One sentence summary: Senescence-associated gene knockdown increases carbon exportation towards sink organs increasing plant yield in Solanum lycopersicum.

List of author contributions:
B.S.L. designed and performed most of the experiments, analysed the data and wrote the article with contributions of all the authors; G.G. designed and performed the confocal and protein-protein interaction experiments and wrote the article with contributions of all the authors; B.T. performed some experiments; F.R.R.A. performed the gas exchange and chlorophyll fluorescence analysis; A.C.D.L. performed some experiments; D.D assisted the preparation of TEM samples and obtained the micrographs; E.P. performed soluble sugar quantification; E.M.S. and G.F.F.S. produced the transgenic plants OEmiR164a; F.T.S.N. supervised E.M.S. and G.F.F.S.; V.P.T. generated the transgenic Atore1 lines overexpressing each SIORE1 putative ortholog under the supervision of S.B.; L.F. designed the experiments, contributed to data analysis and complemented the writing; M.R. designed the experiments, contributed to data analysis and wrote the article with contributions of all the authors.

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Abstract

Senescence is the process that marks the end of leaves lifespan. As it progresses, the massive macromolecular catabolism dismantles the chloroplasts and, consequently, decreases the photosynthetic capacity of these organs. Thus, senescence manipulation is a strategy to improve plant yield by extending the leaf photosynthetically active window of time. However, it remains to be addressed if this approach can improve fleshy fruit production and nutritional quality. One way to delay senescence initiation is by regulating key transcription factors (TFs) involved in triggering this process, such as the NAC TF ORESARA1 (ORE1). Here, three senescence-related NAC TFs from *Solanum lycopersicum* were identified, namely SIORE1S02, SIORE1S03 and SIORE1S06. All three genes showed to be responsive to senescence-inducing stimuli and post-transcriptionally regulated by the microRNA miR164. Moreover, the encoded proteins physically interacted with the chloroplast maintenance related TF SlGLKs. This characterization led to the selection of a putative tomato ORE1 as target gene for RNAi knockdown. Transgenic lines showed delayed senescence and enhanced carbon assimilation that, ultimately, increased the number of fruits and their total soluble solid content. Additionally, the fruit nutraceutical composition was enhanced. In conclusion, the data provide robust evidence that the manipulation of leaf senescence is an effective strategy for yield improvement in fleshy fruit-bearing species.

Keywords: *Solanum lycopersicum*, tomato, ORESARA1, GOLDEN2-LIKE, senescence, yield.
Introduction

The ever-growing demand for food and biofuels has been a significant driving force behind studies focussed on crop yield manipulation. As a complex trait, many strategies can lead to yield improvement, such as manipulation of morphogenetic patterns, source activity, source to sink communication or sink activity (Rossi et al., 2015). Delaying senescence to extend leaf photosynthetic activity is considered one mechanism to strengthen source activity aiming to increase the net carbon availability for sink organs (Thomas & Ougham, 2014; Rossi et al., 2015). The expression of *ISOPENTENYL TRANSFERASE* (*IPT*), which encodes the enzyme responsible for the rate-limiting step in cytokinin biosynthesis, under the control of the *SENESCENCE ASSOCIATED GENE 12* (*SAG12*) promoter has been extensively used for delaying leaf senescence (Gregersen et al., 2013). Once the expression is activated during senescence by the *SAG12* promoter, the increment in cytokinin levels blocks senescence progression thereby retaining photosynthetically active leaves. This approach has proven to successfully block or attenuate both age- and stress-related leaf senescence in different crop species like rice (Liu et al., 2010), cassava (Zhang et al., 2010), wheat (Sykorova et al., 2008) and tomato (Swartzberg et al., 2006).

