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RESEARCH PAPER

Tomato fruit ripening factor NOR controls leaf senescence

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Abstract

NAC transcription factors (TFs) are important regulators of expressional reprogramming during plant development, stress responses, and leaf senescence. NAC TFs also play important roles in fruit ripening. In tomato (*Solanum lycopersicum*), one of the best characterized NACs involved in fruit ripening is NON-RIPENING (NOR), and the *non-ripening* (*nor*) mutation has been widely used to extend fruit shelf life in elite varieties. Here, we show that NOR additionally controls leaf senescence. Expression of *NOR* increases with leaf age, and developmental as well as dark-induced senescence are delayed in the *nor* mutant, while overexpression of *NOR* promotes leaf senescence. Genes associated with chlorophyll degradation as well as senescence-associated genes (SAGs) show reduced and elevated expression, respectively, in *nor* mutants and *NOR* overexpressors. Overexpression of *NOR* also stimulates leaf senescence in *Arabidopsis thaliana*. In tomato, NOR supports senescence by directly and positively regulating the expression of several senescence-associated genes including, besides others, *SlSAG15* and *SlSAG113*, *SlSGR1*, and *SlYLS4*. Finally, we find that another senescence control NAC TF, namely SlNAP2, acts upstream of *NOR* to regulate its expression. Our data support a model whereby NAC TFs have often been recruited by higher plants for both the control of leaf senescence and fruit ripening.

Keywords: Aging, leaf, NAC, non-ripening, NOR, senescence, tomato, transcription factor.

Introduction

Transcription factors (TFs) of the NAC (for NAM, ATAF1/2, and CUC2) family play important roles for development and the response of plants to abiotic and biotic stresses (Puranik *et al.*, 2012; Shao *et al.*, 2015). A prominent process controlled by NAC TFs is leaf senescence, which is a complex physiological process of nutrient recovery to support the development and growth of newly forming organs, including new leaves, flowers, and seeds (Hendelman *et al.*, 2013; Zhong *et al.*, 2016). NAC TFs in diverse dicot and monocot plant species have been shown to control the onset and execution of senescence, for example in *Arabidopsis thaliana* (Guo and Gan, 2006; Kim *et al.*, 2009; Balazadeh *et al.*, 2010, 2014; Wu *et al.*, 2012; Garapati *et al.*, 2015; Kamranfar *et al.*, 2018), rice (*Oryza sativa*; Zhou *et al.*, 2013; Mao *et al.*, 2017), wheat (*Triticum aestivum*; Uauy *et al.*, 2006; Zhao *et al.*, 2015), cotton (*Gossypium hirsutum*; Fan *et al.*, 2015), and tomato (*Solanum lycopersicum*; Lira *et al.*, 2017; Ma *et al.*, 2018).

A master positive regulator of leaf senescence in Arabidopsis is ORE1 (ORESARA1; ANAC092; Kim *et al.*, 2009; Balazadeh *et al.*, 2010). Expression of *ORE1* increases with leaf age, a process regulated at the transcriptional level by the *ORE1* promoter, and post-transcriptionally by the miRNA

miR164 (Kim *et al.*, 2009). ORE1 controls the expression of a number of senescence-associated genes (SAGs) by directly binding to their promoters (Balazadeh *et al.*, 2010) and, accordingly, overexpression or knocking out of *ORE1* promotes or inhibits senescence, respectively (Kim *et al.*, 2009; Balazadeh *et al.*, 2010). Recently, the closest putative orthologs of *ORE1* in tomato (i.e. *SlORE1S02*, *SlORE1S03*, and *SlORE1S06*) were also shown to positively control leaf senescence (Lira *et al.*, 2017). In addition, inhibiting *SlORE1S02* by RNAi not only delayed leaf senescence but also triggered an altered source–sink sugar partitioning, resulting in an increased number of fruits per plant with elevated sugar levels (Lira *et al.*, 2017). Similarly, we recently showed that inhibiting expression of the *SlNAP2* TF gene in transgenic tomato plants delays leaf senescence, which was accompanied by an increased yield of fruits (with elevated sugar content) probably due to extended photosynthesis in aging plants (Ma *et al.*, 2018). *SlNAP2* belongs to the NAP clade of NAC TF genes of which *AtNAP* from Arabidopsis was first studied with respect to leaf senescence (Guo and Gan, 2006) and was later shown also to control silique senescence (Kou *et al.*, 2012). In rice, inhibiting *OsNAP1* delayed leaf senescence but increased seed yield (Liang *et al.*, 2014).

In addition, NAC TFs have been reported, or suggested, to be involved in ripening of fleshy fruits in several species, with a particular emphasis on tomato, an important fleshy fruit-bearing crop that is extensively used as a model vegetable for studies on fruit physiology and development; its nuclear genome has been sequenced (Tomato Genome Consortium, 2012). One of the best characterized examples in tomato is NON-RIPENING (NOR), which also affects fruit shelf life, an important economic trait. Mutations in the *NOR* gene (locus *Solyc10g006880*) lead to the formation of a truncated TF protein (*nor* mutant) or a NAC TF with a single amino acid substitution (*alcobaca* mutant, *alc*) (Giovannoni *et al.*, 2004; Casals *et al.*, 2012). Recently, a further mutation of the *NOR* gene, leading to an early stop codon, was identified in the tomato variety Penjar-1 grown in the Mediterranean area (Kumar *et al.*, 2018). NOR acts upstream of ethylene synthesis and thereby controls fruit ripening (Barry and Giovannoni, 2007). ChIP assays demonstrated that *NOR* is a direct downstream target of RIN (Ripening Inhibitor), a MADS-box TF controlling fruit ripening (Martel *et al.*, 2011; Fujisawa *et al.*, 2013; Ito *et al.*, 2017). Similarly, in melon (*Cucumis melo*), a NOR TF (CmNAC-NOR) was found to be involved in fruit ripening (Rios *et al.*, 2017). In addition, *NOR* homologs control senescence in non-fleshy fruits such as the siliques of Arabidopsis where *NARS1/NAC2* and *NARS2/NAM* redundantly and positively regulate silique senescence while leaf senescence is unaltered compared with the wild type (WT), indicating organ-specific functions of the two NAC TFs (Kunieda *et al.*, 2008).

Besides NOR, other TFs of the NAC family in tomato have been reported to control fruit ripening, including SlNAC4 which positively regulates ripening, possibly through physical interaction with NOR and RIN (shown by yeast two-hybrid studies); furthermore, SlNAC4 was suggested to act as an upstream regulator of *RIN* (Zhu *et al.*, 2014). Evidence for a positive role in regulating fruit ripening was also obtained for SlNAC48 and SlNAC19 (which is identical to SlNAP2) using a virus-induced gene silencing (VIGS) approach. The data suggest that both TFs, SlNAC19 and SlNAC48, act by affecting ethylene biosynthesis and signaling (Kou *et al.*, 2016). *SlNAC3* (recently named *NOR-like1*) shows high expression in fruits and is involved in seed development and fruit ripening (Han *et al.*, 2012, 2014; Gao *et al.*, 2018).

Evidence for an involvement of NAC TFs in fleshy fruit ripening was also obtained from studies performed on developing and ripening fruits of other species, including the octoploid strawberry cultivar *Fragaria* × *ananassa* (Moyano *et al.*, 2018), the Chilean endemic strawberry *Fragaria chiloensis* (Carrasco-Orellana *et al.*, 2018), and bilberry (*Vaccinium myrtillus*; Nguyen *et al.*, 2018).

Taken together, many NAC TFs have been reported to control leaf senescence in different plant species, and some NACs have been firmly proven—or suggested—to control the ripening of fleshy or dry fruits. Considering this, we were interested to investigate whether the so-far best studied fruit ripening control NAC TF in tomato, namely NOR, additionally controls leaf senescence in this plant. Our data show that NOR acts as a positive transcriptional regulator of leaf senescence by directly and positively controlling the expression of several chlorophyll degradation- (CDGs) and senescence-associated genes (SAGs) in this species. The data suggest an evolutionary recruitment of NAC TFs from regulating leaf senescence towards the control of physiology during fruit ripening.

Materials and methods

General

Tomato orthologs of Arabidopsis genes were identified using the PLAZA 3.0 database (http://bioinformatics.psb.ugent.be/plaza/; Proost *et al.*, 2015). Genes were annotated using the PLAZA 3.0 and Sol Genomics (https://solgenomics.net/) databases, and using information extracted from the literature. Oligonucleotide sequences are given in Table S1 at *JXB* online. Primers for quantitative real-time PCR (qRT-PCR) were designed using QuantPrime (www.quantprime.de; Arvidsson *et al.*, 2008).

Plant material and growth conditions

Tomato (*Solanum lycopersicum* L., cultivar Moneymaker) was used as the WT. The *nor* mutant is in the Rutgers genetic background (Tomato Genetics Research Center, https://tgrc.ucdavis.edu; accession number LA3013). The mutant is due to a spontaneous mutation in the *NOR* gene. Seeds were germinated on full-strength Murashige and Skoog (MS) medium containing 2% (w/v) sucrose, and 3-week-old seedlings were transferred to soil containing a mixture of potting soil and quartz sand (2:1, v/v). Plants were grown in a growth chamber at 500 µmol photons m^{-2} s^{-1} (high-pressure sodium vapor lamps; Agrolux, https://www.agrolux.com) and 25 °C under a 14 h/10 h light/dark regime in individual pots (18 cm diameter). For experiments with *Arabidopsis thaliana* (L.) Heynh., accession Col-0 was used as the control. Seeds were germinated in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Altengronau, Germany) in a climate-controlled chamber with a 16 h day length provided by fluorescent light at ~100 µmol m^{-2} s^{-1}, day/night temperature of 20 °C/16 °C, and relative humidity of 60%/75%. After 2 weeks, seedlings were transferred to a growth chamber with a 16 h day (80 µmol m^{-2} s^{-1} or 120 µmol m^{-2} s^{-1}), day/night temperature of 22 °C/16 °C, and 60%/75% relative humidity.

DNA constructs

Primer sequences are listed in Supplementary Table S1. Amplified fragments generated by PCR were sequenced by Eurofins MWG Operon

(Ebersberg, Germany). For *35S:NOR-GFP*, the full-length *NOR* ORF was amplified without its stop codon. The PCR product was cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning kit (Invitrogen). The sequence-verified entry clone was then transferred to the pK7FWG2 vector (Karimi *et al.*, 2002) by LR recombination (Invitrogen). For *NOR-IOE*, the *NOR* coding sequence was cloned into the pER10 vector (Zuo *et al.*, 2002) made GATEWAY compatible. Constructs were transformed into tomato cv. Moneymaker using *Agrobacterium tumefaciens* GV2260, or into Arabidopsis using *A. tumefaciens* GV3101 (pMP90).

The DNA-binding protein–CELD (cellulose D) fusion vector pTacLCELD6xHis was used to construct *NOR-CELD* (Xue, 2005). The NOR coding sequence (without the stop codon) was amplified by PCR with a sense primer (including an *Nhe*I restriction site) and an antisense primer (including a *Bam*HI restriction site) (Supplementary Table S1). The amplified DNA fragment was first inserted into pCR2.1 (Thermo Fisher Scientific) and then inserted N-terminally of CELD using the *Nhe*I and *Bam*HI cloning sites of pTacLCELD6xHis to create an in-frame fusion.

Amino acid sequence alignment

Protein sequences were extracted from PLAZA 3.0 (http://bioinformatics.psb.ugent.be/plaza/). The protein alignment was done using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/); the output set-up was Pearson/FASTA. To highlight conserved regions within the alignments, the Sequence Manipulation Suite (http://www.bioinformatics.org/sms2/color_align_cons.html) was employed.

