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Published in:
Plant Molecular Biology

DOI:
10.1007/s11103-022-01245-0

Publication date:
2022

Plant Molecular Biology (2022) 108:549–563
https://doi.org/10.1007/s11103-022-01245-0

A mutation in Arabidopsis SAL1 alters its in vitro activity against IP_3 and delays developmental leaf senescence in association with lower ROS levels

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Received: 10 August 2021 / Accepted: 21 January 2022 / Published online: 5 February 2022

Abstract

Key message **Our manuscript is the first to find a link between activity of SAL1/OLD101 against IP_3 and plant leaf senescence regulation and ROS levels assigning a potential biological role for IP_3.**

Abstract Leaf senescence is a genetically programmed process that limits the longevity of a leaf. We identified and analyzed the recessive Arabidopsis stay-green mutation *onset of leaf death 101* (*old101*). Developmental leaf longevity is extended in *old101* plants, which coincided with higher peroxidase activity and decreased H_2O_2 levels in young 10-day-old, but not 25-day-old plants. The *old101* phenotype is caused by a point mutation in *SAL1*, which encodes a bifunctional enzyme with inositol polyphosphate-1-phosphatase and 3′ (2′), 5′-bisphosphate nucleotidase activity. SAL1 activity is highly specific for its substrates 3-polyadenosine 5-phosphate (PAP) and inositol 1, 4, 5-trisphosphate (IP_3), where it removes the 1-phosphate group from the IP_3 second messenger. The in vitro activity of recombinant old101 protein against its substrate IP_3 was 2.5-fold lower than that of wild type SAL1 protein. However, the in vitro activity of recombinant old101 mutant protein against PAP remained the same as that of the wild type SAL1 protein. The results open the possibility that the activity of SAL1 against IP_3 may affect the redox balance of young seedlings and that this delays the onset of leaf senescence.

Keywords Leaf senescence · Ageing · Age-related changes · Reactive oxygen species · SAL1 · Arabidopsis

Introduction

Death is the ultimate outcome of plant development, except perhaps for clonal plants which appear to live forever (Thomas 2013; Mitton and Grant 1996; Ally et al. 2010; Vaupel et al. 2004; Arnaud-Haond et al. 2012; Klimešová et al. 2015). Nevertheless, even long-lived plants go through annual cycles whereby a considerable portion of the organism dies, most obviously observed by the massive leaf loss of deciduous trees during autumn. Individual leaves likewise go through developmental stages of which the final stage, leaf senescence, causes the death of this particular plant organ (Keskitalo et al. 2005). Leaf senescence is a highly complex genetically programmed process, which is accompanied by comprehensive morphological, physiological and molecular changes (Woo et al. 2019, 2013; Kim et al. 2018; Wu et al. 2012; Shirzadian Khorramabad 2013), and includes the ordered degradation of macromolecules and remobilization of reclaimed nutrients to benefit the next generation or remainder of the plant (Maillard et al. 2015;

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Distelfeld et al. 2014; Gregersen et al. 2008; Kim et al. 2017; Bresson et al. 2018).

Leaf senescence is induced as part of the developmental program, but can also be induced prematurely by environmental stress. However, stress only induces senescence after individual leaves have reached a certain age. This suggests that the ability to senesce depends on the occurrence of certain age-related changes (ARCs) throughout plant development (Sade et al. 2018; Kanojia and Dijkwel 2018; Kim et al. 2017; Leng et al. 2017; Jibran et al. 2013; Woo et al. 2013; Lim et al. 2007a; Jing et al. 2005). ARCs are defined as any irreversible changes that are rigorously dependent on age and development. The cessation of cell division and leaf expansion are examples of ARCs in leaves (Jibran et al. 2013), but the nature of the ARCs and genes, involved in regulating leaves to acquire the competence to senesce, are poorly understood.

It seems reasonable to assume that hormone treatments altering the developmental program are also able to influence the timing of senescence (Jibran et al. 2013; Kanojia et al. 2021). Hormonal signaling pathways strongly influence senescence and are important internal factors involved in regulating the timing of leaf senescence (Van de Poel et al. 2015; Schippers et al. 2007). In this regard, the plant hormone ethylene has long been considered as a key hormone involved in the regulation of the onset of leaf senescence (Koyama 2018; Schaller 2012; Zacarias and Reid 1990). The induction of leaf senescence by ethylene strictly depends on leaf age (Hensel et al. 1993; Grbić and Bleecker 1995; Jing et al. 2002, 2005) and suggests that ARCs are the master regulators of senescence, while the function of ethylene is to induce senescence within a certain age-window (Jing et al. 2003; Schaller 2012; Jibran et al. 2013; Schippers et al. 2015; Iqbal et al. 2017; Li and Guo 2018).

Identification and characterization of Arabidopsis mutants (*hxk1*/*gen1*; *ahk3*; *ore9*; *dls1*; *ore7*) exhibiting premature or delayed leaf senescence has shown that basic metabolic processes, such as metabolic rate, cytokinin signaling, ubiquitin-mediated degradation, R-transferase activity and chromatin organization, are involved in the regulation of the timing of leaf senescence (Ewbank et al. 1997; Woo et al. 2001; Yoshida et al. 2002; Jing et al. 2003; Moore et al. 2003; Pourtau et al. 2006; Lim et al. 2007b; Watanabe et al. 2013; Kim et al. 2016; Wang et al. 2019).

Changes in oxidative stress and Reactive Oxygen Species (ROS) levels have also been shown to regulate plant development and senescence processes (Kan et al. 2021; Niu et al. 2020; Singh et al. 2016; Rogers and Munné-Bosch 2016; Schippers et al. 2015, 2008). Nevertheless, how ROS regulate senescence and influence ARCs is still largely unknown.

In order to better understand how ARCs regulate senescence, we identified EMS-mutants that showed a delayed onset of leaf senescence (Shirzadian-Khorramabad et al. 2008). Here, we studied the *old101* mutations and we show that the *old101* phenotype results from a mutation in *SAL1* (Quintero et al. 1996), which is also known as *FIERY1* (*FRY1*) (Xiong et al. 2001), *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES2* (*HOS2*) (Xiong et al. 2004), *ALTERED EXPRESSION OF APX2 8* (*ALX8*) (Wilson et al. 2009) and *rotunda1* (*RON1*) (Robles et al. 2010). The *SAL1* gene encodes a bifunctional enzyme with 3′ (2′), 5′-biphosphate nucleotidase activity, which is highly specific for the substrate 3′-polyadenosine 5′-phosphate (PAP), a compound involved in sulfur assimilation. In addition, its polyphosphate 1-phosphatase activity against inositol 1, 4, 5-trisphosphate (IP_3), removes the 1-phosphate group from the IP_3 second messenger (Quintero et al. 1996; Xiong et al. 2001; Lee et al. 2012a). SAL1 regulates a variety of processes, including posttranscriptional gene silencing (Gy et al. 2007), stress responses and plant development (Estavillo et al. 2011; Dichtl et al. 1997; Chen and Xiong 2011, 2010; Chen et al. 2011; Xiong et al. 2001, 2004; Hirsch et al. 2011; Robles et al. 2010). Moreover, it has been suggested that the SAL1-PAP pathway integrates chloroplast retrograde, light, and hormonal signaling in plant growth and development (Phua et al. 2018). While the importance of the SAL1 activity against PAP has been well-established (Phua et al. 2018; Estavillo et al. 2011; Hirsch et al. 2011; Kim and Von Arnim 2009), it is believed that its activity against IP_3 does not have any biological significance (Hirsch et al. 2011; Lee et al. 2012a; Estavillo et al. 2011).