With the increase in the knowledge about the genetic control of senescence, transcription factors (TFs), such as those belonging to the WRKY, MYB and NAM/ATAF1/CUC2 (NAC) families, stand out as regulators of this process (Balzadeh et al., 2008). The extensive NAC family regulates a diversity of processes along plant development (Mitsuda et al., 2005, Olsen et al., 2005, Puranik et al., 2012). Particularly, the *Arabidopsis thaliana* *ORESARA1* gene (*AtORE1*) has received special attention for encoding a master regulator of senescence initiation (Oh et al., 1997, He et al., 2005, Kim et al., 2009, Balazadeh et al., 2010, Kim et al., 2011, Rauf et al., 2013), as *Atore1* mutant exhibits a delayed senescence phenotype (Oh et al., 1997, Kim et al., 2009). Besides being induced by ethylene, salt, darkness and abscisic acid (Kim...
AtORE1 is also post-transcriptionally regulated by microRNA miR164 (Kim et al., 2009). AtORE1 not only induces senescence-related genes, but also physically interacts with and inactivates the chloroplast maintenance-related TF GOLDEN2-LIKE (GLK), shifting the cell from a photosynthetically active program towards senescence (Rauf et al., 2013, Garapati et al., 2015). GLKs have been shown to control chloroplast development in several species, in which usually a pair of paralogs with functional redundancy occurs in the genome of several species (Fitter et al., 2002, Waters et al., 2008, 2009). In tomato, Solanum lycopersicum, the paralogs exhibit organ specificity; while SIGLK1 is mostly expressed in vegetative organs, SIGLK2 is exclusively transcribed in the stylar portion of the fruits (Powell et al, 2012, Nguyen et al., 2014). Interestingly, Slglk2 mutation, which was fixed in many cultivated varieties, negatively impacts fruit chloroplast differentiation; on the other hand, SIGLK2 overexpression increases fruit starch, soluble sugar and carotenoid content (Powell et al., 2012).

Although being a model plant for fleshy fruit physiology, the senescence regulatory network and its impacts on fruit development and quality in tomato remains elusive. Here, putative AtORE1 orthologs, namely SIORE1S02, SIORE1S03 and SIORE1S06, were identified in tomato genome. The genes were functionally characterised regarding their responsiveness to senescence-inducing treatments, regulation by SlmiR164 and capability to physically interact with SIGLkS and to complement Atore1 mutant phenotype. Moreover, SIORE1S02-knockdown plants displayed delayed senescence, enhanced carbon assimilation and altered source-sink sugar partitioning leading to the development of more fruits with increased soluble sugar content. The results obtained provide robust evidences that the manipulation of leaf senescence is a promising strategy for yield improvement in the fleshy fruit-bearing species.
Results

*Solanum lycopersicum* genome harbours three putative senescence-related *AtORE1* orthologs

To identify putative *S. lycopersicum* *AtORE1* orthologs, the protein sequences of all NAC transcriptions factors of *S. lycopersicum* and *A. thaliana*, retrieved from the Plant Transcription Factors Database (http://planttfdb.cbi.pku.edu.cn/), were aligned (Supplemental Table 1). The phylogenetic reconstruction (Supplemental Fig. S1) allowed the selection of three putative NAC domain-containing proteins (Supplemental Fig. S2), named SIORE1S02, SIORE1S03 and SIORE1S06 accordingly to the chromosome localization (Fig. 1). Then, their responsiveness to senescence-inducing treatments was addressed.

The transcript profile of SIORE1S02, SIORE1S03 and SIORE1S06 was evaluated upon different *in vitro* treatments for senescence induction, *i.e.* treatment with ethylene, darkness, jasmonic acid, salicylic acid or salt (Fig. 2). Confirming the induction and progression of senescence, all treatments triggered significant reduction in leaf chlorophyll content as well as down- and up-regulation of SIGLK1 (the chloroplast maintenance-related TF) and SISAG12 (the senescence-associated gene) genes, respectively (Supplemental Fig. S3). The mRNA profile of the three SIORE1s was modulated by the various treatments. While SIORE1S03 was induced in all cases, the level of SIORE1S02 mRNA increased upon the application of ethylene, salicylic acid and salt solution, and SIORE1S06 was upregulated by ethylene and darkness. Thus, although with differences, all three genes were transcriptionally activated by senescence-inducing stimuli.

