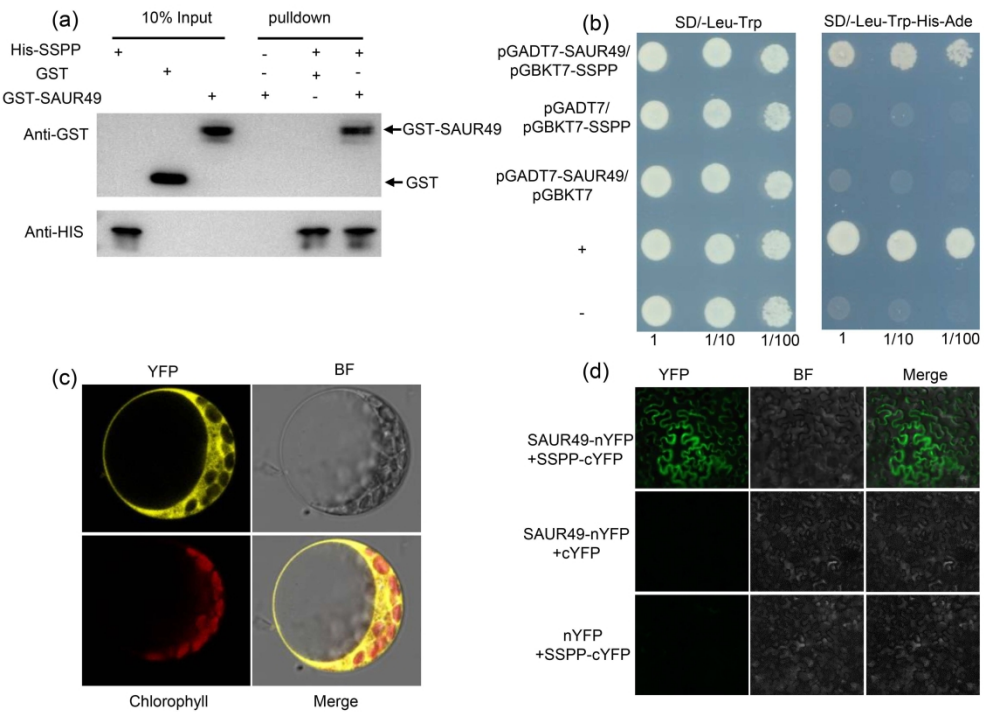

Figure 1. SAUR49 physically interacts with SSPP both *in vitro* and *in planta*

(a) *In vitro* pull-down assay demonstrating the direct interaction between SAUR49 and SSPP. GST-SAUR49 or GST proteins were incubated with His-SSPP-bound Ni-NTA beads. Proteins bound to His-SSPP were pelleted, subjected to 12% (w/v) SDS-PAGE and detected by immunoblot analysis using anti-GST antibody (top) and anti-His antibody (bottom). A solid arrow indicates the migration of each protein.

(b) Y2H assay showing the interaction between SAUR49 and SSPP. pGBK-T7/pGAD-SAUR49 and pGBK-SSPP/pGAD-T7 combinations were used as negative controls. The "+" and "-" represent the positive and negative control samples from Clontech.

(c) Subcellular localization of SAUR49 in *Arabidopsis* protoplasts. The GFP-fused SAUR49 driven by the *35S* promoter (*35S:SAUR49-YFP*) was expressed in *Arabidopsis* protoplasts, and YFP fluorescence was detected at 12 h after transformation.

(d) BiFC analysis of the interaction between SAUR49 and SSPP in *Nicotiana benthamiana* leaves. Fluorescence images of *Nicotiana benthamiana* epidermal cells infiltrated with a mixture of *Agrobacterium tumefaciens* strains harbouring constructs that encode the indicated fusion proteins.