Here we describe the *old101* mutant allele of the *SAL1* gene which causes delayed developmental leaf senescence. The *old101* mutation results in decreased H_2O_2 levels in young mutant plants and a decreased activity of the recombinant old101 protein against its substrate IP3, but not PAP. Therefore, this provides for the possibility that IP_3 signals may regulate ROS levels, which results in a delay of ARCs and onset of leaf senescence in *Arabidopsis*.

Materials and methods

Plant materials and growth conditions

Parental *Arabidopsis thaliana* accessions Landsberg *erecta* (L*er*-0), Columbia (Col-0), *ron1-1* (kindly provided by José Luis Micol) (Robles et al. 2010) and *fry1-6* (SALK_020882) mutant plant lines, were used in this study. The *old101* mutant was obtained from an EMS mutagenized collection (Jing et al. 2002; Shirzadian-Khorramabad et al. 2008). Plants were grown in a growth chamber at 21 °C, 60% relative humidity under ~ 60 µmolm^{-2} s^{-1} fluorescent and incandescent light and a day length of 16 h. An organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) was sterilized and dried and used for plant growth.

Plants, used for ethylene treatment were grown in the same walk-in growth chamber that was used to isolate the *old101* mutant: Air-grown plants were transferred to an ethylene flow-through chamber (AGA, The Netherlands) with a dosage of approximately 10–40 µL/L ethylene for 3 days at 21 °C and ~ 50% relative humidity under ~ 60 µmolm^{-2} s^{-1} fluorescent continuous light. For experiments under sterile conditions, seeds were surface sterilized by soaking in 20% bleach for 5 min, after which they were washed twice with sterile water and plated on Murashige and Skoog medium solidified with 0.8% agar. For the triple response assay, seedlings were grown on MS media containing various concentrations of ACC (1-aminocyclopropane-1-carboxylic acid) in the dark for 5 days (Guzman and Ecker 1990). The hypocotyl lengths of the seedlings were subsequently measured as described by (Dijkwel et al. 1997).

Leaf physiological measurements

The 3rd and 4th rosette leaves were taken from at least 10 seedlings for each data point. For air-treated plants, the leaves were collected from plants grown for the indicated number of days in air. Ethylene treated plants were first grown in air and were subsequently treated with ethylene for 3 days and one additional day in air, before harvest. Prior to measuring the chlorophyll content, samples were incubated overnight in 80% (v/v) aceton at 4 °C in darkness, and the chlorophyll content was quantified spectrophotometrically using the method of (Inskeep and Bloom 1985). For measuring ion leakage, leaf samples were immersed into deionized carbonate-free water, shaken in a 25 °C water bath for 30 min, and the conductivity was measured using a Wissenschaftlich Technische Werkstatten conductivity meter (model KLE1/T, Weilheim, Germany). Samples were subsequently boiled for 10 min and the conductivity was measured again. The percentage of membrane conductivity was calculated as the percentage of the first measurement over the second. Chlorophyll fluorescence emission was measured from the upper surface of the first leaf, at room temperature (23 °C) with a pulse-amplitude modulation portable fluorometer (PAM-2000; H. Walz, Effeltrich, Germany) according to (Maxwell and Johnson 2000). Plants were dark-adapted for 1 to 2 h before experiments to ensure complete relaxation of the thylakoid pH gradient. An attached, fully expanded rosette leaf was placed in the leaf clip, allowing air to circulate freely on both sides of the leaf. At the start of each experiment, the leaf was exposed to 2 min of far-red light (2–4 µmol photons m^{-2} s^{-1}) for determination of Fo (minimum fluorescence in the dark-adapted state). Saturating pulses of white light (8000 µmol photons m^{-2} s^{-1}) were applied to determine Fm or Fm' values. PSII efficiency was calculated as (Fm-Fo)/Fm.

RNA-isolation and RT-PCR

Total plant RNA was isolated using TRIZOL reagents (Sigma –Aldrich now Merck, Germany) according to the manufacturer's protocol. 1.5 µg of RNA was used as template for first-strand cDNA synthesis using 200U of RevertAid H-minus MMuLV reverse transcriptase (Fermentas, USA) and an oligo dT21 primer. Primer pairs for real-time PCR were designed with open-source PCR primer design program PerlPrimer v1.1.10 (Maxwell and Johnson 2000). Real-time PCR amplification was performed with 50 µl of reaction solution, containing 2 µl of tenfold–diluted cDNA, 0.5 µl of a 10 mM stock of each primer (Table S1), 1 µl of 25 mM stock $MgCl_2$, 5 µl PCR buffer + Mg (Roche; Germany), 1 µl of a 1000× diluted SYBR-green stock (Sigma-Aldrich now Merck, Germany), 0.5 µl 100xBSA (New England Biolabs; USA), and 1U of Roche Taq Polymerase. The PCR program was 2′ at 94, 40x (94–10″/58–10″/72–25″), meltcurve. Obtained data was analyzed with Biorad software.

Cloning of the *OLD101* gene

The *old101* mutation was identified as described by (Shirzadian-Khorramabad et al. 2008). For complementation of the *old101* mutation, the coding region of the *OLD101* gene, including 700 bp in front of the start codon, was cloned in the pGreen vector (Hellens et al. 2000). *old101* mutant plants were transformed as described by (Clough and Bent 1998). The presence of the *old101* mutation was confirmed by *BclI* restriction analysis using the primers PrRuG2584 and PrRuG2585 (Table S1). Primers PrRuG557 and PrRuG558 (Table S1) were used to amplify the Basta resistance gene that is present in the T-DNA of transformed plants.

Protein expression, purification and refolding

Expression of SAL1 and old101 recombinant proteins were performed in *E. coli* BL21(DE3) cells. To obtain the complete SAL1 and old101 cDNA versions, Ler-0 and old101 plants were grown at ½ MS medium (Murashige and Skoog 1962) and leaves were harvested for total RNA isolation. RNA was used as template for cDNA synthesis and the complete *SAL1* and *old101* 1.2 kbp coding sequences were amplified using specific primers (Forward-NdeI: GCTTGA***CATATG***ATGTCTATCAATTGT TTTCG and Reverse-NotI: AAA***GCGGCCGC***TCAGAG AGCTGAAGCTTTCTCTTG). The 1.2-kb A-overhang PCR products were ligated into the pTG19-T vector and transformed into DH5α competent cells. The pTG19-T:SAL1 and pTG19-T:old101 constructs were double-digested with *Nde*I and *Not*I, and the 1.2-kb fragments were ligated into *Nde*I/*Not*I-digested pET28a expression vector. Automated nucleotide sequencing confirmed the

fidelity of pET28a:FRY1 and pET28a:old101 expression constructs. The expression constructs were transformed into *E. coli* BL21 (DE3), proteins were expressed in *E. coli* as His-tag fusion proteins under a variety of conditions. Over-expression of the recombinant proteins was confirmed by SDS-PAGE approach. The SDS-PAGE analysis showed that the proteins were present in inclusion bodies that were mostly inactive.