The transcriptional profile of SIORE1s during fruit development and ripening was also assessed (Fig. 3). For all three SIORE1s, the highest expression levels were identified at early immature stages declining towards the ripe stage. Thus, although in leaves these genes were induced by ethylene, mRNA accumulation did not coincide with the ripening-associated climacteric peak of ethylene production typically observed at the breaker (BR) stage. This result indicates that,
although a role in early fruit development cannot be ruled out, a direct function of SIORE1s along fruit ripening seems unlikely, as revealed by the expression pattern in this organ. Moreover, the data suggest an organ-specific ethylene-mediated regulation of SIORE1 genes.
Figure 2

**Figure 2.** Transcript profile of *SIORE1s* in response to senescence-inducing treatments. Transcript profile of *SIORE1s* in leaves of 4-week-old *in vitro*-grown plants after senescence-inducing treatments. Values represent the mean ± SE from at least three biological replicates normalized against the zero hour sample. Each treatment relative transcript ratio is expressed as the ratio between treatment value and the corresponding untreated control (*`). Statistically significant differences relative to the zero hour treatment are represented with closed symbols (*P < 0.05*).

SIORE1s are regulated by SimiR164 and their encoded proteins physically interact with SiGLKs and partially recover the Atore1 phenotype.
In *silico* analysis of the coding sequences revealed putative binding sites for SlmiR164 in all three SiORE1s. However, this site is disrupted by an insertion of 29 bp in SiORE1S02 (Fig. 4A). To ascertain if this insertion affects SlmiR164 recognition, a modified 5’ RACE was performed.
to detect cleaved transcripts, using RNA extracted from non-senescing leaves. This allowed the identification of SlmiR164-guided cleavage transcripts of SIORE1S03 and SIORE1S06, but not of SIORE1S02 (Fig. 4A).
To gain further knowledge about the role of SlmiR164 for SIORE1s regulation, the mRNA of the three genes was profiled in transgenic lines harbouring a 35S:AtMIR164a construct (abbreviated as OEmiR164a) in non-senescent, early senescing and late senescing leaves (Fig. 4B). The mRNA pattern of SIGLK1 and SISAG12 was also assessed as senescence markers. In wild-type plants (WT), leaf senescence was accompanied by a progressive downregulation in SlmiR164 and SIGLK1 and upregulation of SISAG12, SIORE1S02, SIORE1S03 and SIORE1S06. As expected, OEmiR164a lines displayed high levels of SlmiR164 in both, non-senescent and senescent leaves stages. As a result, a lag in SIGLK1 decrease and SISAG12 accumulation, together with the increased chlorophyll levels, were indicative of delayed senescence initiation (Fig. 4C). The marked reduction of SIORE1S03 and SIORE1S06 mRNA levels observed in OEmiR164a lines is in agreement to both being directly regulated by SlmiR164. Interestingly, the levels of SIORE1S02 in non-senescent and senescing leaves were the same as those observed in non-senescent WT leaves, meaning that the maintenance of high levels of SlmiR164 prevented the senescence-associated induction of this gene. Although SlmiR164 is not capable of cleaving SIORE1S02 transcripts, it regulates it by targeting a currently unknown factor that, in turn, drives the senescence-associated mRNA accumulation of this gene. Thus, SlmiR164 directly controls the transcript levels of SIORE1S03 and SIORE1S06 and indirectly regulates the transcript abundance of SIORE1S02, what is in line with the known transcriptional regulation of AtORE1 by AtmiR164 (Kim et al., 2009).