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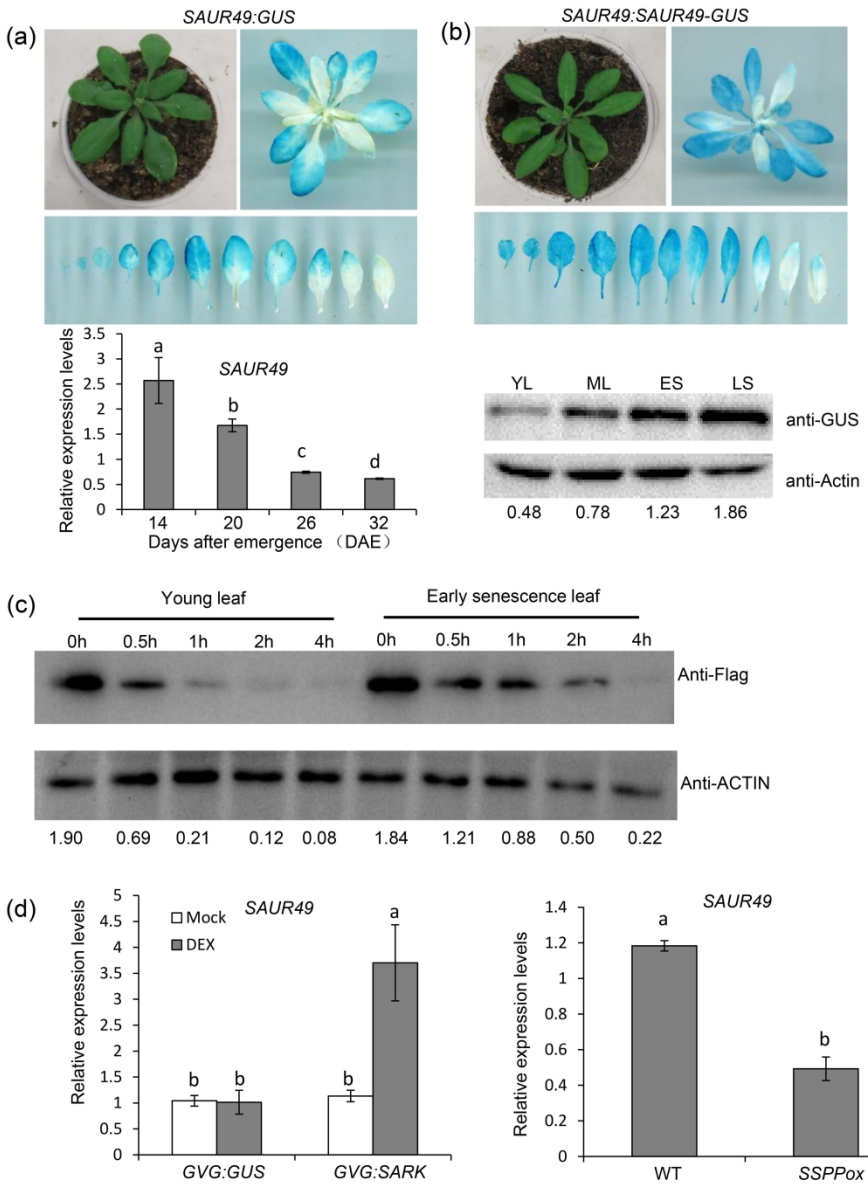

Figure 2. SAUR49 accumulates during leaf senescence and is involved in AtSARK-SSPP-mediated leaf senescence

(a) The spatial and temporal expression of *SAUR49* during leaf development. Top, twenty-eight-day-old *SAUR49:GUS* transgenic *Arabidopsis* plants was sampled for histochemical GUS staining. Leaves from the transgenic plants are shown in order of emergence. Plant shown represents the typical results of three independent lines. Bottom, quantitative RT-PCR (qPCR) analyses of SAUR49 transcript levels in the sixth rosette leaves of wild-type plants harvested at 14, 20, 26 and 32 days after emergence (DAE). *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

(b) Protein accumulation of SAUR49 during leaf development. Top, 28-day-old *SAUR49:SARU49-GUS* transgenic *Arabidopsis* plants were sampled for histochemical GUS staining. Leaves from the transgenic plants are shown in order of emergence. Plant shown represents the typical results of three independent lines. Bottom, western blot analysis of SAUR49 protein accumulation in the fifth and sixth leaves of *SAUR49:SARU49-GUS* at four different developmental stages. Actin was used as an internal control.