For in vitro refolding of SAL1 and old101 proteins, cells were sonicated and re-suspended in 20 mM Tris/10 mM EDTA buffer, pH = 8.0. The suspension was layered on top of 50% glycerol and then centrifuged at 6500 rpm for 25 min. Pellets were first re-suspended in the above buffer and proteins present in inclusion bodies denatured in a 20 mM Tris, 8 M urea, 10 mM DTT, pH = 8.0. Refolding was performed by diluting the dissolved proteins tenfold in 20 mM Tris, pH = 8.0 containing 10% glycerol at 4 °C and subsequent incubation for 1 h and finally an additional overnight dialysis against 20 mM Tris, 0.5 mM DDT, 10% glycerol, pH = 8.0 at 4 °C.

Inositol polyphosphate 1-phosphatase activity of purified recombinant old101 and SAL1 proteins against the 1,4,5-trisphosphate (IP_3) substrate was performed by measuring released Pi, based on spectrometric detection of the phosphate; molybdate: malachite green complex at 630 nm (Baykov et al. 1988). Phosphatase activity of the proteins against PAP was assayed by measuring the substrate utilization, using reversed-phase HPLC as described (Murguia et al. 1995; Xu et al. 2012). A standard curve for estimation of PAP concentration was performed by reversed phase HPLC (Suppl. Figure 6).

H_2O_2 staining, H_2O_2 and peroxidase quantification

Leaf samples were harvested from 10, 15 and 25-day-old air-grown L*er*-0 and *old101* plants for DAB staining and H_2O_2 and Peroxidase measurements. Foliar H_2O_2 levels were visualized by 3,3'-diaminobenzidine (DAB) staining (Thordal-Christensen et al. 1997). Additionally, leaf H_2O_2 was quantified using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen; USA). Approximately 100 mg of leaf tissue was flash frozen in liquid nitrogen, ground, and extracted with 500 µL of 20 mM K_2HPO_4, pH 6.5. The slurry was cleared by centrifugation at 16,000×*g* for 15 min at 4 °C. The supernatant was incubated with 0.1 mM Amplex Red reagent and 0.2 units/mL horseradish peroxidase (HRP) at room temperature for 30 min in the dark (final reaction volume of 100 µL). Finally, absorbance was measured at 560 nm using a mQuant plate reader (BioTek Instruments) with Gen5 software, and the concentration of H_2O_2 was calculated using a standard curve.

Statistical analyses

The data statistical analysis and Student's t tests were carried out with Microsoft Excel version 2016 and GraphPad Software Edition 2021 (https://www.graphpad.com/quickcalcs). Each data point was expressed as mean ± standard deviation (SD) of at least 3 biological independent experiments. Significance between wild type and *old101* was evaluated at the level of $P < 0.05$ (*) and $P < 0.01$ (**).

Results

The *old101* mutation delays germination and developmental leaf senescence

Arabidopsis mutants with an increased lifespan were selected by treating an EMS mutagenized population of Arabidopsis L*er*-0 seedlings with ethylene and selecting for plants that did not show cotyledon senescence (Shirzadian-Khorramabad et al. 2008; Jing et al. 2002). Among selected mutants, the *onset of leaf death101* (*old101*) stayed green for a considerably longer time than the wild type, segregated as a monogenic recessive trait, and was selected for further study (Sturre et al. 2009; Shirzadian-Khorramabad et al. 2008). First, the *old101* growth progression for up to 30 days was compared between mutant and wild type. When wild type and *old101* seeds were sown onto wetted filter paper, the *old101* seeds germinated about one day later than the wild type, resulting in a reduced number of seedlings with green cotyledons after 6 days of growth (Fig. 1a, b). Furthermore, wild type and mutant plant were grown for 30 days and five soil-based growth stages (Boyes et al. 2001) were measured. The stages were 1.02 (Two rosette leaves > 1 mm), 1.04 (Four rosette leaves > 1 mm), 1.07 (Seven rosette leaves > 1 mm), 1.10 (Ten rosette leaves > 1 mm) and 5.1 (First flower buds become visible). Figure 2a shows that growth development was 1 to 2 days delayed in *old101* plants in all stages in comparison with wild type plants. Subsequent growth revealed that 42-day-old *old101* plants exhibited delayed senescence: while old101 plants had only a few plants bearing a single senescent cotyledon, wild type plants had 4–5 yellow leaves. Consistent with the delayed senescence and in contrast with wild type 3rd and 4th leaves, membrane ion leakage of the *old101* corresponding leaves did not increase and the photochemical efficiency of photosystem II remained stable (Fig. 2b, c, d, e). Thus, *old101* mutants germinate and grow slower and show delayed senescence as compared to the wild type.

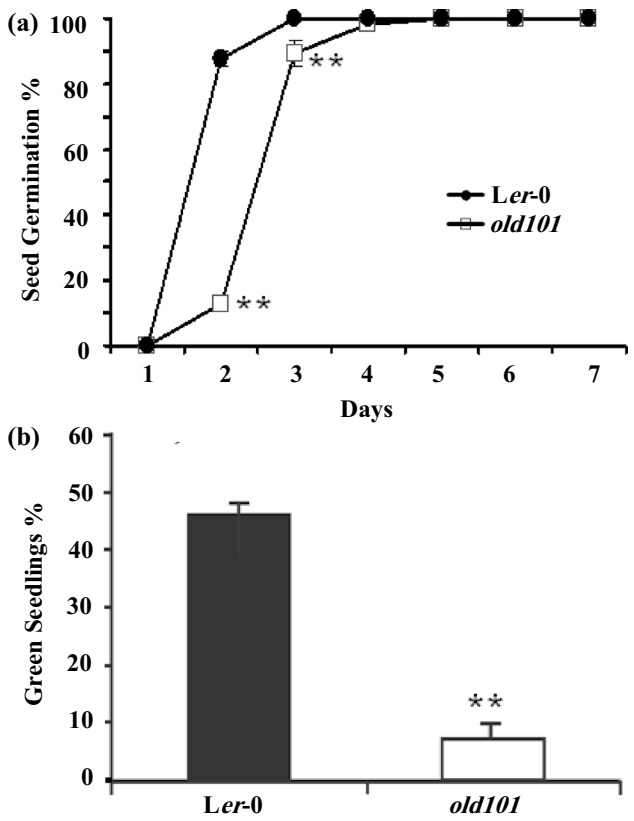


Fig. 1 Wild type and *old101* seedling development. **a** Seeds were placed on wet filter paper and incubated at 4 °C for 72 h before being allowed to germinate at 23 °C. Seeds were considered to have germinated when radicles penetrated the seed coat. **b** After 6 days of growth at 23 °C, the number of seedlings having two green cotyledons was counted and expressed as percentage of total germinated seedlings. Values shown represent means ± SD of three separate experiments each using 50–100 seeds. Values that are significantly different between wild type and *old101* at $P < 0.05$ (*) and $P < 0.01$ (**), using Student's t test, are indicated

The leaf senescence phenotype is delayed in *old101* plants after ethylene treatment