Treatments

For estradiol (EST) induction, 3-week-old *NOR-IOE* seedlings were incubated in sterile water containing 15 μM EST [control treatment: 0.15% (v/v) ethanol]. The seedlings were kept on a rotary shaker for 6 h and then immediately frozen in liquid nitrogen. For abscisic acid (ABA) treatment, 3-week-old WT seedlings and detached young leaves from 10-week-old WT and *NOR* transgenic plants were placed in sterile water containing 40 μM ABA with constant light. The seedlings were kept on a rotary shaker for 2, 4, 8, or 16 h and harvested in liquid nitrogen. For dark-induced leaf senescence experiments, detached young leaves from 10-week-old WT and *NOR* transgenic plants were placed on moist filter papers in Petri dishes with the adaxial side facing upwards. The plates were kept in darkness at 22 °C for 2 weeks. Filter papers were changed every 5 d. Gene expression levels were determined by qRT-PCR.

Gene expression analysis

Total RNA was extracted using Trizol reagent (Life Technologies). Synthesis of cDNA and qRT-PCR using SYBR Green were performed as described (Balazadeh *et al.*, 2008). PCR was performed using an ABI PRISM 7900HT sequence detection system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; *Solyc04g009030*) served as the reference gene for data analysis. Statistical significance was determined using Student's *t*-test.

DNA-binding site selection

In vitro binding site selection was performed using the CELD fusion method with the pTacNOR-LCELD6xHis construct, employing biotin-labeled double-stranded oligonucleotides (Xue, 2005). The DNA binding activity of NOR–CELD was measured using methylumbelliferyl β-D-cellobioside as substrate (Xue, 2002). DNA binding assays with a biotin-labeled single-stranded oligonucleotide or a biotin-labeled double-stranded oligonucleotide without a target binding site were used as controls.

Chromatin immunoprecipitation

ChIP-qPCR was performed using leaves of mature *35S:NOR-GFP* plants, and the WT served as control. ChIP was performed as described (Kaufmann *et al.*, 2010) using anti-green fluorescent protein (GFP) antibody to immunoprecipitate protein–DNA complexes. qPCR primers were designed to flank the NOR-binding sites within the promoter regions of potential target genes. Primers annealing to a promoter region of *Solyc04g009030* lacking a NOR-binding site were used as a negative control. Primers used for qPCR are listed in Supplementary Table S1.

Chlorophyll measurements

Chlorophyll content was determined using a SPAD analyser (N-tester; Hydro Agri). Alternatively (Fig. 4D), frozen leaf powder was suspended in 5 ml of 80% (v/v) acetone in water and homogenized for 1 min. Chlorophyll content was determined with a spectrophotometer at 663 nm and 646 nm as described by Arnon (1949).

Ion leakage measurements

Ion leakage in leaves was determined as reported in Thirumalaikumar *et al.* (2018). Briefly, ion leakage during senescence was measured in control and dark-treated leaves of WT and *NOR* transgenic plants. Detached leaves were shaken in distilled water overnight at 4 °C and the initial conductivity of the samples was determined using an ion conductivity meter (SI Analytics, Mainz, Germany). Subsequently, the samples were boiled at 100 °C for 30 min and then kept at room temperature to cool down to 25 °C, after which the total conductivity was measured. Data are shown as the percentage of total conductivity.

Accession numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: *NOR* (NM_001247723.2); *SlNAC3* (NM_001279348.2); *SlNAP2* (XM_004236996.2); *SlSAG15* (XM_010320381.2); *SlSAG113* (XP_004239911.1); *SlSGR1* (NP_001234723.1); *SlPPH* (XM_004229633.3); *SlPAO* (NP_001234535.2); *SlYLS4* (XM_004245218); *SlERT1B* (NM_001361347); *SlKFB20* (XM_010320257); *SlABCG40* (XP_004247842.1); and *SlGA2ox5* (NP_001234757.1).

Results

NOR *is up-regulated during leaf senescence*

NOR encodes a tomato NAC TF that harbours a conserved DNA-binding NAM domain at its N-terminus (Fig. 1A; Supplementary Fig. S1). At the protein level, NOR is closely related to SlNAC3/NOR-like1 from tomato, and to NARS1 and NARS2 from Arabidopsis (Fig. 1B; Supplementary Fig. S1). As shown below, NOR binds to the promoters of several direct target genes, demonstrating it to be a nuclear-localized protein.

NOR is hardly expressed in young leaves, but its expression increases during developmental and dark-induced senescence (Fig. 1C, D), indicating a possible function of the tomato TF for regulating leaf senescence. Of note, *NOR* shows high expression not only in senescent leaves, but also in flowers and fruits of different developmental stages (Fig. 1C).

Overexpression of NOR *promotes leaf senescence*

To test whether NOR indeed regulates leaf senescence, we first generated transgenic tomato (*S. lycopersicum* cv. 'Moneymaker') plants constitutively expressing *NOR* under the control of the *Cauliflower mosaic virus* (CaMV) *35S* promoter. We selected two lines (hereafter, *OX-L5* and *OX-L19*; Supplementary Fig.

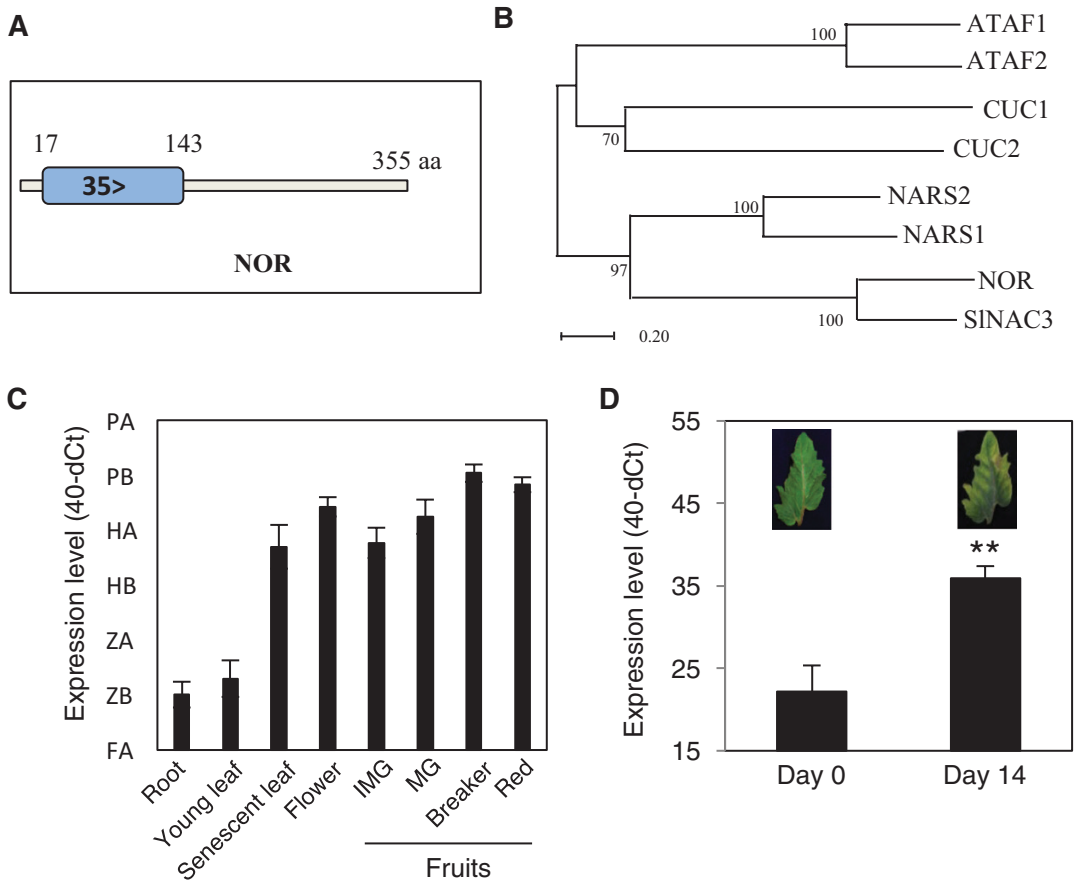

Fig. 1. Relationship of NOR to other NAC factors and *NOR* expression during senescence. (A) Schematic presentation of the NAM domain of NOR. Numbers indicate amino acid positions. (B) Phylogenetic analysis of selected NAC proteins. The phylogenetic tree was constructed by MEGA 5.05 software using the Neighbor–Joining method with the following parameters: bootstrap analysis of 1000 replicates, Poisson model, and pairwise deletion. NOR and SlNAC3 are two tomato TFs and the others are from Arabidopsis. Gene codes of the Arabidopsis TFs are: *ATAF1*, *At1g01720*; *ATAF2*, *At5g08790*; *NARS1*, *At3g15510*; *NARS2*, *At1g52880*; *CUC1*, *At3g15170*; and *CUC2*, *At5g53950*. (C) *NOR* transcript abundance in different tissues of wild-type tomato plants (cv. Moneymaker). The *y*-axis indicates the expression level (40-dCt). Data are means ±SD of three biological replicates. (D) Expression of *NOR* in young detached leaves of 8-week-old WT plants before (day 0) and after 14 d of dark treatment. Leaves were excised from the top part of the stem. Data are means ±SD (n=3). Asterisks denote a significant difference from Day 0 (Student's t-test, $^{**}P \leq 0.01$).

S2A) for further analysis. Notably, *NOR* overexpression lines showed early leaf senescence (Fig. 2A). The ratio of yellow leaves (defined as leaves with >50% yellowing) to all leaves of 12-week-old *OX* plants was significantly higher in *OX-L5* and *OX-L19* plants than in the WT (Fig. 2B). Furthermore, the chlorophyll content of leaves from the same position (leaf no. 3) dropped faster during development in *OX* than in the WT (Fig. 2C). The precocious senescence of *NOR* overexpressors was not accompanied by an early flowering (days after sowing; Supplementary Fig. S3A), although the start of flowering was observed at a slightly reduced leaf number compared with the WT (Supplementary Fig. S3B). We did not observe a difference in flowering time between *nor* mutant and WT plants (Supplementary Fig. S3).

We also observed a generally reduced shoot height of *NOR* overexpressors compared with the WT, while the *nor* mutant appeared slightly taller under our growth conditions (Fig. 2A). The reduced growth of *NOR* overexpressors (see also Supplementary Fig. S4A–C) may be due to increased expression of *SlGA2ox5* (Supplementary Fig. S4D), a gibberellic acid (GA) degradation gene whose induction may result in reduced GA levels and thereby a reduced shoot elongation (Davière and Achard; 2013). *SlGA2ox5* expression was also enhanced in plants expressing *NOR* from an EST-inducible promoter (hereafter, *NOR-IOE*), shortly (6 h) after EST treatment, suggesting *SlGA2ox5* as a direct target of NOR, in accordance with the presence of a NOR-binding site in its promoter (Supplementary Table S3). Of note, expression of other GA metabolism genes was not significantly affected by NOR (Supplementary Table S4).