Numbers below the blots indicate the intensity ratios of the SAUR49-GUS band to the β-actin control band in each lane. YL, young leaves with leaf area approximately 50% of mature leaves; ML, fully expanded mature leaves; ES, early-senescent leaves, with <25% yellowing leaf area; LS, late-senescent leaves, with >50% yellowing leaf area.

(c) Stability of SAUR49-Flag protein. Time-course analysis of protein abundance in the sixth true leaves of *35S:SAUR49-Flag* plants at young and early senescent stages after treatment with 100 μM cycloheximide (CHX). Numbers below the blots indicate the intensity ratios of the SAUR49-Flag band to the β-actin control band in each lane.

(d) Relative expression levels of *SAUR49* in *GVG:SARK* and *SSPPox* transgenic *Arabidopsis*. Left, 21-day-old *GVG:SARK* plants were treated with 30 μM DEX to induce the overexpression of *AtSARK* or treated with a mock solution as a control. The fifth and sixth leaves were harvested at 24 h after treatment for RNA extraction and subsequent quantitative RT-PCR. Right, relative expression levels of *SAUR49* in *SSPPox* transgenic *Arabidopsis*. Fifth and sixth rosette leaves from developmental stage 5.10 were used to analyse the *SAUR49* transcript accumulation. The expression of *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

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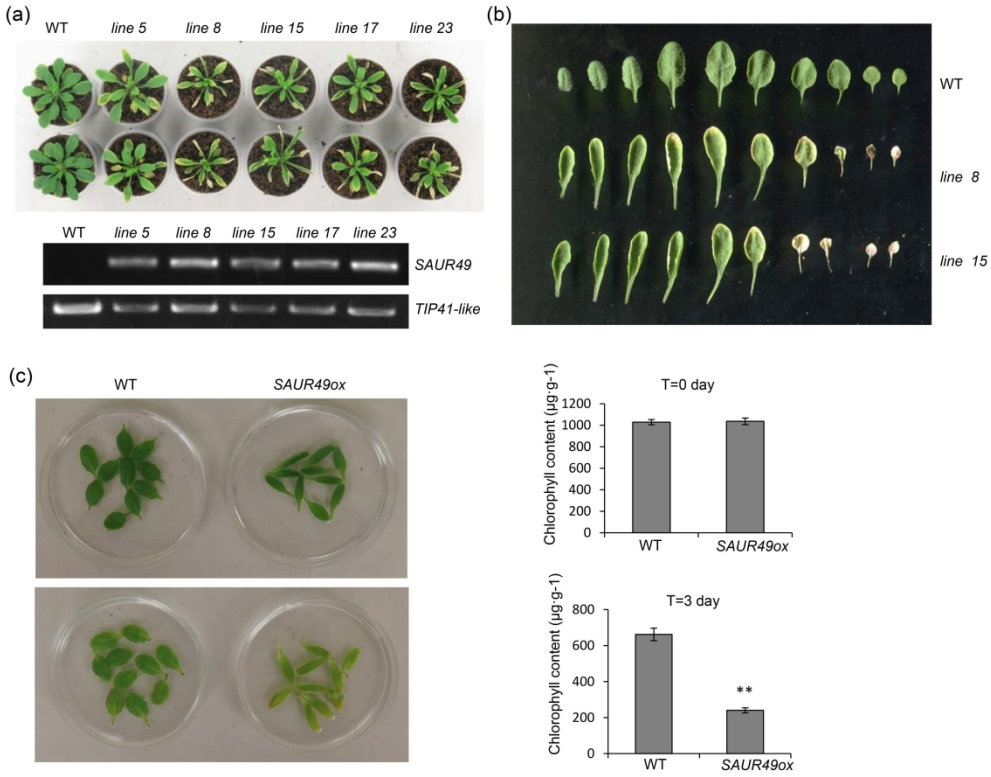

Figure 3. Ectopic expression of *SAUR49* causes premature leaf senescence

(a) Five independent transgenic lines of *35S:SAUR49-Flag* (lines 5, 8, 15, 17, and 23) and their WT controls were cultivated under long-day conditions. An image was taken at 28 d. The *SAUR49* transcript levels in the fifth and sixth leaves of the five different lines were determined by semi-quantitative RT-PCR. The *TIP41-like* gene was used as an internal control.