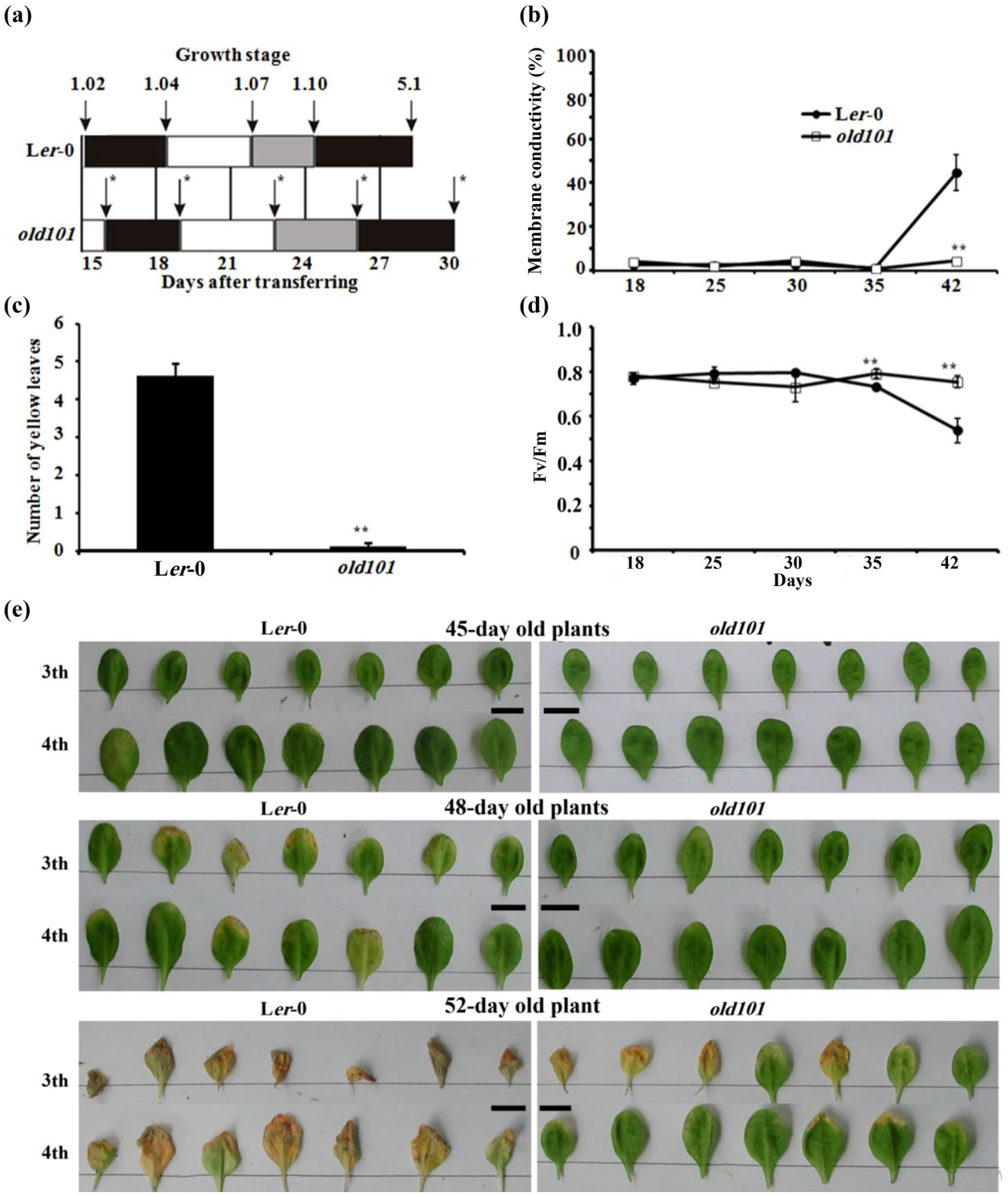
Ethylene is a strong inducer of plant senescence in Arabidopsis (Jing et al. 2005; Zacarias and Reid 1990). Since the effect of exogenous ethylene application on leaf senescence strictly depends on leaf age, ethylene treatment can provide a measure of leaf age (Jing et al. 2002, 2005). Therefore, *old101* plants were treated with ethylene and leaf yellowing was quantified.

The effect of ethylene on leaf yellowing was measured in wild type L*er*-0 and *old101* plants, grown for 14 to 38 days in air, then for 3 days in air supplemented with ethylene and subsequently one additional day in air. Figure 3a shows a 30-day old wild type and *old101* plant that had been treated with ethylene at day 26. Wild type plants showed 2 yellow cotyledons and around 4 yellow leaves, while mutants only exhibited yellow cotyledons. Different aged plants were treated with ethylene and the *old101* mutation caused a delay in ethylene-induced leaf senescence of between 3 to 12 days (Fig. 3b). The delayed senescence phenotype was further assessed by studying typical senescence-associated physiological markers in the 3rd and 4th rosette leaves of ethylene-treated plants. Measurement of chlorophyll content, photochemical efficiency of photosystem II (PSII) and ion leakage of leaf 3 and 4 of ethylene-treated plants showed that senescence in *old101* was delayed by approximately 12 days (Fig. 3b, c; Suppl. Fig. 1).

The delayed senescence phenotype may be a result of reduced ethylene biosynthesis, signaling and response. Therefore, the responsiveness of *old101* mutants to the ethylene precursor ACC was determined in etiolated seedlings. ACC treatment induced the triple response in both mutant and wild type plants and quantification of hypocotyl length showed that *old101* seedlings did not show a reduced response to ACC (Fig. 3d). The obtained results show that *old101* plants respond to ethylene treatment normally, but ethylene-induced senescence is delayed as compared to the wild type.

old101 contains a point mutation in *SAL1*

The old101 mutation was identified by a map-based strategy, combined with sequencing long-range PCR products of the mapped region as described by (Sturre et al. 2009). The *old101* mutation caused a G to A change in the second exon of AT3G63980 at position 338 (Fig. 4a). AT5G63980 has previously been annotated as *SAL1*/*FRY1* and encodes a bifunctional enzyme with 3′ (2′), 5′-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase activities (Xiong et al. 2001). The mutation resulted in an aspartate (GAT) to asparagine (AAT) residue substitution at position 38 in the encoded protein and is located between the first and second α-helix of the protein (York et al. 1995; Quintero et al. 1996; Xiong et al. 2001, 2004) (Fig. 4a). The presence of the *old101* mutation was confirmed by *BclI* restriction enzyme resulting in one 127 bp PCR product for *old101* DNA, and 103 and 24 bp fragments in the case of L*er*-0 DNA (Suppl. Figure 2). To confirm that *OLD101* is identical to *SAL1*, a 5.6 kb L*er*-0 genomic fragment, containing the *SAL1* coding sequence and a 0.7-kb promoter sequence (AGRIS database) (Davuluri et al. 2003), was cloned and transformed to *old101* plants (Fig. 4b). The *old101* phenotype of the T2 population of three independent transformants segregated in a 3:1 ratio of wild type to mutant. To confirm the T-DNA presence in *old101* and *old101* + *OLD101/SAL1* plants, amplification of the Basta resistance gene was applied. The wild type complementing phenotype strictly correlated with the presence of



the T-DNA in *old101* + *OLD101/SAL1* plants, while no T-DNA was detected in T_2 plants that showed the *old101* phenotype (Suppl. Fig. 3). These results demonstrate that *OLD101* is identical to *SAL1*. Furthermore, since the *old101* phenotype is much subtler under standard growth conditions than the phenotype of *ron1-1* (a L*er*-0 *SAL1* allele) (Robles et al. 2010), the *old101* mutation is not likely to cause a complete disruption of protein function.