The tomato nor *mutant exhibits retarded leaf senescence*

Dark treatment is an efficient way to induce senescence in plants, as shown in many reports (Biswal and Mohanty, 1976; Chen and Kao 1991; Weaver *et al.*, 2001). Here, we tested the effect of darkness on detached leaves which represent an excellent model to study senescence-related signaling events (e.g. Sakuraba *et al.*, 2014); we examined the phenotypes of *nor*, WT, and *OX-L19* after 14 d of dark treatment. Detached leaves from the overexpression line showed earlier de-greening in extended darkness than those of the WT. In contrast, leaves of the *nor* mutant remained green longer in darkness and their

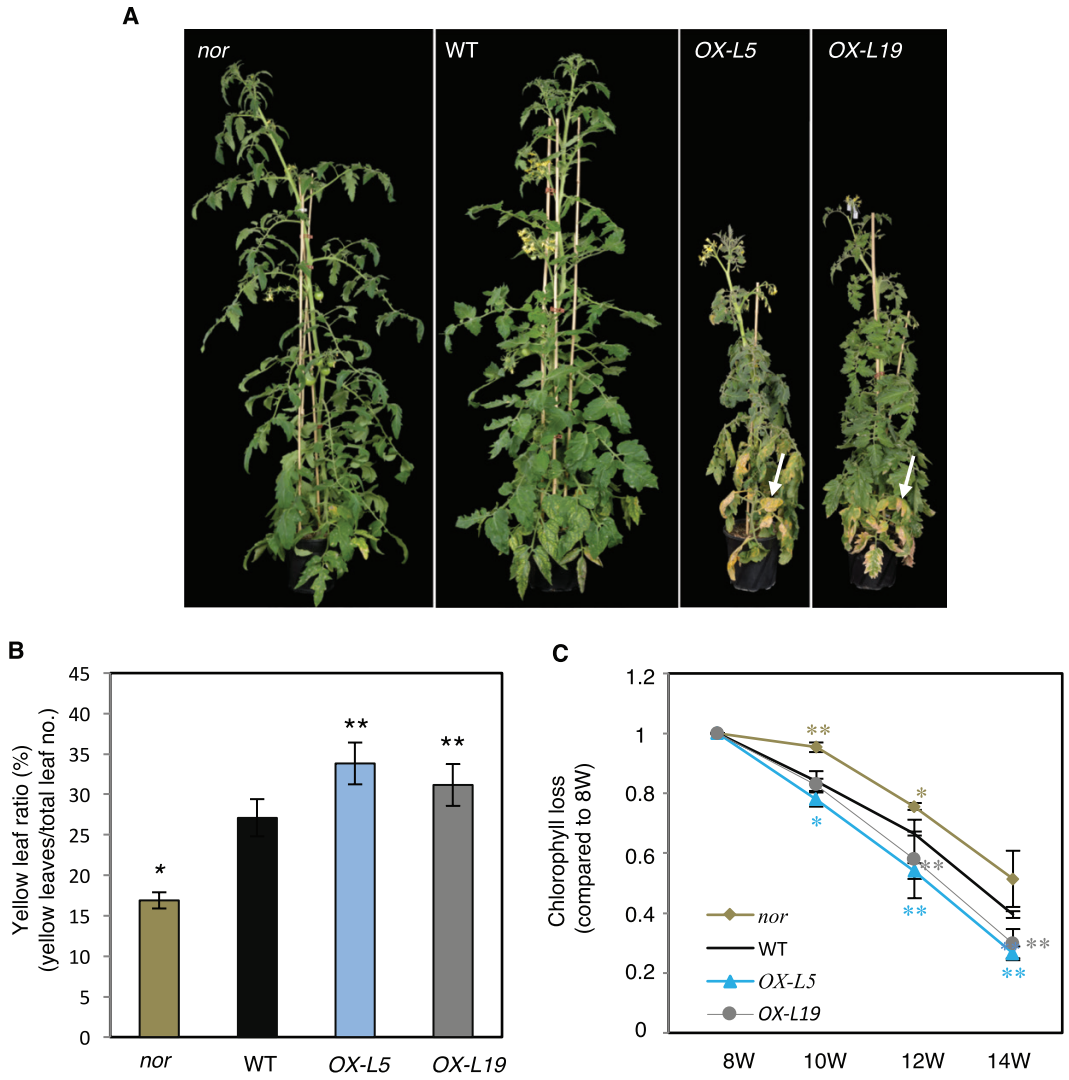

Fig. 2. NOR promotes leaf senescence in tomato. (A) Phenotype of 12-week-old *nor*, WT, *OX-L5*, and *OX-L19* plants. Note the early leaf senescence in *NOR* overexpressors (white arrows). (B) Yellow leaf ratio of 12-week-old WT, *OX-L5*, *OX-L19*, and *nor* plants. Yellow leaves showing >50% yellowing were counted and divided by the total number of leaves. Data are means ±SD (n=5). (C) Chlorophyll loss of the third true leaf (counted from the bottom of the stem) of 8- (8W), 10- (10W), 12- (12W), and 14-week-old (14W) WT, *OX-L5*, *OX-L19*, and *nor* plants. Chlorophyll content was measured by a SPAD meter and at each time point compared with 8W for each genotype (set to 1). Data are means ±SD of three biological replicates. Asterisks in (B) and (C) indicate significant differences from the WT (Student's t-test, $^{*}P\leq0.05$; $^{**}P\leq0.01$).

chlorophyll content remained high after treatment compared with the WT and *OX-L19* (Fig. 3A, B). Moreover, ion leakage, an indicator of senescence often associated with membrane damage (Hou *et al.*, 2013; Y.S. Kim *et al.*, 2013; Bresson *et al.*, 2018), was significantly elevated in *OX-L19* compared with the WT, while it was reduced in *nor* (Fig. 3C). In accordance with this, expression of various SAGs and CDGs was up-regulated in *OX-L19* plants compared with the WT, but down-regulated in *nor* (Fig. 3D; Supplementary Table S2). To substantiate the conclusion that NOR affects senescence-related genes, we also tested their expression during age-dependent and dark-induced senescence in WT tomato plants. As shown in Supplementary Fig. S5, all genes are significantly up-regulated in both senescence processes.

To further examine the function of NOR in regulating senescence, we generated *NOR* knock down lines by artificial miRNA (*ami-NOR*) in tomato cultivar Moneymaker. The *ami-NOR* construct targets 21 nucleotides (TGTACCATAGTTTGAAGGCTG) ~200 bp close to the 3' end of the *NOR* coding sequence. This region encodes the transactivation domain of the TF. We selected two lines (*ami-L2* and *ami-L35*) with a reduced *NOR* transcript abundance as determined by end-point PCR (Supplementary Fig. S6A). The *ami-NOR* lines exhibited delayed senescence during dark treatment, similar to the *nor* mutant (Supplementary Fig. S6B, C).

NOR overexpression promotes leaf senescence in Arabidopsis

To test whether *NOR* also induced early leaf senescence in a heterologous species, we overexpressed it in transgenic *A. thaliana* plants. We selected two Arabidopsis lines expressing *NOR* for further analysis (hereafter, *OX-L6* and *OX-L8*;

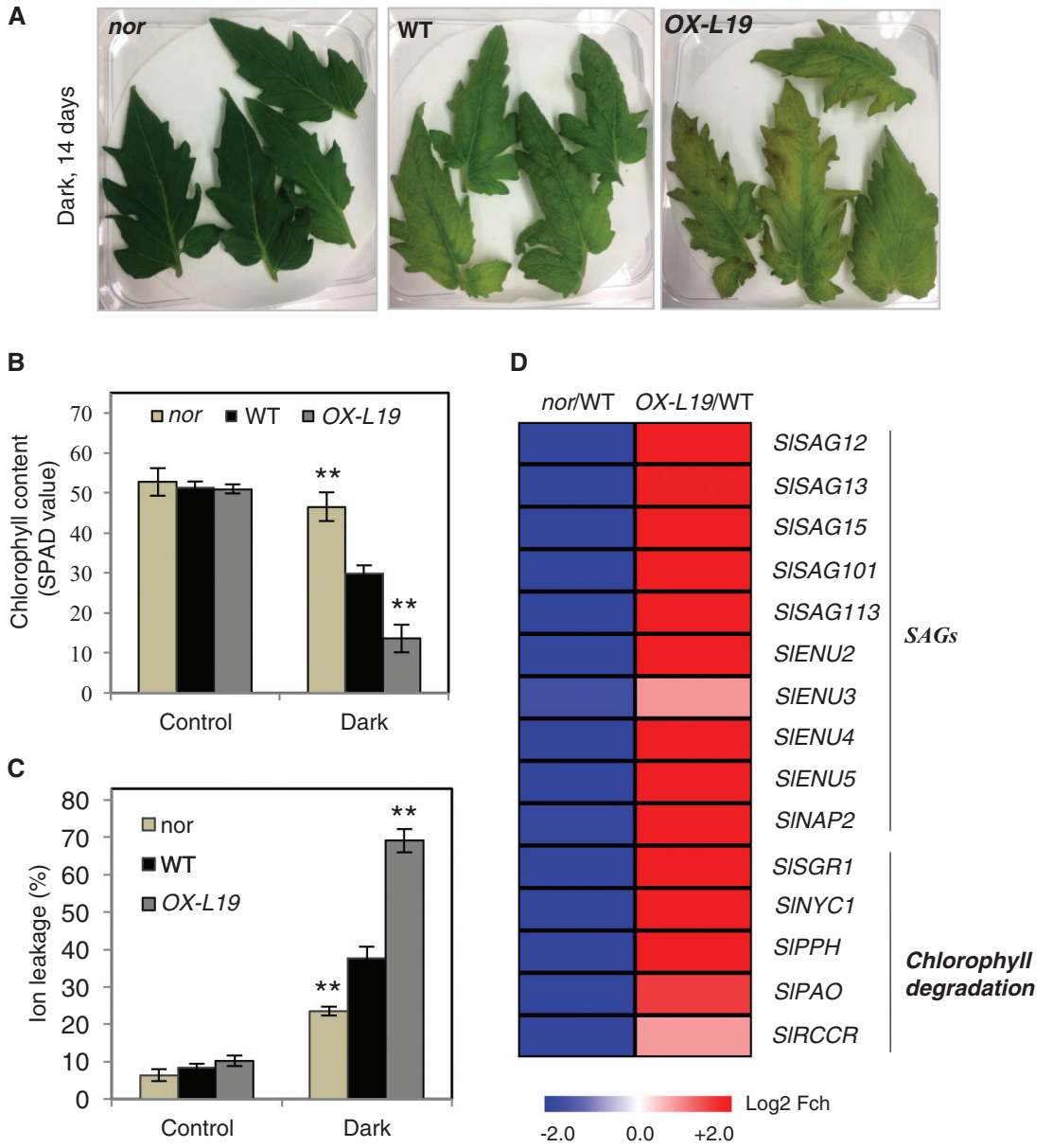

Fig. 3. Dark-induced leaf senescence in *NOR*-modified plants. (A) Detached leaves of 8-week-old *nor*, WT, and *OX-L19* plants after dark treatment. Young leaves from the top of the stem were detached and subjected to darkness for 14 d (Dark). (B) Chlorophyll content of leaves before darkness (control) and of dark-treated leaves. Chlorophyll content was measured using a SPAD meter. (C) Ion leakage of leaves before (control) and after dark treatment. (D) Heat map showing the fold change (log_2) of the expression of *SAG*s and chlorophyll degradation genes in detached leaves of 8-week-old *nor* and *OX-L19* plants, after dark treatment, compared with the WT. The full data are given in Suppelementary Table S2. In (B) and (C), asterisks indicate significant differences from the WT (Student's *t*-test; $^{**}P \leq 0.01$).

Fig. 4A). As in tomato, overexpression of *NOR* promoted early leaf senescence in Arabidopsis (Fig. 4A), indicating functional conservation across species. *OX* plants had a higher ratio of yellow to all leaves than the WT at the same age (5 weeks) (Fig. 4B).