(b) Rosette leaves detached from 25-day-old WT and line 8 and line 15 of *35S:SAUR49-Flag* are shown in the reverse order of emergence.

(c) Overexpression of *SAUR49* accelerates dark-induced leaf senescence. The sixth leaves of 21-day-old wild-type plants and *35S:SAUR49-Flag* transgenic plants (*SAUR49ox*) were detached and incubated for three days in darkness. Total chlorophyll contents were monitored before and after the dark treatment. Data are mean ± SE from three biological replicates. Asterisks indicate statistically significant difference in student's t-test (**p < 0.01).

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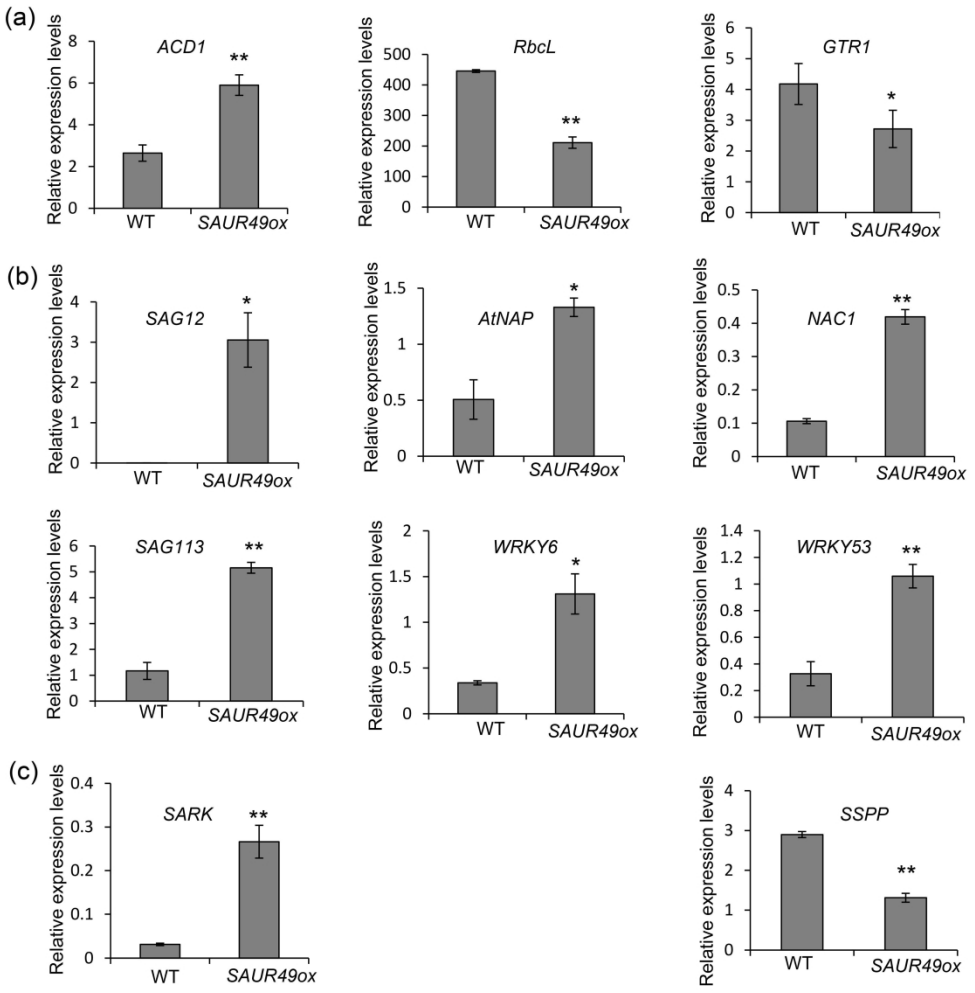