◂**Fig. 2** Development of air-grown wild type and *old101* plants. **a** Soil-based-analysis of growth progression of wild type and *old101* plants was determined for 5 stages (Boyes et al. 2001) including 1.02 (Two rosette leaves > 1 mm), 1.04 (Four rosette leaves > 1 mm), 1.07 (Seven rosette leaves > 1 mm), 1.10 (Ten rosette leaves > 1 mm) and 5.1 (First flower buds visible). Arrows indicate the time (Days after transfer of the seeds from 4 to 23 °C) at which L*er*-0 and *old101* plants had reached the indicated growth stages. The results shown are the means of at least 25 plants. **b** Wild type and mutant plants were grown for 18 to 42 days in air and the membrane ion leakage of the 3rd and 4th leaves was determined. **c** Wild type and *old101* plants were grown for 42 days in air and the number of yellow leaves including cotyledons was counted. **d** Maximum quantum yield of PSII electron transport of the 3rd and 4th leaves was determined at various time points from 18 to 42 days. **e** Photographs of L*er*-0 and *old101* 3th and 4th vegetative leaves of plants that were grown for 45-, 48- and 52-days in air. For **b**, **c** and **d**, the results are shown as mean ± SD of at least three biological replicates. Values that are significantly different between wild type and *old101* at $P < 0.05$ (*) and $P < 0.01$ (**), using Student's t test, are indicated. Scale is 10 mm

The *old101* mutation does not change SAL1 in vitro activity against PAP

The SAL1 nucleotide phosphatase activity against PAP is of biological significance (Estavillo et al. 2011; Phua et al. 2018). Therefore, we first hypothesized that the *old101* mutation would affect this activity. The wild type and *old101 SAL1* cDNAs were cloned and transformed into *E. coli* BL21 (DE3) cells for recombinant protein expression (Suppl. Fig. 4). After purification of the denatured recombinant protein, the SAL1 and old101 proteins were refolded as described in "Materials and methods" section. Following purification and refolding, the proteins were analyzed by circular dichroism (CD) technique (Ranjbar and Gill 2009) as an estimation for refolding. The far-UV CD spectra of the refolded proteins revealed a typical spectra of α/β and α + β classes (Albert et al. 2000) and also showed substantial similarities with those of the crystallized AtSAL1 protein "PDB code: 5ESY (Chan et al. 2016) (Chan et al. 2016)" HAL2 "PDB code: 1KA1" and old101 predicted model (Suppl. Fig. 5). SAL1 in vitro 3′ (2′), 5′-bisphosphate nucleotidase activity against the substrate PAP was subsequently measured as described (Murguia et al. 1995; Xu et al. 2012). The specific activity of the recombinant wild type SAL1 protein was 1.8 µmol/min/mg protein (Fig. 5), which is broadly similar to what was found previously (Chan et al. 2016; Quintero et al. 1996). Surprisingly, the specific activity of the recombinant old101 protein was not significantly different from the wild type recombinant SAL1 protein. The results suggest that the *old101* mutation does not affect the nucleotide phosphatase activity of its encoded protein.

The old101 protein has reduced inositol polyphosphate 1-phosphatase activity

The SAL1 protein also has inositol polyphosphate 1-phosphatase activity, however, the biological function of this activity is unclear. Refolded old101 and SAL1 proteins were tested for their ability to hydrolyze inositol 1,3,4-triphosphate (IP_3). The activity of these proteins against IP_3 was measured by quantifying released Pi based on the malachite green method (molybdate: malachite green complex at 630 nm) (Baykov et al. 1988). The in vitro activity of old101 against IP_3 was 2.5 times lower than that of SAL1 (Fig. 5) and this suggests that the *old101* mutation affects the polyphosphate 1-phosphatase activity of the SAL1 protein.

The *old101* mutation is associated with lower ROS accumulation in young plants

Early senescence is associated with increased ROS levels (Kan et al. 2021; Guo et al. 2017; Schippers et al. 2008; Jing et al. 2008; Kanojia and Dijkwel 2018). Since *old101* plants show a delayed onset of senescence, we hypothesized that this may be a result of lower ROS levels. First, we stained leaves with DAB: a deep brown polymerization product was formed between DAB and H_2O_2, which could be visualized after elimination of leaf chlorophyll (Orozco-Cardenas and Ryan 1999; Thordal-Christensen et al. 1997). Leaves of 10, 15 and 25-day-old soil grown plants were stained and are shown in Fig. 6a, b and c. Wild type leaves appeared darker brown than mutant leaves, especially in young plants and this suggests lower ROS levels in the mutant. We subsequently quantified H_2O_2 levels and Fig. 6d shows that total H_2O_2 levels of 10 and 15-day-old *old101* plants were lower than those of the mutant. Twenty-five-day-old mutants, however, had H_2O_2 levels that were insignificantly lower than those of wild type plants. The lower H_2O_2 levels in *old101* plants furthermore were accompanied with over fourfold lower expression of the oxidative stress marker gene *DEFL* in 15-day-old plants (Fig. 6f). Lower H_2O_2 levels could be a result of decreased production or increased scavenging. We measured activity of the H_2O_2 scavenger peroxidase in 10, 15 and 25-day-old mutant and wild type plants. Figure 6e shows that 10 and 15-day-old mutant plants had significantly increased peroxidase levels as compared to the wild type, while at day 25 no significant difference was found. Thus, the delayed ageing phenotype of *old101* plants is associated with lower H_2O_2 levels and increased peroxidase activity in young plants.