Moreover, AtORE1 is known to physically interacts with AtGLKs in the nucleus, preventing the activity of these chloroplasts maintenance TFs (Rauf et al., 2013). Then, it was first addressed whether SIORE1s and SIGLKs are nucleus-targeted proteins. Confocal analysis of Nicotiana tabacum leaves transiently expressing transcription factor - GFP fusion proteins revealed that SIORE1S02, SIORE1S03, SIORE1S06, SIGLK1 and SIGLK2 were localized in the nucleus as the GFP fluorescence in all cases co-localized with the fluorescence of the DAPI marker (Supplemental Fig. S4). For the latter, the result confirmed that previously reported in tomato protoplasts by...
Figure 5. Analysis of SIGLks and SIORE1s protein-protein interaction by Bimolecular Fluorescent Complementation (BiFC). VYNE and VYCE fusion proteins were transiently expressed in *N. tabacum* leaves by infiltration with *A. tumefaciens*. VYNE-Cnx6/VYCE-Cnx6 and AIORE1/AtGLK2 (Gehl et al., 2009) were used as technical and biological positive controls, respectively. VYNE-Cnx6/SLGLK2-VYCE and VYCE-Cnx6/SIORE1SO2-VYNE interactions were used as negative controls. SIGLks/SIGLks interaction was confirmed as evidenced by the YFP fluorescence detected. YFP, DAPI nuclear marker, bright-field and merged signals are indicated above the panels. Bars = 20 μm, except in VYNE-Cnx6/VYCE-Cnx6 images = 40 μm.

Tang et al. (2015). Next, bimolecular fluorescence complementation (BiFC) was used to test the physical interaction. The C-terminal of SIORE1s and SIGLks were fused to the N- (VYNE) and C-terminal (VYCE) of the Venus YPF protein, respectively (Fig. 5). The interaction between
AtORE1 and AtGLK2 was used as positive control. In all interactions tested, fluorescence was detected in the nucleus, thus, all SlORE1s physically interact with both SIGLKs. Of note, a strong fluorescence signal was observed in the nucleolus in all SlORE1-SIGLK interactions, and also for AtORE1 and AtGLK2.

It has been suggested that the ubiquitin proteasome system may be active in the nucleolus, where ubiquitin and proteasome were immunolocalized (Stępiński, 2012). In addition, this compartment provides a site for transient inactivation of enzymatic or regulatory proteins (Stępiński, 2014). As SIGLK2 was shown to be degraded by the ubiquitin-proteasome system (Tang et al., 2015), one may speculate that the ORE1-GLK complex is transferred to the nucleolus where GLKs are inactivated by ubiquitination and subsequently degraded in this compartment.

These results show similarities between SlORE1s and AtORE1 regulation. To address whether SlORE1s were able to recover the Atore1 delayed-senescence phenotype, each SlORE1s was constitutively overexpressed in the Arabidopsis mutant background, Atore1-OE:SlORE1S02, Atore1-OE:SlORE1S03 and Atore1-OE:SlORE1S06. In line with AtORE1 overexpression in Col-0 background (Rauf et al., 2013), no visible phenotype was observed in young plants. Dark-induced senescence was assayed in detached leaves. After seven days, while Atore1 leaves remained green, the leaves overexpressing any of the SlORE1s acquired a yellowish tone indicative of ongoing senescence, but not to the same extent as the Col-0 genotype (Fig. 6). Thus, all three SlORE1s were able to partially recover the senescence delay in Atore1 mutant.

SlORE1S02-knockdown in tomato increases photosynthesis and delays dark-induced senescence

Among the three SlORE1s, SlORE1S02 was chosen for stable RNA interference (RNAi) knockdown as this is the closest ortholog to AtORE1 in the phylogenetic reconstruction, its transcript levels are highly modulated along senescence and indirectly regulated by Smlir164,
and the encoded protein physically interacts with SIGLks and partially complement the Atore1 mutant. Five tomato transgenic lines were obtained and three lines displaying over 70% silencing (Supplemental Fig. S5), namely L1, L3 and L6, were chosen for further phenotyping. 

Figure 6. SIOREIs partially recover Atore1 senescence impairment. Detached leaves from three-month-old Col-0, Atore1 mutant, Atore1-OE:SIORE1S02, Atore1-OE:SIORE1S03 and Atore1-OE:SIORE1S06 plants were kept in darkness for seven days to induce senescence. While Atore1 displayed no signs of senescence, the yellowish colour of the genotypes overexpressing SIOREIs hints an ongoing senescence program, yet not to the extent observed in the Col-0 genotype. Bar = 1 cm.
Interestingly, *SIORE1S03* and *SIORE1S06* were also reduced in leaves of *SIORE1S02*-knockdown lines, but not to the same extent as *SIORE1S02*. Since there is an extremely low degree of similarity among the RNAi target region of *SIORE1S02* and the coding sequences of *SIORE1S03* and *SIORE1S06*, the possibility of cross-silencing can be ruled-out. Instead, it is likely that *SIORE1S02* is an upstream positive regulator of *SIORE1S03* and *SIORE1S06* (Supplemental Fig. S6).