To test whether *NOR* overexpression promotes senescence in darkness, we detached leaves from the Arabidopsis *OX-L6* and *OX-L8* lines, and, after 6 d of dark incubation, observed much stronger senescence than in leaves of the WT control (Fig. 4C). Chlorophyll content after dark treatment was more strongly reduced in these lines than in the WT (Fig. 4D). Expression of the senescence-associated marker gene *AtSAG12* (Noh and Amasino, 1999) was significantly up-regulated in these lines in comparison with the WT (Fig. 4E). From these results, we conclude that NOR positively regulates leaf senescence in both tomato and Arabidopsis.

A recent report by Kim *et al.* (2018) shows that expression of the *NOR*-related Arabidopsis genes *NARS1* (also called *ANAC056*) and *NARS2* (*ANAC018*) is elevated in aging leaves of Arabidopsis (see Supplementary fig. 1 of Kim *et al.*). Additionally, both genes show a relatively high expression in seeds (eFP browser). However, while silique senescence was delayed in the *nars1/nars2* double mutant, no delay was observed for leaf senescence by Kunieda *et al.* (2008), indicating that NARS1 and NARS2 act differently from NOR.

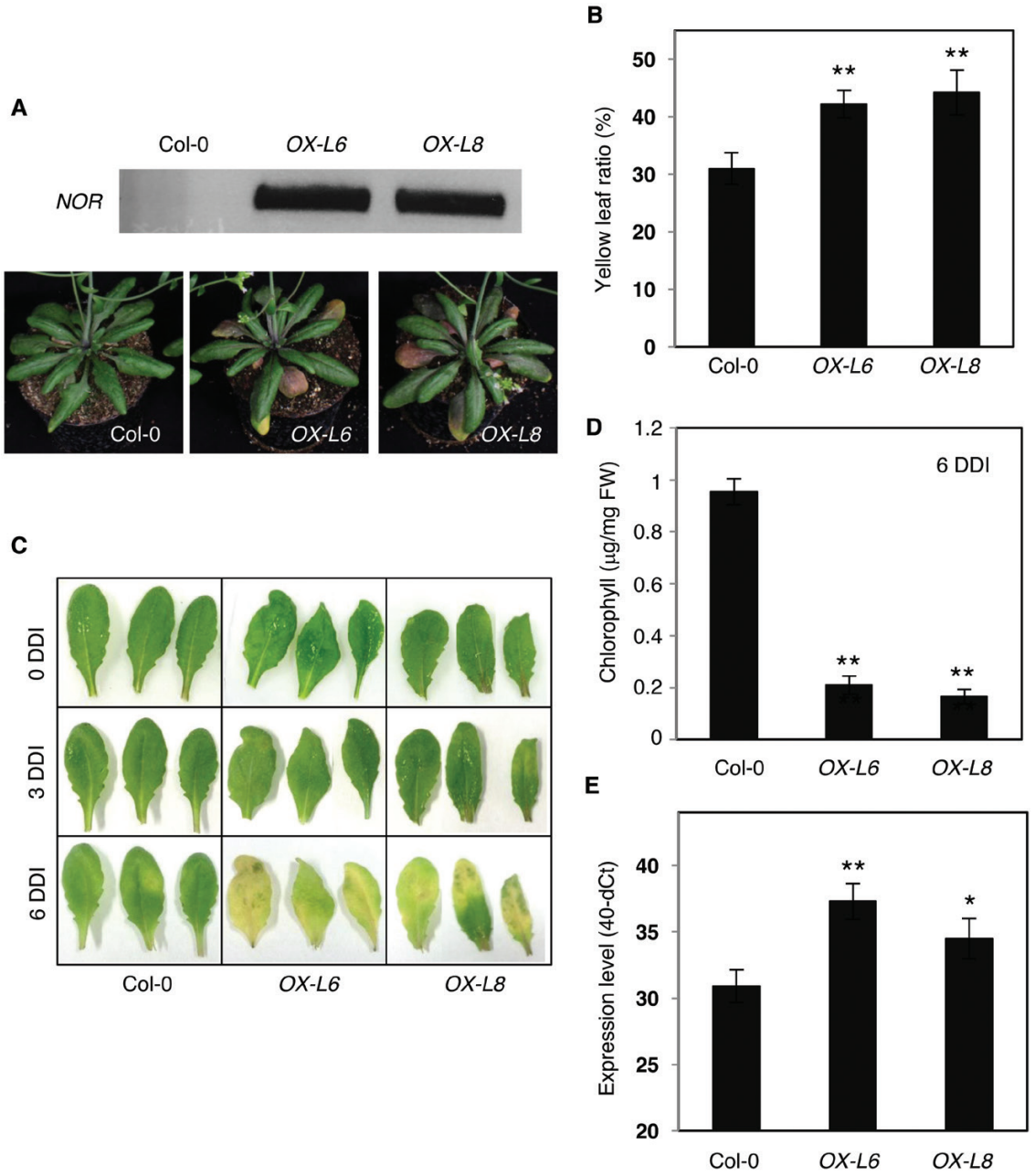

Fig. 4. Overexpression of *NOR* in Arabidopsis promotes leaf senescence. (A) Phenotype of Arabidopsis Col-0 wild-type and *NOR* overexpression plants. The upper panel shows *NOR* transcript abundance in *OX-L6* and *OX-L8* plants, determined by end-point PCR; as expected, no *NOR* transcript is observed in the Arabidopsis WT. The lower panel shows the phenotype of 5-week-old plants (Col-0 and *NOR* overexpressors). (B) Yellow leaf ratio of 5-week-old Col-0, *OX-L6*, and *OX-L8* plants. Yellow leaves showing >50% yellowing were counted and compared with the total leaf number. Data are means ±SD (n=5). (C) Dark-induced senescence. DDI, days after dark incubation. Note the more pronounced senescence in the two *NOR* overexpressors compared with Col-0 at 6 DDI. Leaves 5–7 detached from the various plants (separated by black vertical lines) were used in the experiment. (D) Chlorophyll content of (C), at 6 DDI of Col-0, *OX-L6*, and *OX-L8* genotypes (n=5). (E) Expression of *AtSAG12* in detached leaves 5–7 of Col-0, *OX-L6*, and *OX-L8* plants at 6 DDI. The y-axis indicates the expression level (40-dCt). Data are means ±SD of three biological replicates. Asterisks in (B), (D), and (E) indicate a significant difference from the Col-0 wild type (Student's t-test; *P≤0.05; **P≤0.01).

Identification of the consensus DNA-binding sequence of NOR

Knowledge about the DNA-binding motif(s) of a TF under analysis strongly assists in unraveling the wider gene regulatory network it controls. We therefore performed an *in vitro* binding site selection assay using the earlier reported CELD fusion method (Xue, 2005) to identify NOR-binding sites. We first analyzed the binding activity of NOR towards 16 randomly selected TaNAC69 motifs, S1–S16, bound by the TaNAC69 TF from wheat (*T. aestivum*) (Supplementary Fig. S7A), considering the following rationale. Both NOR and TaNAC69 belong to the NAP subfamily of NAC TFs; the two proteins share ~53% overall amino acid similarity. Within their NAC domains, NOR and TaNAC69 share 79.4% similarity and 66.7% identify, suggesting that the two NACs might

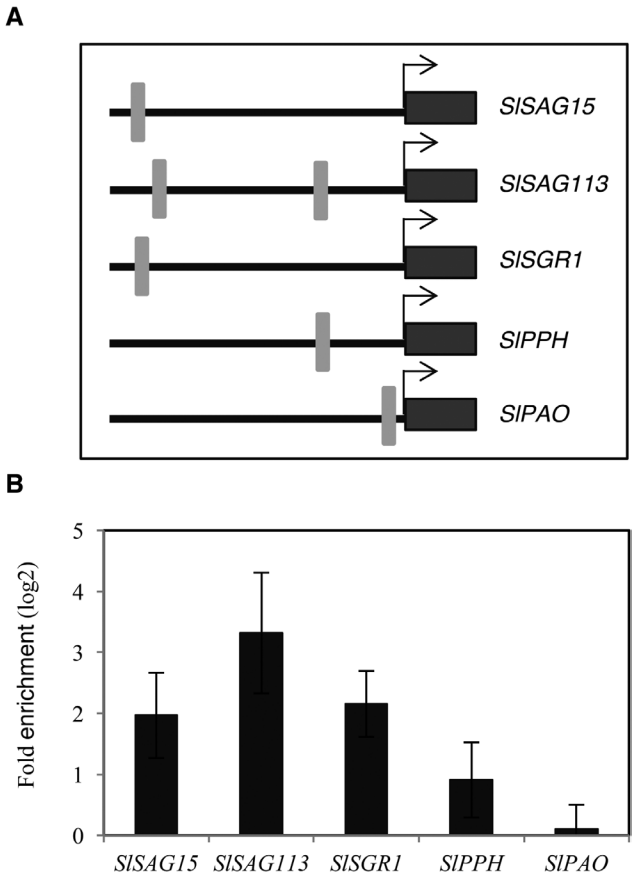

Fig. 5. Direct regulation of SAGs by NOR. (A) Schematic diagram showing positions of NOR-binding sites in 1 kb promoters of selected genes. Arrows indicates the ATG translational start codon. Gray boxes indicate the NOR-binding sites and black boxes indicate the coding regions of the genes. Sequences of the gene promoters including the NOR-binding sites tested in the ChIP experiments are given in Supplementary Table S3. (B) ChIP-qPCR shows enrichment of *SlSAG15*, *SlSAG113*, *SlSGR1*, and *SlPPH* promoter (1 kb) regions containing the NOR-binding site. Eight-week-old *NOR-GFP* plants (mature leaves ~3–5) were harvested for the ChIP experiment. qPCR was performed to quantify the enrichment of the promoter regions. In the case of *SlSAG113*, which has two potential NOR-binding sites in its promoter (see A), we tested binding of NOR to the sequence proximal to the *ATG* start codon. Values were normalized to the values for *Solyc04g009030* (promoter lacking a NOR-binding site). Data are the means ±SD of two independent biological replicates, each determined in three technical replicates.

have a similar binding site. Previously, it was shown that S1 is a high-affinity binding sequence of TaNAC69 (Xue *et al.*, 2006). In our results, NOR showed strong binding affinity for S1, with affinity decreasing progressively with substitutions. Overall, NOR bound to TaNAC69-selected motifs containing the YACG (or CGTR) core sequence (Supplementary Fig. S7A). Further analysis of the specificity of binding through base substitution, insertion, or deletion revealed that the mutation of nucleotides in the core motifs (e.g. S1m3 and S1m9) resulted in a strong reduction of NOR binding activity (Supplementary Fig. S7B). Taken together, our data suggest two high-affinity binding sites of NOR, CG(Y/C)(G/C)(5-7n)N(A/G)CGn(A/C/G)(A/C/T) and (C/T)ACGn(A/C)(A/T)(C/G/T)(C/T), as motif I and motif II, respectively.

Identification of NOR target genes

Although NOR is a TF well known for its function in fruit ripening, no direct target genes have, to our knowledge, been reported so far. Therefore, based on the results presented in Fig. 3D, we selected individual genes for further analysis to test whether they might be direct downstream targets of NOR. To this end, we chose several genes harboring the NOR-binding site within their 5' upstream regulatory regions, including *SlSAG15*, *SlSAG113*, *SlSGR1*, *SlPPH*, and *SlPAO* (Fig. 5A). ChIP-qPCR using tomato plants expressing NOR–GFP protein in the nucleus (Supplementary Fig. S2C) revealed direct binding of the NOR TF to the promoters of all genes except *SlPAO* (Fig. 5B). The absence of a detectable *in planta* binding of NOR to the *SlPAO* promoter might have several causes. One likely model is that the binding of NOR requires an open chromatin status which may not have been prevalent under the conditions in which we performed the experiment.