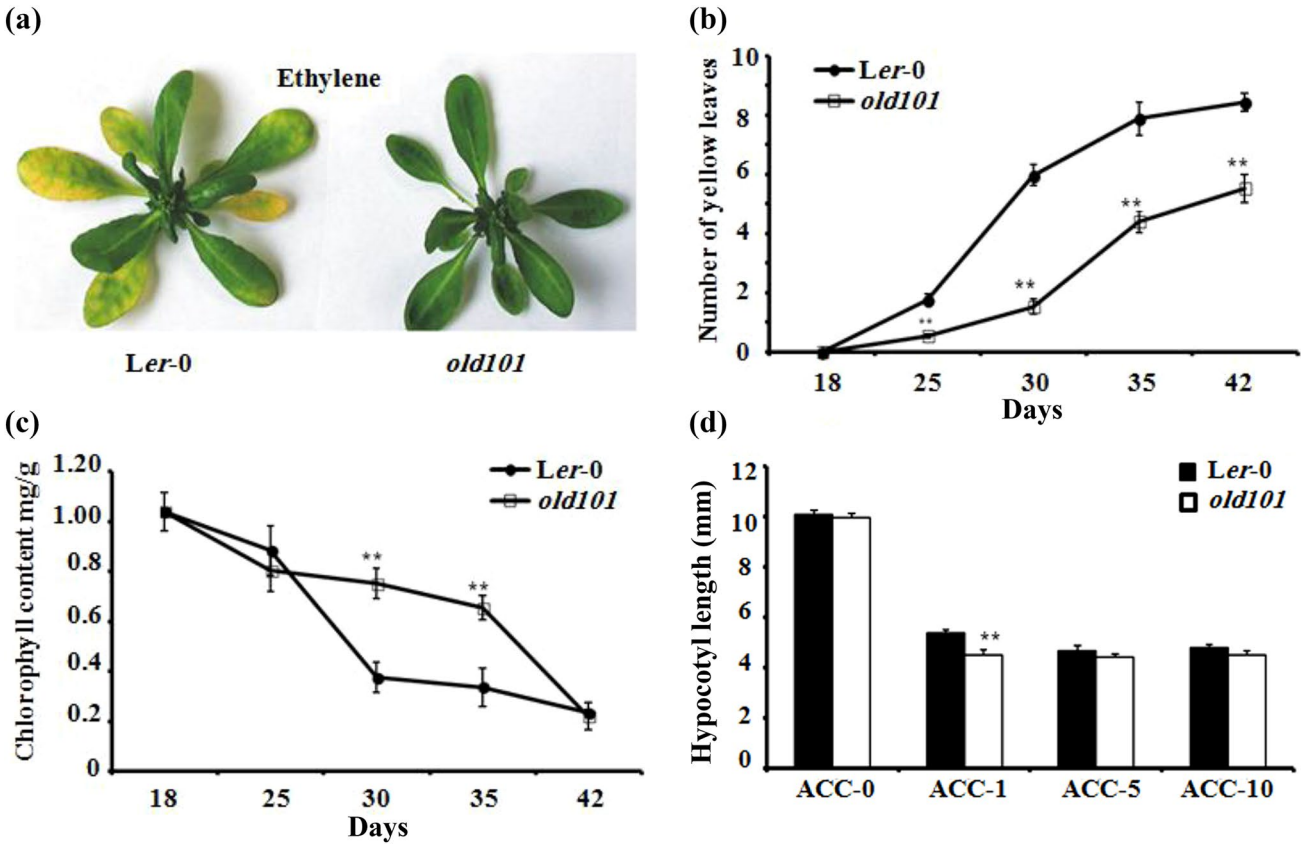


Fig. 3 Effect of ethylene on wild type and *old101* plants. **a** Photographs of representative L*er*-0 and *old101* plants that were grown in air for 26 days, followed by 3 days of growth in air supplemented with ethylene and one additional day of growth in air. **b** The average number of yellow leaves per plant in ethylene-treated L*er*-0 and *old101* plants of different developmental stages ranging from 18 to 42 days. **c** Chlorophyll content in leaves of ethylene-treated L*er*-0 and *old101* plants. Wild type and mutant plants were soil-grown in air for up to 4 days before the indicated times, and subsequently treated with ethylene for 3 days and grown for one additional day in air before measurements were taken. **d** Hypocotyl lengths of wild type (L*er*-0) and *old101* seedlings grown on MS medium containing 0, 1, 5 or 10 µM ACC in darkness were measured 5 days after transfer to 23 °C. Values represent means ± SD of at least three replicates of 20–30 plants. Values that are significantly different between wild type and *old101* at $P < 0.05$ (*) and $P < 0.01$ (**), using Student's t test, are indicated

Discussion

The *old101* mutation regulates age related changes

Arabidopsis leaves growing under environmentally favorable conditions undergo the process of leaf senescence as its final developmental stage. This often-called developmental leaf senescence process is believed to be a consequence of age-dependent or age-related changes (ARCs). However, early leaf senescence can be induced by adverse environmental conditions or by treatments with hormones that typically play a role in the plants' stress response, such as Jasmonic acid and ethylene (Hensel et al. 1993; Kim et al. 2015; Jing et al. 2005; Jibran et al. 2013). Crucially, however, these hormones were not able to induce leaf senescence in very young leaves (Hensel et al. 1993; Jing et al. 2002), suggesting that leaves need to acquire the competence to senesce before this destructive process can be induced (Jing et al. 2005; Kanojia and Dijkwel 2018). Thus, also stress-induced senescence appears to be dependent on the occurrence of ARCs, although these may include different ARCs than those required for the induction of developmental senescence. The Arabidopsis *old101* mutant was isolated as a mutant in which leaves acquire the competence to senesce later than wild type leaves (Shirzadian-Khorramabad et al. 2008) and ethylene treatment induced leaf senescence on average 9 days later in the mutant than in the wild type (Fig. 3). Because *old101* mutants show a normal triple response and since ethylene does induce senescence, albeit at a later stage, this mutant does not have a defective ethylene-signaling pathway. Nevertheless, also when grown in air under favorable conditions, *old101* mutants show slightly delayed development throughout life, starting with an approximately 1-day delay in germination and a more marked delay in developmental

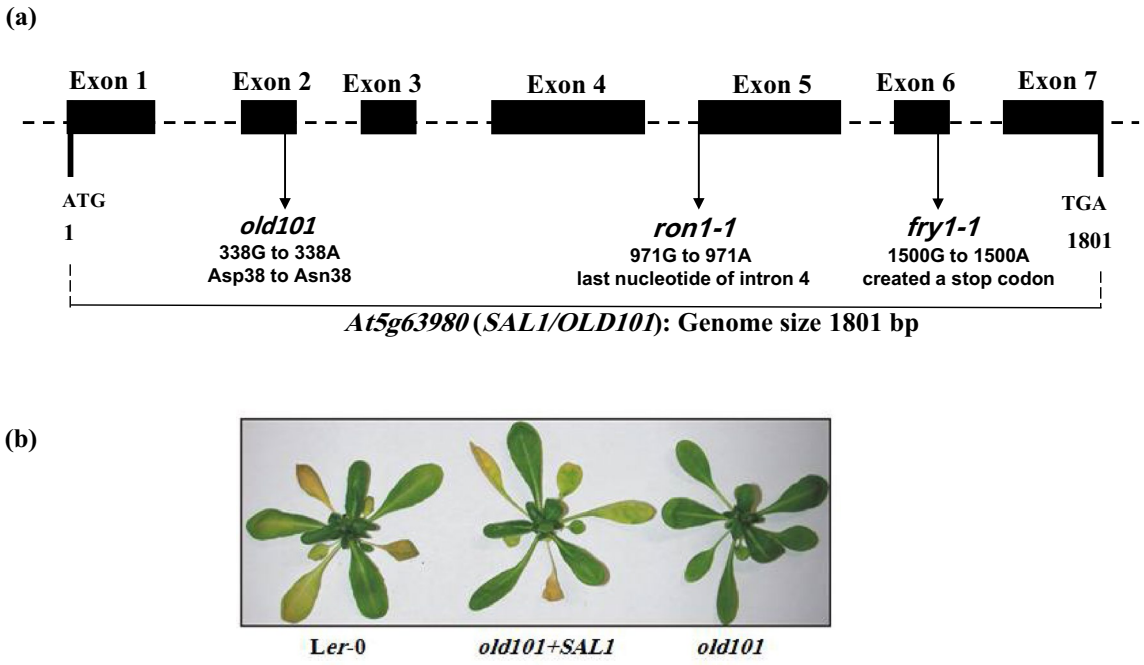


Fig. 4 Location of the *old101*, *ron1-1* and *fry1-1* mutations in *SAL1* and genetic complementation of the *old101* mutant. **a** The *old101* mutation causes a G to A point mutation at position 338 resulting in an aspartate (GAT) to asparagine (AAT) residue substitution at position 38 of the encoded protein between the first and second α-helix of the protein (York et al. 1995; Xiong et al. 2001; Robles et al. 2010). **b** The *SAL1* genomic region, including the promoter area, was transformed to *old101* plants as described in "Materials and methods" section. Representative 30-day-old L*er*-0 (Left), *old101* carrying the homozygous *SAL1* gene (Middle), and *old101* (Right) plants after ethylene treatment are shown

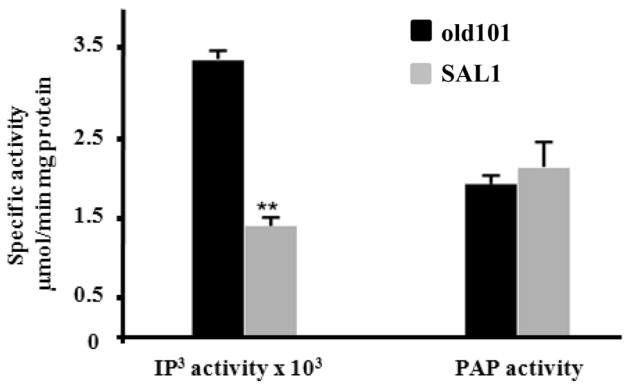


Fig. 5 Specific activities of recombinant refolded SAL1 and old101 proteins against substrates (3′-phosphoadenosine 5′-phosphate) and IP_3 (inositol 1,4,5-triphosphate). The phosphatase activity of the proteins against PAP was assayed by measuring substrate utilization using reversed-phase HPLC as described in "Materials and methods" section. The concentration of phosphate liberated from IP_3, was measured by the molybdate-malachite green method as described in "Materials and methods" section. Values are expressed as mean ± SD of three replicates. Values that are significantly different between SAL1 and old101 at $P < 0.05$ (*) and $P < 0.01$ (**), using Student's t test, are indicated

leaf senescence (Fig. 2e). This is consistent with the idea that occurrence of ARCs is delayed throughout the development of *old101* mutant plants.