Gas exchange and chlorophyll fluorescence parameters were measured by using an infra-red gas analyser (IRGA) at two developing stages of the plant: (I) before the beginning of flowering stage, in the fifth leaf of 70-day-old plants (referred as young plant leaves - YPL), and (II) in fruit-bearing stage, in the sixth leaf of 120-day-old plants (referred as mature plant leaves - MPL). No differences were found in photosynthetic parameters in YPL. However, MPL from *SIORE1S02*-knockdown plants showed increased carbon assimilation (A) in comparison to the untransformed control genotype (MT), while all other measured parameters remained unaltered (Table 1).

Chlorophyll levels in YPL and MPL were higher in transgenic plants than in equivalent leaves of the MT genotype (Fig. 7A). Whereas, at MPL stage, the density of chloroplasts per mesophyll cell was slightly higher in transgenic lines (Fig. 7B), the ultrastructure of this organelle was not affected by the downregulation of *SIORE1S02* (Supplemental Fig. S7). Accordingly, *SIGLK1* mRNA levels were higher in both YPL and MPL of transgenic plants (Fig. 7C). Interestingly, while the senescence marker *SISAG12* was undetectable in YPL, lower levels were detected in transgenic MPL compared to the corresponding MT control (Fig. 7C). Together these results suggest a delay in senescence in *SIORE1S02*-knockdown plants. To further corroborate this hypothesis, the eighth leaf of 120-day-old plants was detached and kept in the dark for 10 days. While MT leaves turned yellowish, transgenic ones kept greenness, supporting the conclusion that *SIORE1S02* deficiency delays senescence (Fig. 7D).
**Figure 7.** SIORE1S02-knockdown affects leaf aging. A, Chlorophyll content in leaves. Values represent the mean ± SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control (P < 0.05). YPL: young plant leaves; MPL: mature plant leaves. B, Number of chloroplasts per mesophyll cell of MPL. Values represent the mean ± SE from at least 45 cells. Asterisks denote statistically significant differences to MT control (P < 0.05). C, SIGLK1 and SISAG12 transcript ratio in YPL and MPL leaves. Values represent the mean ± SE from at least three biological replicates normalised against the respective sample from MT control. Asterisks denote statistically significant differences to MT control (P < 0.05). The relative transcript values are available in Supplemental Table S4. D, SIORE1S02-knockdown effect on dark-induced senescence in tomato leaves. Detached leaflets from 120-day-old plants kept ten days in darkness retain greenness, while MT control leaflets turned yellow. Bar = 5 cm.

**SIORE1S02-knockdown** in tomato increases yield and brix

As transgenic plants assimilate carbon for an extended period of time, the impact of **SIORE1S02-knockdown** over yield was evaluated. Transgenic plants produced more fruits than...
MT control plants, rendering a significant increase in harvest index (HI), as no difference was detected in aerial shoot mass (Fig. 8A). The transgenic fruits showed no alteration in the shape, weight and diameter (Supplemental Fig. S8). Besides, SIORE1S02-knockdown lines showed...
higher levels of soluble solids in ripe fruits as reflected by the increment in brix units (Fig. 8B), which is also an important trait in tomato, especially for industrial processing.

Enhanced HI and brix are typically due to increased levels of glucose, fructose and sucrose and are indicative of altered carbohydrate metabolism. Thus, the content of starch and soluble sugars in leaves and fruits was determined (Fig. 9). The fruits of transgenic plants showed an increased content of glucose in all analysed stages and of fructose in both ripening stages, which explains the increment in brix units. Moreover, starch content in mature green and breaker fruits of SIORE102-knockdown lines were also higher when compared to MT control. As expected, six days after the breaker stage, starch was totally degraded in all genotypes. In leaves, while YPL of transgenic plants accumulated higher starch and sucrose levels than MT control genotype; when fruits are developing, in the MPL, the allocation of fixed carbon as starch diminished, whereas sucrose still accumulated at higher levels. The upregulation in the leaf carbohydrate levels triggered by SIORE102-knockdown is consistent with the above-mentioned increase in carbon assimilation observed in the transgenic lines. Moreover, considering that in tomato carbon is mostly translocated as sucrose (Barker et al., 2000), this carbohydrate pattern suggests an enhanced carbon export from leaves towards sink organs in SIORE102-deficient plants.