We next selected additional genes known to be regulated by natural or dark-induced senescence in tomato, or induced by abiotic stresses that trigger senescence (based on literature reports), and checked whether their promoters harbor a NOR-binding site. Considering that NOR regulates fruit ripening (Giovannoni *et al.*, 2004; Casals *et al.*, 2012; Kumar *et al.*, 2018), we also included a few genes reported to control this process. We then tested whether expression of these genes is affected in tomato *NOR-IOE* plants. As shown in Supplementary Fig. S2B, expression of *NOR* was strongly enhanced in 3-week-old *NOR-IOE* seedlings 6 h after treatment with 15 μM EST, as expected. Similarly, all selected NOR-binding site-containing genes except two showed enhanced expression when *NOR* was induced (Fig. 6A; Supplementary Table S2).

Among the genes up-regulated by NOR are the senescence-related genes *SlYLS4*, *SlKFB20*, and *SlSRG1*. *SlYSL4* (*Solyc08g068330*), a homolog of Arabidopsis *YLS4* (*YELLOW LEAF SPECIFIC4*), is expressed in a senescence-specific manner; the gene encodes an aspartate aminotransferase possibly involved in remobilizing leaf nitrogen during senescence (Yoshida *et al.*, 2001). *SlKFB20* (*Solyc03g120320*), a gene induced in tomato leaves during senescence, is a homolog of Arabidopsis *AT1G80440*, which encodes a kelch-repeat F-box protein targeting type-B ARR (Arabidopsis Response Regulator) proteins for degradation in the negative regulation of the cytokinin response (H.J. Kim *et al.*, 2013*a*, *b*). Notably, cytokinins delay senescence (Hwang *et al.*, 2012). *SlSRG1* (*Solyc02g071430*) is closely related to *SENESCENCE-RELATED GENE1* (*SRG1*) from Arabidopsis, which encodes a member of the Fe (II)/ascorbate oxidase gene family and is highly induced under low nitrogen conditions and during sucrose-induced senescence (Pourtau *et al.*, 2006). *SlABCG40* (*Solyc09g091670*), which encodes a protein belonging to the ATP-binding cassette (ABC) transporters, is one of the most up-regulated genes after EST treatment. It is induced by >120-fold after induction of *NOR* with EST in *NOR-IOE* lines. In Arabidopsis, *ABCG40* encodes an ABC transporter protein involved in the cellular uptake of ABA (Kang *et al.*, 2010), a phytohormone that triggers stomatal closure upon water shortage and stimulates leaf senescence in various species (Zhang *et al.*, 2012; Zhao *et al.*, 2017).

Three other genes analyzed, namely *SlERT1B*, *SlADH2*, and *SlACS2*, are involved in fruit ripening, and all are up-regulated after EST treatment in *NOR-IOE* plants. *SlERT1B* (*Solyc10g085230*) encodes a putative UDP-glycosyltransferase potentially involved in glycoalkaloid biosynthesis in tomato fruits (Itkin *et al.*, 2013; Alseekh *et al.*, 2015). *SlADH2* (*ALCOHOL DEHYDROGENASE2*; *Solyc06g059740*) participates in the biosynthesis of volatiles and, accordingly, its transcript abundance increases during fruit ripening (Speirs *et al.*, 1998); it is a direct target of RIN (Qin *et al.*, 2012). *SlACS2* (*1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE2*; *Solyc01g095080*) encodes an ethylene biosynthesis gene highly expressed during fruit ripening. Down-regulation of *SlACS2* lowers ethylene production and delays fruit ripening (Oeller *et al.*, 1991).

We included further genes with likely functions in fruit ripening or leaf senescence in our analysis. One is *SlCEL7* (*Solyc11g040340*), which encodes a putative endo-β-1,4-glucanase of the glycosyl hydrolase 9 (cellulase E) family (www.uniprot.org); *SlCEL7* has been suggested to play a specific role for regulating the loosening of cell walls during fruit growth (Catalá *et al.*, 2000). As seen in Fig. 6A (and Supplementary Table S2), expression of *SlCEL7* was significantly elevated in *NOR-IOE* plants after EST induction, suggesting it to be a downstream target of NOR. In addition, the hormone-related genes *ETHYLENE-RESPONSIVE TRANSCRIPTION FACTOR B.2* (*SlERF.B.2*, *Solyc02g077360*), *SlERF.C.5* (*Solyc02g077370*), *SlERF13* (*Solyc01g090340*), and *SlERF17* (*Solyc12g009240*) were also significantly up-regulated after EST induction of the NOR TF (Fig. 6A), suggesting them to be downstream targets of NOR.

As the phytohormone auxin is involved in controlling leaf senescence and fruit ripening (Kim *et al.*, 2011; Breitel *et al.*, 2016), we also included three auxin-related genes in our analysis, namely *SlGH3_4* (*Solyc02g092820*), which encodes a putative indole-3-acetic acid amido synthetase, an enzyme conjugating auxin to an inactive form thereby reducing cellular free auxin levels, and small auxin up-regulated RNA67 (*SlSAUR67*; *Solyc08g079140*). Expression of various *GH3* genes has previously been shown to increase in leaves during developmental and dark-induced senescence, consistent with the decrease of free auxin levels in senescing leaves (Buchanan-Wollaston *et al.*, 2005; van der Graaff *et al.*, 2006; Kim *et al.*, 2011). While *SlGH3_4* was significantly up-regulated upon induction of *NOR*, *SlSAUR67* was not affected. The expression of the third auxin-related gene *SlSAUR74* (*Solyc10g052550*) was significantly reduced after *NOR* induction (Fig. 6A).

We next analyzed expression of the selected genes by qRT-PCR in *ami-NOR* lines. Almost all genes that were up-regulated in *NOR-IOE* plants after EST induction were down-regulated in *ami-NOR*, confirming the transcription activation role of NOR toward these genes (Fig. 6A).

Finally, we employed ChIP-qPCR to test binding of NOR to the promoters of selected downstream targets *in vivo*, including *SlABCG40*, *SlERT1B*, *SlKFB20*, and *SlYLS4*. As shown in Fig. 6B, NOR binds to all four promoters.

SlNAP2 affects NOR *expression*

We previously reported that SlNAP2, a tomato NAC TF, functions as a positive regulator of leaf senescence by directly controlling the expression of various SAGs as direct targets. In addition, SlNAP2 controls the expression of several ABA-related genes (Ma *et al.*, 2018). SlNAP2 has two related DNA-binding sites, called BS1 and BS2, which are present in the promoters of its direct gene targets (Ma *et al.*, 2018). As previous work on Arabidopsis indicated regulatory connectivity between different NAC TFs to control senescence (e.g. Garapati *et al.*, 2015; Kim *et al.*, 2018), we here thought to investigate the possibility that *NOR* is a downstream affected gene target of SlNAP2. In accordance with this model, sequence analysis of the *NOR* promoter identified an SlNAP2 BS1-binding site 403 bp upstream of the ATG start codon (Fig. 7A). Furthermore, expression of *NOR* significantly increased in transgenic tomato plants expressing *SlNAP2* from an EST-inducible promoter (*SlNAP2-IOE*; Ma *et al.*, 2018) 6 h after EST treatment (Fig. 7B). Finally, SlNAP2 directly binds to the *NOR* promoter, as shown by ChIP-qPCR (Fig. 7C). Collectively, our data thus show that SlNAP2 functions as an upstream regulator of *NOR*.

NOR affects ABA-induced leaf senescence

The observation that *SlABCG40*, encoding a potential ABA transporter in tomato, is highly up-regulated upon *NOR* induction and the fact that SlNAP2 affects expression of *NOR* suggested an involvement of a SlNAP2–NOR signaling cascade in ABA-induced leaf senescence. To test this hypothesis, we analyzed the expression of *SlNAP2* and *NOR* during dark-induced senescence, and after ABA treatment in non-senescent leaves. As shown in Supplementary Fig. S8A, expression of both genes increases during age-dependent senescence. In addition, expression of *SlNAP2* increases rapidly (within 2–4 h) after ABA treatment, while expression of *NOR* shows a delayed response to ABA treatment, with a detectable increase only after 16 h of treatment (Supplementary Fig. S8B), in accordance with the observation that SlNAP2 acts upstream of *NOR*. We next tested the effect of ABA on *NOR* expression in tomato plants over- or underexpressing *SlNAP2*. As shown in Supplementary Fig. S8C, *NOR* was more highly expressed after ABA treatment in *SlNAP2* overexpressors (*SlNAP2-OX* plants) than in non-ABA-treated *SlNAP2-OX* plants. In contrast, *NOR* expression was less affected by ABA treatment in WT plants and *SlNAP2* knockdown plants (i.e. RNAi lines reported by Ma *et al.*, 2018). Furthermore, ABA treatment accelerated leaf senescence more strongly in *NOR* overexpressors (*OX-L19*) than in WT and *ami-NOR* plants (Supplementary Fig. S8D, E). Taken together, our data (together with those of Ma *et al.*, 2018) support the model that SlNAP2 and NOR act together to promote ABA-induced leaf senescence in tomato.

Discussion

NOR is a NAC TF well characterized for its role in fruit ripening in tomato (Barry and Giovannoni, 2007; Casals *et al.*, 2012;