Figure 4. Overexpression of *SAUR49* alters the transcript levels of several senescence-associated marker genes and the transcript levels of genes involved in chlorophyll metabolism and chloroplast functions in *Arabidopsis*

(a) Overexpression of *SAUR49* alters the expression levels of genes involved in chlorophyll metabolism and chloroplast function in *Arabidopsis*.

(b) Overexpression of *SAUR49* increased the transcript levels of several senescence-related marker genes in *Arabidopsis*.

(c) Relative expression levels of *SSPP* and *SARK* in WT and *35S:SAUR49-Flag* transgenic plants (*SAUR49ox*). The fifth and sixth leaves of 28-day-old *SAUR49ox* and WT control plants were sampled. The transcript levels of the marker genes were determined by quantitative RT-PCR, with *TIP41-like* used as an internal control. Data are mean ± SE from three biological replicates. Asterisk indicates statistically significant differences in student's t-test (**p < 0.01; *p<0.05).

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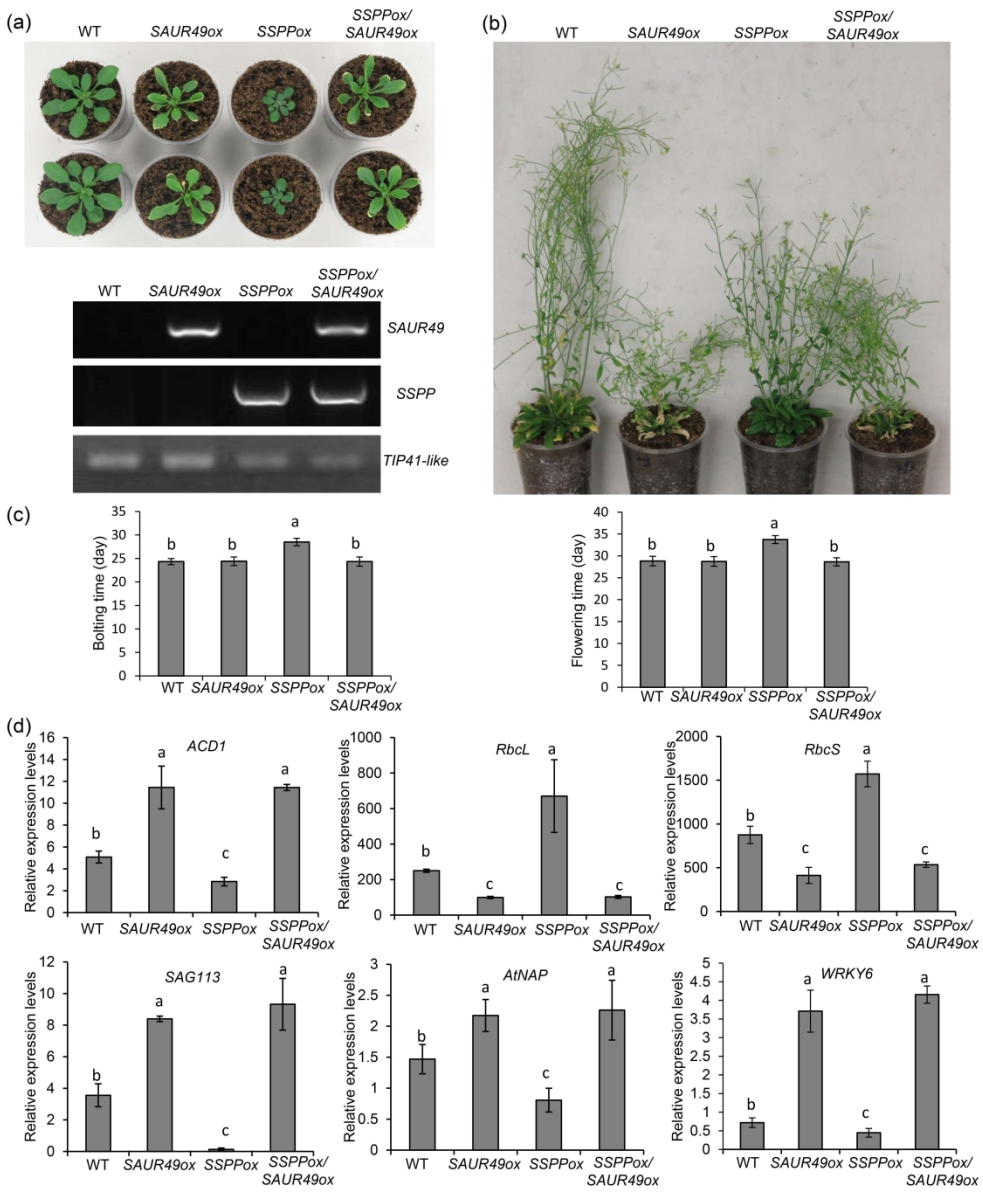