Positional cloning revealed that the *old101* mutation results in the Asp^{38} to Asn^{38} amino acid substitution of the SAL1 protein. SAL1, also known as FRY1, HOS2, ALX8, RON1 and FOU8, was previously found to be involved in regulating multiple plant developmental and stress signaling pathways, including ABA-mediated drought and cold stresses, light response, flowering time, leaf shape, and root architecture (Quintero et al. 1996; Xiong et al. 2001, 2004; Kim and Von Arnim 2009; Robles et al. 2010; Hirsch et al. 2011; Estavillo et al. 2011; Gy et al. 2007; Rodríguez et al. 2010). The phenotypes of plants carrying the null alleles *fry1-6* and *ron1-1*, which are from the Col-0 and L*er*-0 background, respectively, are strikingly different from their respective wild types: although the *fry1-6* phenotype is more apparent than that of *ron1-1*, both alleles confer a severe dwarf phenotype, crinkly rounded leaves and delayed flowering (Gy et al. 2007; Robles et al. 2010). In stark contrast, the *old101* phenotype is only subtly different from the wild type and this demonstrates that the *old101* allele is either leaky or has an ectopic activity.

SAL1 possesses two activities: 3′ (2′), 5′-bisphosphate nucleotide phosphatase activity which is highly specific

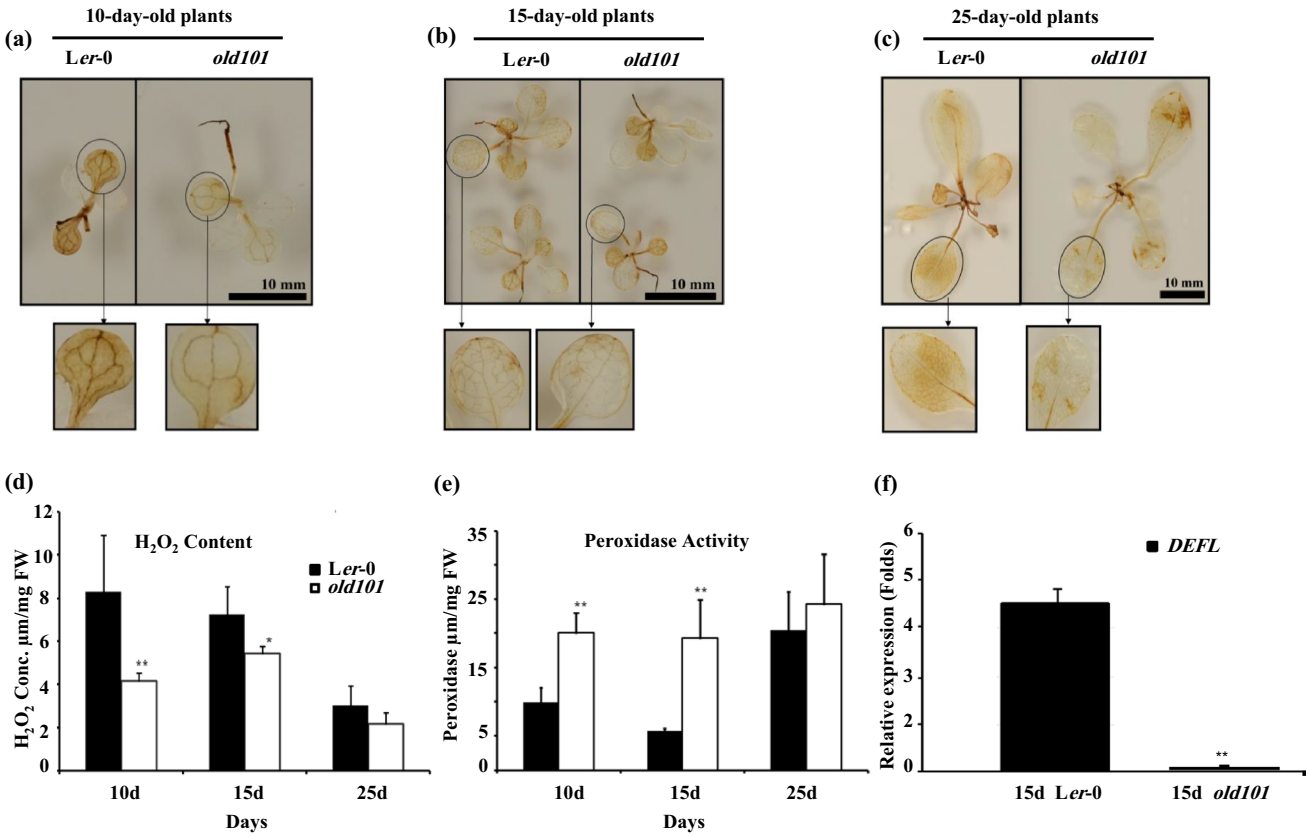


Fig. 6 Oxidative stress symptoms in wild type and *old101* mutant plants. **a–c** Representative images of wild type L*er*-0 and mutant *old101* plants that were grown for the indicated number of days on soil, after which tissues were stained with DAB to visualize H_2O_2 accumulation as a brown precipitate. **d** Quantification of leaf H_2O_2 in 10, 15 and 25-day-old soil-grown wild type and *old101* leaves using the Amplex Red method. **e** Peroxidase activity in 10, 15 and 25-day-old soil-grown wild type and *old101* mutant plants. **f** Relative expression of oxidative stress marker gene *DEFL* in 15-day old L*er*-0 and *old101* plants. Values shown represent the means ± SD of three replicates. Values that are significantly different between L*er*-0 and *old101* at $P < 0.05$ (*) and at $P < 0.01$ (**), using Student's t test, are indicated

for its substrate 3′-polyadenosine 5′-phosphate (PAP), and inositol polyphosphate 1-phosphatase activity that can remove the 1-phosphate group from the second messenger inositol 1, 4, 5-trisphosphate (IP_3). Defects in its activity against PAP is well-established to be responsible for several of the hitherto-described phenotypes (Phua et al. 2018; Estavillo et al. 2011; Hirsch et al. 2011; Lee et al. 2012a; Kim and Von Arnim 2009). Constitutive PAP accumulation in *sal1* mutants resulted in drought tolerance, but also affected growth and changed leaf morphology (Phua et al. 2018). While its biological role in IP_3 metabolism has not been clarified, it may play a role in auxin transport (Zhang et al. 2011). Moreover, a soybean SAL1 homologue, GmSAL1, only hydrolyzes IP_3, and its overexpression in transgenic Arabidopsis resulted in reduction of IP_3 signals (Ku et al. 2013). We show here that the recombinant old101 protein does not change its in vitro activity against PAP. In contrast, its in vitro activity against IP_3 is just 40% of that of SAL1 (Fig. 5). These results are consistent with the idea that the *old101* phenotype is a result of a leaky mutation in *SAL1* that affects its activity against IP_3, and that this activity has biological roles. Appropriate regulation of steady-state IP_3 and PIP_2 levels is necessary for regular plant growth and development (Gillaspy 2013; Xue et al. 2009). Furthermore, phospholipase C involvement in plant development was confirmed in two T-DNA mutants *plc3-2* and *plc3-3*, which exhibited reduced lateral root initiation and germinated more slowly (Zhang et al. 2018). We would like to speculate that the SAL1 phosphoinositol 1-phosphatase activity may regulate the occurrence of ARCs and hence Arabidopsis developmental leaf senescence. However, the measured in vitro activities are not necessarily representative of the in vivo activities and the *old101* mutation may affect protein stability or interaction with other factors and as such its in vivo activity against PAP.