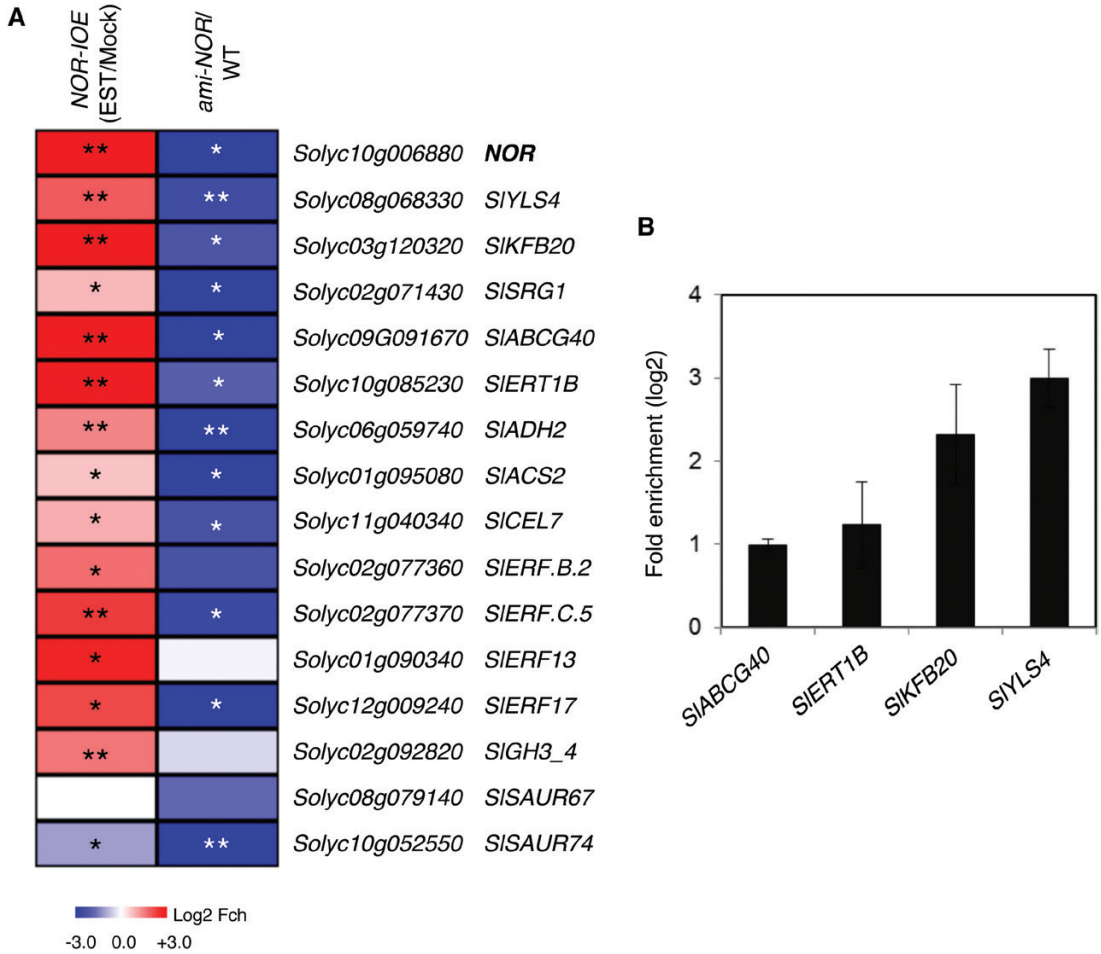

Fig. 6. Heat map of differentially expressed genes in *NOR-IOE* and *ami-NOR* plants. (A) Gene expression was analyzed by qRT-PCR in *NOR-IOE* seedlings treated with EST (15 µM) for 6 h and compared with expression in mock-treated [ethanol, 0.15% (v/v)] seedlings (left column), or in *ami-NOR* seedlings compared with wild-type (WT) seedlings. Seedlings were 3 weeks old. The color code indicates the $\log_2$ scale of the fold change; blue, down-regulated; red, up-regulated. Data represent means of three biological replicates. Data are means ±SD of three biological replicates. Asterisks indicate a significant difference from mock-treated samples (for *NOR-IOE* samples) or from the WT (for *ami-NOR* samples). Student's *t*-test; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$). The full data are given in Supplementary Table S2. (B) ChIP–qPCR shows enrichment of *SlABCG40*, *SlERT1B*, *SlKFB20*, and *SlYLS4* promoter regions containing the NOR-binding site within the 1 kb upstream promoter regions of the corresponding genes. Experimental conditions were as described in the legend to Fig. 5B. Sequences of the gene promoters including the NOR-binding sites tested in the ChIP experiments are given in Supplementary Table S3. Data are the means ±SD of two independent biological replicates, each determined in three technical replicates.

Kumar *et al.*, 2018). Also in melon (*Cucumis melo*), a NOR homolog has been shown to affect fruit ripening (Ríos *et al.*, 2017). Recently, several other NAC TFs have been reported to control fruit ripening in tomato, including, for example, SlNAC4 (Zhu *et al.*, 2014), SlNAC19 and SlNAC48 (Kou *et al.*, 2016), and SlNAC3 (NOR-like1), the latter of which also functions in seed development (Han *et al.*, 2012, 2014; Gao *et al.*, 2018). With the data available so far, it appears that NAC TFs—in conjunction with TFs of other families—form interconnected regulatory networks to control fruit aging. For example, RIN, a long-known regulator of tomato fruit ripening of the MADS-box TF family, directly regulates *NOR* by binding to its promoter, as revealed by ChIP assay (Martel *et al.*, 2011; Fujisawa *et al.*, 2013). In addition, expression of *NOR* and *RIN* is reduced in *SlNAC4* RNAi lines, which might indicate that it acts as an upstream regulator of *NOR* and *RIN* (Zhu *et al.*, 2014). Furthermore, yeast two-hybrid assays revealed an interaction of SlNAC4 protein with NOR and RIN, although the functional relevance of this interaction *in planta* was not demonstrated (Zhu *et al.*, 2014). Recently, experimental evidence showed that the basic leucine zipper (bZIP) TF SlAREB1 which, at the transcript level, is induced by ABA, may function as an upstream regulator of *NOR*, although direct *in planta* binding of SlAREB1 to the *NOR* promoter by, for example, ChIP was not demonstrated (Mou *et al.*, 2018).

Although increasing evidence suggests an involvement of multiple NAC factors in tomato fruit development, a role for NACs in the regulation of leaf senescence in this vegetable crop has rarely been demonstrated despite the fact that NACs play diverse roles in the control of leaf senescence in other species (Podzimska-Sroka *et al.*, 2015; Leng *et al.*, 2017; Ma *et al.*, 2018; Yang and Udvardi, 2018). Particularly detailed knowledge about the NAC-controlled senescence networks is available for Arabidopsis where multiple NAC TFs have been shown to positively or negatively regulate leaf senescence by

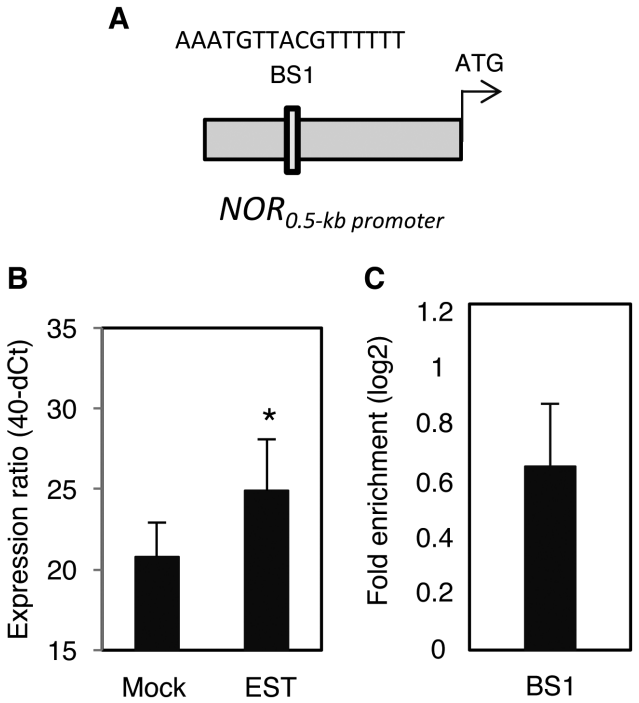

Fig. 7. SlNAP2 acts as an upstream regulator of *NOR*. (A) Schematic presentation of SlNAP2-binding site 1 (BS1) within the *NOR* promoter. The sequence of the binding site, which is located in the forward strand of the promoter, is indicated. (B) Expression of *NOR* in 3-week-old *SlNAP2-IOE* seedlings treated with estradiol (EST; 15 μM) for 6 h compared with ethanol [0.15% (v/v)]-treated seedlings (Mock). Gene expression was determined by qRT-PCR. Data represent means of three biological replicates. Asterisks indicate a significant difference from mock-treated plants (Student's *t*-test; $*P \leq 0.05$). (C) ChIP-qPCR shows enrichment of the *NOR* promoter region containing the SlNAP2-binding site 1 (BS1). Mature leaves (nos 3–5) harvested from 8-week-old *SlNAP2-GPF* plants were used for the ChIP experiment. Values were normalized to the values for *Solyc04g009030* (promoter lacking a SlNAP2-binding site). Data are means ±SD of two independent biological replicates, each performed with three technical replicates.

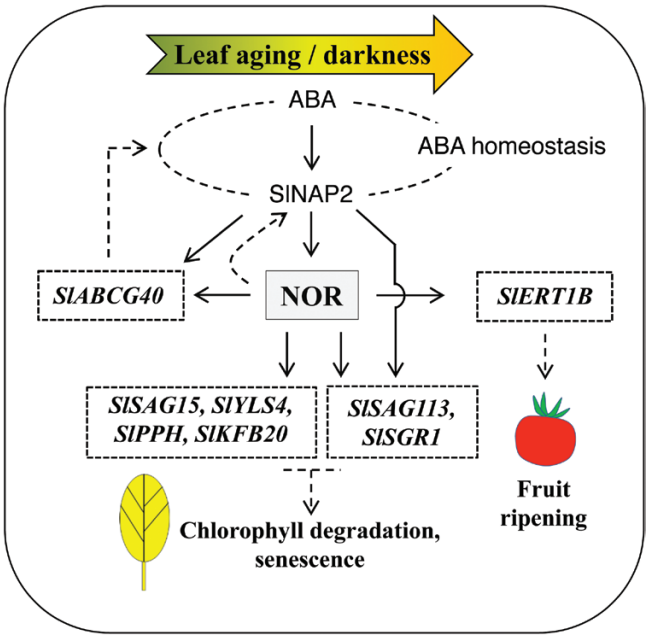

Fig. 8. Model for the regulation of leaf senescence by NOR. NOR positively controls leaf senescence in tomato by directly regulating various senescence-associated genes including, besides others, *SlSAG15*, *SlYLS4*, *SlPPH*, *SlKFB20*, *SlSAG113*, and *SlSGR1*. It also directly regulates the expression of *SlABCG40*, an ABA transporter-encoding gene. The NAC transcription factor SlNAP2 enhances *NOR* expression by directly binding to its promoter and, together with NOR, it jointly regulates *SlSAG113*, *SlSGR1*, and *SlABCG40*. In addition, NOR enhances *SlNAP2* expression, suggesting a positively acting feed-forward loop involving the two NAC factors. SlNAP2 has previously been reported to contribute to establishing ABA homeostasis during leaf senescence (Ma *et al.*, 2018), to which the activation of *SlABCG40* by NOR may contribute. NOR also directly and positively regulates the expression of the fruit ripening-related gene *SlERT1B*, consistent with its well-known role in this process. Solid lines indicate direct binding of the transcription factor (SlNAP2 or NOR) to target gene promoters, while dashed lines indicate indirect physiological connections.

binding to the promoters of diverse target genes to control different physiological processes underlying the complex syndrome of senescence (Guo and Gan, 2006; Kim *et al.*, 2009; Wu *et al.*, 2012; Garapati *et al.*, 2015; Sakuraba *et al.*, 2016; Oda-Yamamizo *et al.*, 2016; Kamranfar *et al.*, 2018; Kim *et al.*, 2018; Li *et al.*, 2018).

The situation is less clear in tomato, although aging in fruits and leaves may at least in part share identical TFs and gene regulatory networks. Recently, Lira *et al.* (2017) found that orthologs of *ORE1*, a central positive regulator of leaf senescence in Arabidopsis (Kim *et al.*, 2009; Balazadeh *et al.*, 2010), control leaf senescence in tomato leading to extended greenness upon down-regulation of *SlORE1* gene expression. The increased fruit yield in such plants might be due to an extended photosynthetic lifetime of the leaves, providing carbon for fruit (sink) growth over a longer period than in WT plants, although another possibility is that *SlORE1* genes directly control ripening processes in fruits. Similarly, down-regulation of the expression of the NAC TF gene *SlNAP2* delays leaf senescence in tomato followed by an increased fruit yield (Ma *et al.*, 2018). SlNAP2 binds to the promoters of several senescence-related genes, including *SlSAG113* (*Solanum lycopersicum SENESCENCE-ASSOCIATED GENE113*) and the chlorophyll degradation genes *SlSGR1* (*S. lycopersicum senescence-inducible chloroplast stay-green protein 1*) and *SlPAO* (*S. lycopersicum pheide a oxygenase*). SlNAP2 also directly controls the expression of several ABA-related genes including ABA transport, biosynthesis, and degradation genes, suggesting that it has an important function in controlling ABA homeostasis in senescing tomato leaves (Ma *et al.*, 2018).