Figure 5. Overexpression of *SAUR49* reverses the delayed senescence phenotype of *SSPPox*

(a) Twenty-eight-day-old *35S:SAUR49-Flag* (*SAUR49ox*), *35S:SSPP* (*SSPPox*), and *SSPPox/SAUR49ox* plants and their WT control plants were imaged. The transcript levels of *SAUR49* and *SSPP* in the fifth and sixth rosette leaves of transgenic plant at developmental stage 5.10 were analysed by semi-quantitative RT-PCR. The *TIP41-like* gene was used as an internal control. The experiment was performed three times, each yielding similar results.

(b) The *SAUR49ox, SSPPox,* and *SSPPox/SAUR49ox* plants and their WT controls were cultivated under long-day conditions for up to 50 days.

(c) The bolting time and flowering time of *SAUR49ox, SSPPox,* and *SSPPox/SAUR49ox* plants and their WT controls under long-day conditions. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

(d) Overexpression of *SAUR49* effectively reverses the changes in the expression levels of genes involved in chloroplast function and the downregulated expression of senescence-associated marker genes in *SSPPox*. Quantitative RT-PCR was used to determine the expression levels of *SAG113, AtNAP, WRKY6, ACD1, RbcL,*

and *RbcS* in the fifth and sixth leaves of *SAUR49ox, SSPPox,* and *SSPPox/SAUR49ox* plants as well as their control plants at developmental stage 5.10. *TIP41-like* was used as an internal control. Data are mean ± SE from three biological replicates. Different letters indicate statistically significant differences based on analysis of variance (ANOVA) ($p < 0.05$).