Roles of SAL1 in the regulation of oxidative stress and leaf senescence

The *old101* allele of *SAL1* conferred higher levels of proxidase activity and decreased H_2O_2 accumulation in leaves of young 10-day-old, but not of 25-day-old plants. This was accompanied with lower expression of the ROS marker gene *DEFL* (*AT2G43510*) (Gadjev et al. 2006; Guo et al. 2017) (Fig. 6f). ROS is a by-product of metabolism but also functions as an important signaling molecule in the regulation of growth and development and abiotic and biotic stress responses (Mhamdi and Van Breusegem 2018; Choudhury et al. 2017; Jajic et al. 2015; Lai et al. 2012; Mittler et al. 2004). The analysis of Arabidopsis mutants with an altered leaf senescence phenotype has provided strong evidence for a connection between oxidative stress and senescence. Several delayed senescence mutants including *ore1, ore3, and ore9 and ore4-1* exhibited enhanced tolerance to oxidative stress (Woo et al. 2002, 2004), indicating that the ability to effectively cope with oxidative stress, rather than ROS levels per se, may regulate ageing. The *ore4-1* mutation caused a reduction in expression of the plastidic gene *PRPS17* leading to reduced photosynthetic activity and delayed leaf senescence (Woo et al. 2002). The late senescence phenotype of *ore4-1* plants is probably due to a decrease in metabolic rate, indicating that energy expenditure is a major factor in the regulation of leaf senescence. The accumulation of oxidative stress in chloroplasts was associated with the ageing process in *Cistus clusii* plants (Munné-Bosch and Alegre 2002) and knockout lines of the NADH chlororespiratory gene *ndhF* resulted in decreased ROS levels and delayed leaf senescence in tobacco (Zapata et al. 2005). Mutations in *NAC075*, *WRKY42*, *NTL4*, *WRKY55* and *WORKY75* caused delayed senescence due to altered ROS levels (Kan et al. 2021; Niu et al. 2020; Lee et al. 2012b; Guo et al. 2017). NAC075 works as a negative regulator of leaf aging in *Arabidopsis*. T-DNA insertional loss of function *nac075* mutants demonstrated a considerable decrease in expression levels of antioxidant enzymes in coincidence with accelerated leaf senescence, while overexpression of NAC075 reduced the accumulation of ROS and prolonged leaf longevity in *Arabidopsis* (Kan et al. 2021). *WRKY42* was highly induced prior and during plant senescence and a *WRKY42* loss-of-function mutant showed delayed senescence, while increased *WRKY42* expression caused early leaf senescence. *WRKY42* regulates plant senescence by enhancing SA biosynthesis and ROS levels through activation of the promoters of *ISOCHORISMATE SYNTHASE 1* (*ICS1*), *OXIDASE HOMOLOG F* (*RbohF*) and several SAGs (Niu et al. 2020). While over-expression of the NAC-transcription factor-encoding *NTL4* in drought-stressed transgenic plants resulted in higher ROS accumulation and early leaf senescence, *ntl4* mutant plants showed lower ROS, delayed leaf senescence and increased drought tolerance. It was suggested that NTL4 functions as a molecular switch that enhances ROS production in dry conditions, in order to induce leaf senescence and remobilization of nutrients from aging leaves to developing organs (Lee et al. 2012b). Moreover, *WRKY55* contributed to ROS and SA accumulation, accelerating senescence and causing resistance to bacterial pathogens. T-DNA mutation lines of WRKY55 prolonged leaf senescence in association with low levels of ROS (Wang et al. 2020). While knocked-down and knocked-out plants of *WRKY75* displayed delayed developmental leaf senescence, increased *WRKY75* expression accelerated the initiation of leaf senescence, and this was dependent on both *WRKY75*-induced salicylic acid (SA) production and increased ROS levels. *WRKY75* increased ROS via inhibition of *CATALASE 2* (*CAT2*) expression and induced SA production by triggering *SA INDUCTION-DEFICIENT2* (*SID2*) transcription (Guo et al. 2017). In contrast to delayed leaf senescence mutants, in the early leaf senescence mutants oxidative stress levels were found to be elevated. Early senescence in the rice *psl85* mutant and Arabidopsis *cpr5* and *old5* mutants coincided with increased ROS levels (He et al. 2018; Jing et al. 2008; Schippers et al. 2008). The *old5* mutation caused a leaky mutation in the quinolinate synthase, resulting in increased NAD levels and higher respiration. These observations are consistent with the idea that metabolic rate, ROS and ageing are linked. Such a reduced metabolic rate coincides with lower ROS levels and delayed, or slowed ageing, while a higher metabolic rate causes higher ROS levels and early ageing. Importantly, the altered ROS levels or metabolic rate occurred prior to leaf senescence symptoms became apparent. This suggests that ROS levels or metabolic rate functions as an ARC. This would explain why the lower ROS levels in young *old101* plants ultimately delay ageing in 'old' *old101* plants. Importantly, a direct correlation between SAL1 function and decreased ROS levels has been reported in the *alx8* allele, where the loss of SAL1 function resulted in increased *APX2* expression and decreased H_2O_2 levels (Pornsiriwong et al. 2017; Estavillo et al. 2011; Rossel et al. 2006; Wilson et al. 2009).

In summary, the leaky, EMS-induced, *old101* mutation exposes a potential specific function for SAL1 activity against IP_3 in the regulation of plant development and ROS levels in young leaves. We propose that the *old101* mutation may slightly slow development and metabolism throughout plant development, resulting in reduced H_2O_2 levels in young leaves and delayed occurrence of ARCs, ultimately leading to delayed leaf senescence.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11103-022-01245-0.

Acknowledgements We would like to thank Bert Venema and Margriet Ferwerda for their excellent technical support, and Anne de Jong from the University of Groningen for the use of lab equipment.

Author contributions The authors contributed to the study as follows: the material preparation, data collection, analysis and writing the first draft of the manuscript were performed by RS-K. The IP3 and PAP experiments were done by TM. Supervising the protein assay experiments was performed by RHS. The mutant population was created by H-CJ. JH was the promotor of the first part of the work in GBB (Groningen Biomolecular Sciences and Biotechnology Institute). PD supervised the whole work and revised the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This work was funded in part by a grant from the Ministry of Science, Research and Technology of Islamic Republic of Iran, University of Guilan to RSHK, a Massey University Research Fund grant to PPD.

Availability of data and materials All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Conflict of interest The authors declare no conflict of interest.

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