Here, we report that the long-known tomato fruit ripening factor NOR controls leaf senescence, thereby identifying a novel role for NOR for controlling development (see model in Fig. 8). Of note, overexpression of *NOR* in both transgenic tomato and Arabidopsis plants promotes developmental leaf senescence as well as dark-induced senescence. The role of NOR in regulating leaf senescence is related to changes in the expression of SAGs and CDGs. Expression of various senescence-responsive genes was enhanced in constitutive or EST-inducible *NOR* overexpressors, and we demonstrated binding of the NOR TF to their promoters by ChIP. As direct *in vivo* targets of NOR, we identified *SlSAG15*, *SlSAG113*,

SlSGR1, and *SlPPH*, as well *SlABCG40*, *SlERT1B*, *SlKFB20*, and *SlYLS4*. Of note, *SlSAG113*, *SlSGR1*, and *SlABCG40* were previously also identified as direct targets of SlNAP2 (Ma *et al.*, 2018), strongly suggesting functional overlap of NOR and SlNAP2 in regulating leaf senescence-associated genes in tomato (Fig. 8). In accordance with this, both TFs belong to the same clade (the NAP clade) of NAC factors (Kou *et al.*, 2014). This clade also includes the *AtNAP* gene, a well-known regulator of leaf senescence in *A. thaliana* (Guo and Gan, 2006). Interestingly, however, *SlPAO* did not appear to be a direct downstream target gene of NOR (this report; Fig. 5B), while we previously found it to be a direct target of SlNAP2 (Ma *et al.*, 2018), indicating partial, but not complete, functional redundancy of both TFs with respect to the control of leaf senescence. Such a redundancy of NAC TFs for the control of senescence was recently highlighted for Arabidopsis by Li *et al.* (2018).

Another important finding of our study is that *SlNAP2* itself is affected, at the expression level, by NOR; more specifically, as shown in Fig. 3D, expression of *SlNAP2* is significantly reduced in leaves of the *nor* mutant, while it is elevated in the *NOR* overexpressor line *OX-19*, suggesting that NOR acts upstream of *SlNAP2*. On the other hand, we found that expression of *NOR* is enhanced in *SlNAP2-IOE* plants shortly (6 h) after EST treatment (Fig. 7B), consistent with a model that places SlNAP2 upstream of *NOR*. In accordance with this, we found that ABA treatment triggered an increased expression of *NOR* in *SlNAP2* overexpressors, and it supported a stronger leaf senescence in *NOR* overexpressors than in WT and *ami-NOR* plants (Supplementary Fig. S8). We previously found evidence that SlNAP2 plays an important role in establishing ABA homeostasis during the process of leaf senescence (Ma *et al.*, 2018); the new data reported here about the involvement of NOR add further insights into this regulatory system (Fig. 8).

Collectively, the available experimental data therefore suggest that NOR and SlNAP2 together form a positively acting regulatory loop whereby the expressional activity of each *NAC* gene is enhanced by the other NAC TF. However, we note that unraveling the details of this regulatory interaction requires further detailed investigation in the future.

Together, the available data strongly suggest that NAC TFs controlling leaf senescence also affect age-dependent senescence (or ripening) of fleshy and non-fleshy fruits, across species. This observation raises a number of interesting questions, including the following. (i) How do NAC TFs exert their specific aging-related functions in photosynthetic leaves compared with those in fruits; that is, how are the target genes prevalent or specific for leaf senescence selected compared with target genes involved in fruit ripening? (ii) Related to this: do NAC TFs interact with other different TFs in leaves versus fruits to exert their molecular functions? (iii) To what extent do epigenetic marks affect which genes are primary targets of the senescence-related NACs in leaves versus fruits? (iv) In which way has evolution shaped the gene regulatory landscape of age-related NAC TFs in leaves compared with fruits? These questions lead to an even wider perspective which addresses the diversification of NAC functions at the organ, tissue, and cellular levels, an aspect not well understood at present. Future research clearly has to address this aspect in more detail.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Amino acid sequence alignment of selected NAC transcription factors.

Fig. S2. Selection of *NOR* transgenic lines.

Fig. S3. Flowering time of tomato wild-type and *NOR*-modified plants.

Fig. S4. Phenotype of *NOR* overexpressors at seedling stage.

Fig. S5. Heat map of the expression of senescence-related tomato genes during age-dependent senescence and dark-induced senescence.

Fig. S6. Dark-induced senescence in *ami-NOR* plants.

Fig. S7. Identification of the binding sequences of NOR.

Fig. S8. NOR affects ABA-induced leaf senescence.

Table S1. Oligonucleotide sequences.

Table S2. Data for results shown in heat maps.

Table S3. Promoters of NOR target genes.

Table S4. Expression of GA metabolism genes.

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References

Alseekh S, Tohge T, Wendenberg R, *et al*. 2015. Identification and mode of inheritance of quantitative trait loci for secondary metabolite abundance in tomato. The Plant Cell **27**, 485–512.

Arnon DI. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiology **24**, 1–15.

Arvidsson S, Kwasniewski M, Riaño-Pachón DM, Mueller-Roeber B. 2008. QuantPrime—a flexible tool for reliable high-throughput primer design for quantitative PCR. BMC Bioinformatics **9**, 465.

Balazadeh S, Riaño-Pachón DM, Mueller-Roeber B. 2008. Transcription factors regulating leaf senescence in *Arabidopsis thaliana*. Plant Biology **10(Suppl 1)**, 63–75.

Balazadeh S, Schildhauer J, Araújo WL, Munné-Bosch S, Fernie AR, Proost S, Humbeck K, Mueller-Roeber B. 2014. Reversal of senescence by N resupply to N-starved *Arabidopsis thaliana*: transcriptomic and metabolomic consequences. Journal of Experimental Botany **65**, 3975–3992.

Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Köhler B, Mueller-Roeber B. 2010. A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. The Plant Journal **62**, 250–264.

Barry CS, Giovannoni JJ. 2007. Ethylene and fruit ripening. Journal of Plant Growth Regulation **26**, 143–159.

Biswal UC, Mohanty P. 1976. Dark stress-induced senescence of detached barley leaves. II. Alteration in absorption characteristic and photochemical activity of chloroplasts isolated from senescing leaves. Plant Science Letters **7**, 371–379.

Breitel DA, Chappell-Maor L, Meir S, *et al*. 2016. AUXIN RESPONSE FACTOR 2 intersects hormonal signals in the regulation of tomato fruit ripening. PLoS Genetics **12**, e1005903.

Bresson J, Bieker S, Riester L, Doll J, Zentgraf U. 2018. A guideline for leaf senescence analyses: from quantification to physiological and molecular investigations. Journal of Experimental Botany **69**, 769–786.

Buchanan-Wollaston V, Page T, Harrison E, *et al*. 2005. Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in Arabidopsis. The Plant Journal **42**, 567–585.

Carrasco-Orellana C, Stappung Y, Mendez-Yañez A, Allan AC, Espley RV, Plunkett BJ, Moya-Leon MA, Herrera R. 2018. Characterization of a ripening-related transcription factor FcNAC1 from *Fragaria chiloensis* fruit. Scientific Reports **8**, 10524.

Casals J, Pascual L, Canizares J, Cebolla-Cornejo J, Casanas F, Nuez F. 2012. Genetic basis of long shelf life and variability into Penjar tomato. Genetic Resources and Crop Evolution **59**, 219–229.

Catalá C, Rose JK, Bennett AB. 2000. Auxin-regulated genes encoding cell wall-modifying proteins are expressed during early tomato fruit growth. Plant Physiology **122**, 527–534.

Chen CT, Kao CH. 1991. Senescence of rice leaves XXX. Levels of endogenous polyamines and dark-induced senescence of rice leaves. Plant & Cell Physiology **32**, 935–941.

Davière JM, Achard P. 2013. Gibberellin signaling in plants. Development **140**, 1147–1151.

Fan K, Bibi N, Gan S, Li F, Yuan S, Ni M, Wang M, Shen H, Wang X. 2015. A novel NAP member GhNAP is involved in leaf senescence in *Gossypium hirsutum*. Journal of Experimental Botany **66**, 4669–4682.

Fujisawa M, Nakano T, Shima Y, Ito Y. 2013. A large-scale identification of direct targets of the tomato MADS box transcription factor RIPENING INHIBITOR reveals the regulation of fruit ripening. The Plant Cell **25**, 371–386.

Gao Y, Wei W, Zhao X, *et al*. 2018. A NAC transcription factor, NOR-like1, is a new positive regulator of tomato fruit ripening. Horticultural Research doi: 10.1038/s41438-018-0111-5.

Garapati P, Xue GP, Munné-Bosch S, Balazadeh S. 2015. Transcription factor ATAF1 in Arabidopsis promotes senescence by direct regulation of key chloroplast maintenance and senescence transcriptional cascades. Plant Physiology **168**, 1122–1139.

Giovannoni JJ, Tanksley SD, Vrebalov J, Noensie E. 2004. *NOR* gene for use in manipulation of fruit quality and ethylene response. US Patent No. 5,234,834 issued 13 July 2004.

Guo Y, Gan S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. The Plant Journal **46**, 601–612.

Han Q, Zhang J, Li H, Luo Z, Ziaf K, Ouyang B, Wang T, Ye Z. 2012. Identification and expression pattern of one stress-responsive NAC gene from *Solanum lycopersicum*. Molecular Biology Reports **39**, 1713–1720.

Han QQ, Song YZ, Zhang JY, Liu LF. 2014. Studies on the role of the *SlNAC3* gene in regulating seed development in tomato (*Solanum lycopersicum*). Journal of Horticultural Science and Biotechnology **89**, 423–429.

Hendelman A, Stav R, Zemach H, Arazi T. 2013. The tomato NAC transcription factor SlNAM2 is involved in flower-boundary morphogenesis. Journal of Experimental Botany **64**, 5497–5507.

Hou K, Wu W, Gan SS. 2013. SAUR36, a small auxin up RNA gene, is involved in the promotion of leaf senescence in Arabidopsis. Plant Physiology **161**, 1002–1009.

Hwang I, Sheen J, Müller B. 2012. Cytokinin signaling networks. Annual Review of Plant Biology **63**, 353–380.

Itkin M, Heinig U, Tzfadia O, *et al*. 2013. Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. Science **341**, 175–179.

Ito Y, Nishizawa-Yokoi A, Endo M, Mikami M, Shima Y, Nakamura N, Kotake-Nara E, Kawasaki S, Toki S. 2017. Re-evaluation of the rin mutation and the role of RIN in the induction of tomato ripening. Nature Plants **3**, 866–874.

Kamranfar I, Xue GP, Tohge T, Sedaghatmehr M, Fernie AR, Balazadeh S, Mueller-Roeber B. 2018. Transcription factor RD26 is a key regulator of metabolic reprogramming during dark-induced senescence. New Phytologist **218**, 1543–1557.

Kang J, Hwang JU, Lee M, Kim YY, Assmann SM, Martinoia E, Lee Y. 2010. PDR-type ABC transporter mediates cellular uptake of the phytohormone abscisic acid. Proceedings of the National Academy of Sciences, USA **107**, 2355–2360.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends in Plant Science **7**, 193–195.

Kaufmann K, Muiño JM, Østerås M, Farinelli L, Krajewski P, Angenent GC. 2010. Chromatin immunoprecipitation (ChIP) of plant transcription factors followed by sequencing (ChIP-SEQ) or hybridization to whole genome arrays (ChIP-CHIP). Nature Protocols **5**, 457–472.