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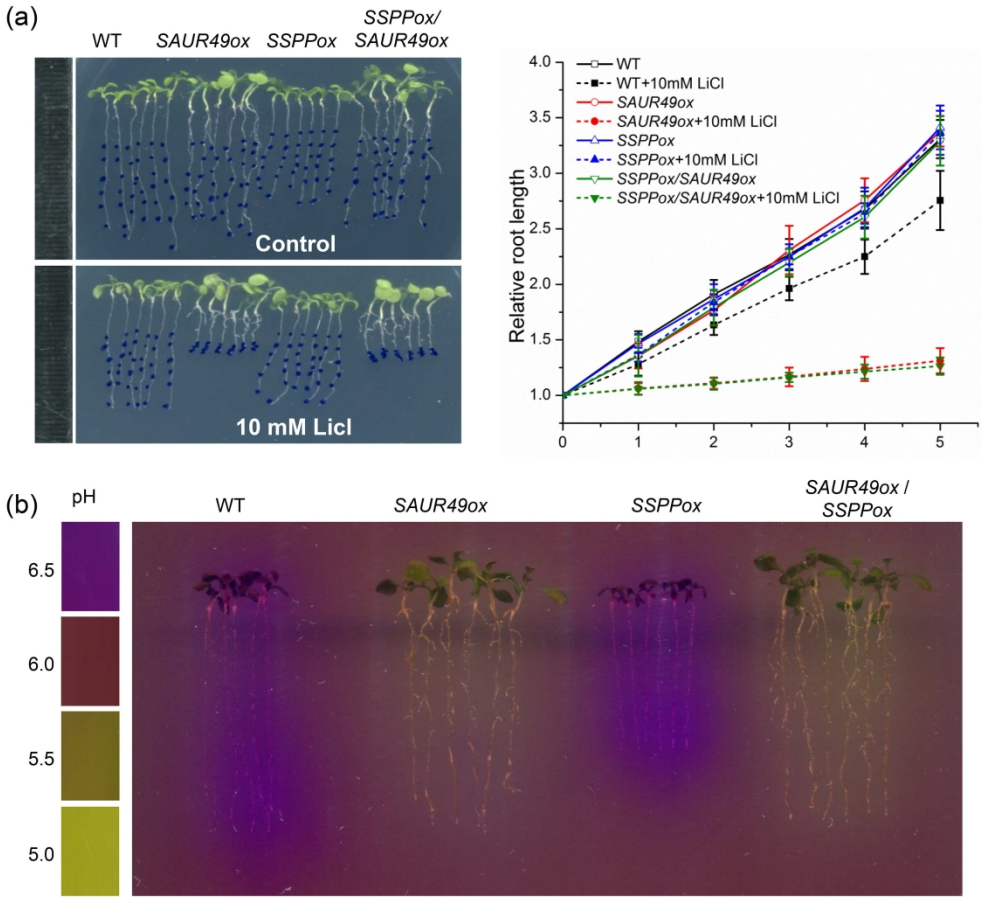

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(a) Left, 4-day-old *35S:SAUR49-Flag* (*SAUR49ox*), *35S:SSPP* (*SSPPox*), *SSPPox/SAUR49ox* and their WT control seedlings were transferred to media that contained 10 mM LiCl or a mock control solution and grown for 5 days. The blue points indicate root tip positions every day. Right, comparison of relative root length of each transgenic line with and without LiCl treatment every day for a total of five days.
(b) Medium acidification around the root system. Twelve-day-old seedlings of *SAUR49ox, SSPPox, SSPPox/SAUR49ox* and their WT controls were transferred to plates containing the pH indicator dye bromocresol purple, and colour changes were recorded after 8 h.

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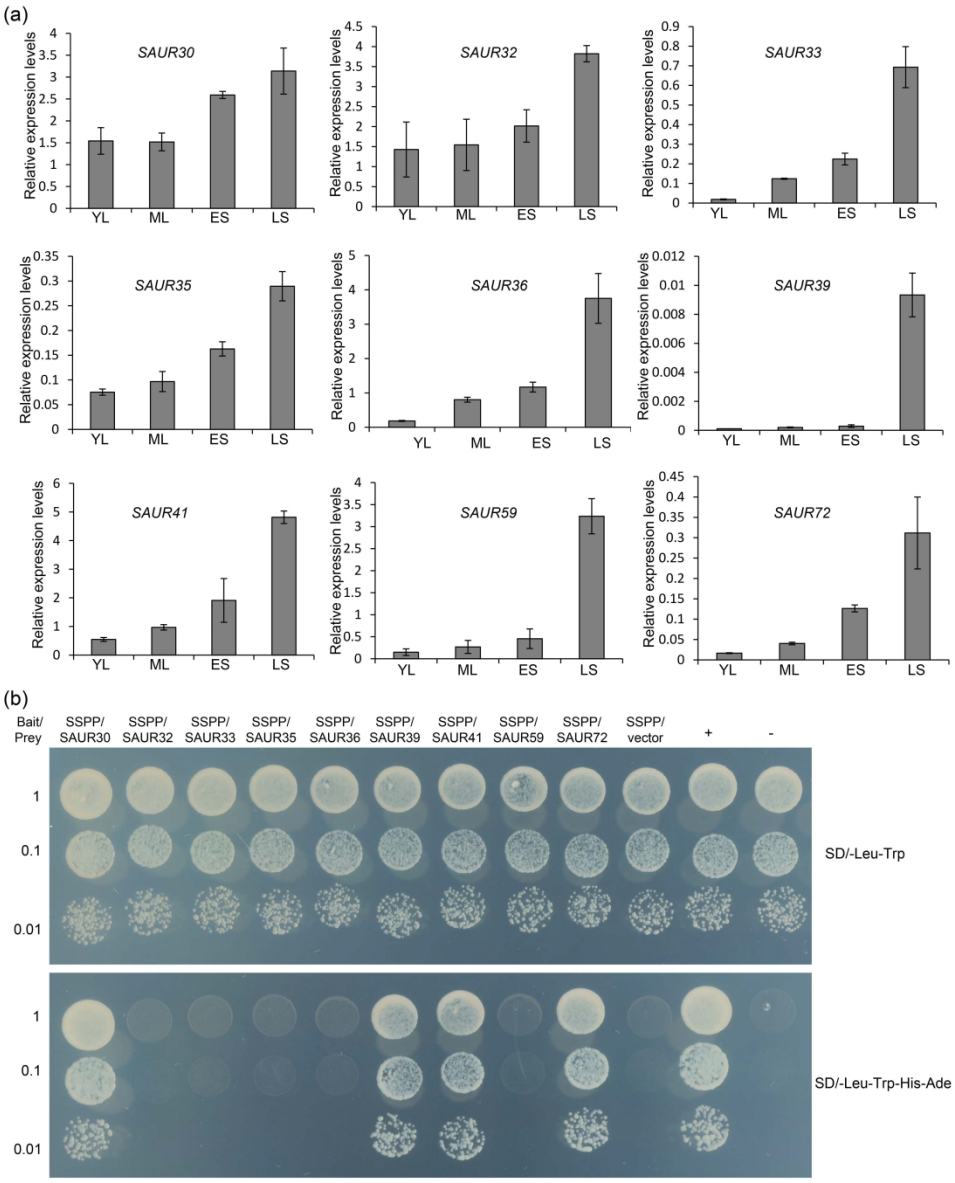

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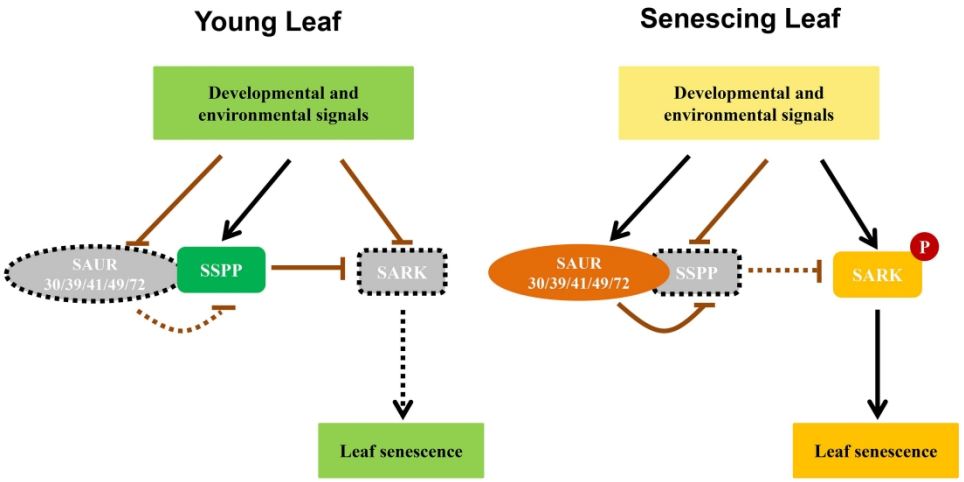

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