Kim HJ, Chiang YH, Kieber JJ, Schaller GE. 2013*a*. SCFKMD controls cytokinin signaling by regulating the degradation of type-B response regulators. Proceedings of the National Academy of Sciences, USA **110**, 10028–10033.

Kim HJ, Kieber JJ, Schaller GE. 2013*b*. The rice F-box protein KISS ME DEADLY2 functions as a negative regulator of cytokinin signalling. Plant Signaling & Behavior **8**, e26434.

Kim HJ, Park JH, Kim J, *et al*. 2018. Time-evolving genetic networks reveal a NAC troika that negatively regulates leaf senescence in Arabidopsis. Proceedings of the National Academy of Sciences, USA **115**, E4930–E4939.

Kim JI, Murphy AS, Baek D, Lee SW, Yun DJ, Bressan RA, Narasimhan ML. 2011. *YUCCA6* over-expression demonstrates auxin function in delaying leaf senescence in *Arabidopsis thaliana*. Journal of Experimental Botany **62**, 3981–3992.

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, Hwang D, Nam HG. 2009. Trifurcate feed-forward regulation of age-dependent cell death involving *miR164* in Arabidopsis. Science **323**, 1053–1057.

Kim YS, Sakuraba Y, Han SH, Yoo SC, Paek NC. 2013. Mutation of the Arabidopsis NAC016 transcription factor delays leaf senescence. Plant & Cell Physiology **54**, 1660–1672.

Kou X, Liu C, Han L, Wang S, Xue Z. 2016. NAC transcription factors play an important role in ethylene biosynthesis, reception and signaling of tomato fruit ripening. Molecular Genetics and Genomics **291**, 1205–1217.

Kou X, Wang S, Wu M, Guo R, Xue Z, Meng N, Tao X, Chen M, Zhang Y. 2014. Molecular characterization and expression analysis of NAC family transcription factors in tomato. Plant Molecular Biology Reporter **32**, 501–516.

Kou X, Watkins CB, Gan SS. 2012. Arabidopsis AtNAP regulates fruit senescence. Journal of Experimental Botany **63**, 6139–6147.

Kumar R, Tamboli V, Sharma R, Sreelakshmi Y. 2018. *NAC-NOR* mutations in tomato Penjar accessions attenuate multiple metabolic processes and prolong the fruit shelf life. Food Chemistry **259**, 234–244.

Kunieda T, Mitsuda N, Ohme-Takagi M, Takeda S, Aida M, Tasaka M, Kondo M, Nishimura M, Hara-Nishimura I. 2008. NAC family proteins NARS1/NAC2 and NARS2/NAM in the outer integument regulate embryogenesis in Arabidopsis. The Plant Cell **20**, 2631–2642.

Leng Y, Ye G, Zeng D. 2017. Genetic dissection of leaf senescence in rice. International Journal of Molecular Sciences **18**, 2686.

Li Z, Woo HR, Guo H. 2018. Genetic redundancy of senescence-associated transcription factors in Arabidopsis. Journal of Experimental Botany **69**, 811–823.

Liang CZ, Wang YQ, Zhu YN, Tang JY, Hu B, Liu LC, Ou SJ, Wu HK, Sun XH, Chu JF, Chu CC. 2014. OsNAP connects abscisic acid and leaf senescence by fine-tuning abscisic acid biosynthesis and directly targeting senescence-associated genes in rice. Proceedings of the National Academy of Sciences, USA **111**, 10013–10018.

Lira BS, Gramegna G, Trench BA, *et al*. 2017. Manipulation of a senescence-associated gene improves fleshy fruit yield. Plant Physiology **175**, 77–91.

Ma X, Zhang Y, Turečková V, Xue GP, Fernie AR, Mueller-Roeber B, Balazadeh S. 2018. The NAC transcription factor SlNAP2 regulates leaf senescence and fruit yield in tomato. Plant Physiology **177**, 1286–1302.

Mao C, Lu S, Lv B, Zhang B, Shen J, He J, Luo L, Xi D, Chen X, Ming F. 2017. A rice NAC transcription factor promotes leaf senescence via ABA biosynthesis. Plant Physiology **174**, 1747–1763.

Martel C, Vrebalov J, Tafelmeyer P, Giovannoni JJ. 2011. The tomato MADS-box transcription factor RIPENING INHIBITOR interacts with promoters involved in numerous ripening processes in a COLORLESS NONRIPENING-dependent manner. Plant Physiology **157**, 1568–1579.

Mou W, Li D, Luo Z, Li L, Mao l, Ying T. 2018. SIAREB1 transcriptional activation of *NOR* is involved in abscisic acid-modulated ethylene biosynthesis during tomato fruit ripening. Plant Science **276**, 239–249.

Moyano E, Martínez-Rivas FJ, Blanco-Portales R, Molina-Hidalgo FJ, Ric-Varas P, Matas-Arroyo AJ, Caballero JL, Muñoz-Blanco J, Rodríguez-Franco A. 2018. Genome-wide analysis of the NAC transcription factor family and their expression during the development and ripening of the *Fragaria×ananassa* fruits. PLoS One **13**, e0196953.

Nguyen N, Suokas M, Karppinen K, Vuosku J, Jaakola L, Häggman H. 2018. Recognition of candidate transcription factors related to bilberry fruit ripening by de novo transcriptome and qRT-PCR analyses. Scientific Reports **8**, 9943.

Noh YS, Amasino RM. 1999. Identification of a promoter region responsible for the senescence-specific expression of *SAG12*. Plant Molecular Biology **41**, 181–194.

Oda-Yamamizo C, Mitsuda N, Sakamoto S, Ogawa D, Ohme-Takagi M, Ohmiya A. 2016. The NAC transcription factor ANAC046 is a positive regulator of chlorophyll degradation and senescence in Arabidopsis leaves. Scientific Reports **6**, 23609.

Oeller PW, Lu MW, Taylor LP, Pike DA, Theologis A. 1991. Reversible inhibition of tomato fruit senescence by antisense RNA. Science **254**, 437–439.

Podzimska-Sroka D, O'Shea C, Gregersen PL, Skriver K. 2015. NAC transcription factors in senescence: from molecular structure to function in crops. Plants **4**, 412–448.

Pourtau N, Jennings R, Pelzer E, Pallas J, Wingler A. 2006. Effect of sugar-induced senescence on gene expression and implications for the regulation of senescence in Arabidopsis. Planta **224**, 556–568.

Proost S, Van Bel M, Vaneechoutte D, Van de Peer Y, Inzé D, Mueller-Roeber B, Vandepoele K. 2015. PLAZA 3.0: an access point for plant comparative genomics. Nucleic Acids Research **43**, D974–D981.

Puranik S, Sahu PP, Srivastava PS, Prasad M. 2012. NAC proteins: regulation and role in stress tolerance. Trends in Plant Science **17**, 369–381.

Qin G, Wang Y, Cao B, Wang W, Tian S. 2012. Unraveling the regulatory network of the MADS box transcription factor RIN in fruit ripening. The Plant Journal **70**, 243–255.

Ríos P, Argyris J, Vegas J, *et al*. 2017. ETHQV6.3 is involved in melon climacteric fruit ripening and is encoded by a NAC domain transcription factor. The Plant Journal **91**, 671–683.

Sakuraba Y, Han SH, Lee SH, Hörtensteiner S, Paek NC. 2016. Arabidopsis NAC016 promotes chlorophyll breakdown by directly upregulating STAYGREEN1 transcription. Plant Cell Reports **35**, 155–166.

Sakuraba Y, Jeong J, Kang MY, Kim J, Paek NC, Choi G. 2014. Phytochrome-interacting transcription factors PIF4 and PIF5 induce leaf senescence in Arabidopsis. Nature Communications **5**, 4636.

Shao H, Wang H, Tang X. 2015. NAC transcription factors in plant multiple abiotic stress responses: progress and prospects. Frontiers in Plant Science **6**, 902.

Speirs J, Lee E, Holt K, Yong-Duk K, Steele Scott N, Loveys B, Schuch W. 1998. Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruit affects the balance of some flavor aldehydes and alcohols. Plant Physiology **117**, 1047–1058.

Thirumalaikumar VP, Devkar V, Mehterov N, Ali S, Ozgur R, Turkan I, Mueller-Roeber B, Balazadeh S. 2018. NAC transcription factor JUNGBRUNNEN1 enhances drought tolerance in tomato. Plant Biotechnology Journal **16**, 354–366.

Tomato Genome Consortium. 2012. The tomato genome sequence provides insights into fleshy fruit evolution. Nature **485**, 635–641.

Uauy C, Distelfeld A, Fahima T, Blechl A, Dubcovsky J. 2006. A NAC gene regulating senescence improves grain protein, zinc, and iron content in wheat. Science **314**, 1298–1301.

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flügge UI, Kunze R. 2006. Transcription analysis of arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiology **141**, 776–792.

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

Wu A, Allu AD, Garapati P, *et al*. 2012. JUNGBRUNNEN1, a reactive oxygen species-responsive NAC transcription factor, regulates longevity in Arabidopsis. The Plant Cell **24**, 482–506.

Xue GP. 2002. Characterisation of the DNA-binding profile of barley HvCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. Nucleic Acids Research **30**, e77.

Xue GP. 2005. A CELD-fusion method for rapid determination of the DNA-binding sequence specificity of novel plant DNA-binding proteins. The Plant Journal **41**, 638–649.

Xue GP, Bower NI, McIntyre CL, Riding GA, Kazan K, Shorter R. 2006. TaNAC69 from the NAC superfamily of transcription factors is up-regulated by abiotic stresses in wheat and recognises two consensus DNA-binding sequences. Functional Plant Biology **33**, 43–57.

Yang J, Udvardi M. 2018. Senescence and nitrogen use efficiency in perennial grasses for forage and biofuel production. Journal of Experimental Botany **69**, 855–865.

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

Zhang K, Xia X, Zhang Y, Gan SS. 2012. An ABA-regulated and Golgi-localized protein phosphatase controls water loss during leaf senescence in Arabidopsis. The Plant Journal **69**, 667–678.

Zhao D, Derkx AP, Liu DC, Buchner P, Hawkesford MJ. 2015. Overexpression of a NAC transcription factor delays leaf senescence and increases grain nitrogen concentration in wheat. Plant Biology **17**, 904–913.

Zhao Y, Gao J, Im Kim J, Chen K, Bressan RA, Zhu JK. 2017. Control of plant water use by ABA induction of senescence and dormancy: an overlooked lesson from evolution. Plant & Cell Physiology **58**, 1319–1327.

Zhong J, Powell S, Preston JC. 2016. Organ boundary NAC-domain transcription factors are implicated in the evolution of petal fusion. Plant Biology **18**, 893–902.

Zhou Y, Huang WF, Liu L, Chen TY, Zhou F, Lin YJ. 2013. Identification and functional characterization of a rice NAC gene involved in the regulation of leaf senescence. BMC Plant Biology **13**.

Zhu M, Chen G, Zhou S, Tu Y, Wang Y, Dong T, Hu Z. 2014. A new tomato NAC (NAM/ATAF1/2/CUC2) transcription factor, SlNAC4, functions as a positive regulator of fruit ripening and carotenoid accumulation. Plant & Cell Physiology **55**, 119–135.

Zuo J, Niu QW, Frugis G, Chua NH. 2002. The *WUSCHEL* gene promotes vegetative-to-embryonic transition in Arabidopsis. The Plant Journal **30**, 349–359.