Isoprenoid metabolism is affected by SIORE102-knockdown tomato plants

It was shown that lowering SIORE102 transcript abundance affects chlorophyll accumulation and carbon assimilation and partitioning. To better understand the origin of these metabolic alterations and their impact of fruit chemical composition, isoprenoid-derived compounds (i.e. chlorophyll, tocopherols and carotenoids, Supplemental Table S3) were quantified and the mRNA levels of chlorophyll-, tocopherol- and carotenoid-metabolism associated genes profiled (Supplemental Table S4) (Fig. 10). The analysed genes were those that showed to be transcriptionally regulated key points of the biosynthetic pathways (Quadrana et al., 2013;
Figure 9. SIORE1S02 knockdown alters sugar profile in leaves and fruits. Content of starch and soluble sugars in leaves and fruits of SIORE1S02 knockdown lines. Values represent the mean ± SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control genotype (P < 0.05). ND: Not detected. YPL, young plant leaves; MPL, mature plant leaves; MG, mature green stage; BR, breaker; BR6: 6 days after BR, respectively.

Almeida et al., 2015, 2016). The transcript abundance of those paralogs that, based on previous reports, were not directly involved in the analysed pathways in the sampled organs was not addressed. This is the case of SIPHL2 and SIPHL3, which are extremely low
expressed (Lira et al., 2015) and, differently from \textit{SIPPHL1}, are not functionally characterised (Lin et al., 2016); \textit{SlPSY3} is involved in root carotenogenesis (Liu et al., 2015); and \textit{SlLCY\textbeta{}2} (Solyc10g079480) and \textit{SlGGDR1} (Solyc01g088310) are poorly expressed in the analysed organs (Solyc10g079480) and \textit{SIPPHL1}, are not functionally characterised (Lin et al., 2016); \textit{SlPSY3} is involved in root carotenogenesis (Liu et al., 2015); and \textit{SlLCY\textbeta{}2} (Solyc10g079480) and \textit{SlGGDR1} (Solyc01g088310) are poorly expressed in the analysed organs.
In MPL of SIORE1S02-knockdown plants, the chlorophyll recycling/biosynthesis was enhanced, as evidenced by the increase in CHLOROPHYLL SYNTHASE (SlCHLG), PHEOPHYTINASE-LIKE 1 (SIPHL1) and PHYTOL KINASE (SlPPHL1) transcript abundance. Although the mRNA levels of PHYTOENE SYNTHASE 1 (SIPSY1) and PHYTOENE DESATURASE (SIPDS) were increased in YPL, no differences were found in leaf carotenoid contents, except for a slight reduction in lutein from MPL. Regarding tocopherol synthesis, YPL showed similar levels of tocopherol in both genotypes, which is in line with the expression of the enzyme encoding genes that supply phytol diphosphate (PDP) precursor, such as GERANYLGERANYL DIPHOSPHATE REDUCTASE (SIGGDR), SlVTE5 and PHYTOL PHOSPHATE KINASE (SlVTE6). However, in SIORE1S02-knockdown MPL, due to chlorophyll biosynthesis enhancement, PDP availability for tocopherol synthesis is reduced compared to MT control resulting in reduced levels of this compound. It is worth mentioning that the most abundant form of vitamin E is α-tocopherol that perfectly correlates with the amount of total-tocopherol (Supplemental Table S3). Apparently, the maintenance of a highly active photosynthetic apparatus in MPL requires less amounts of antioxidant tocopherol compounds.

The impact of SIORE102-knockdown on fruit isoprenoid profile was distinct from those observed in leaves. There was no difference in fruit chlorophyll content between transgenic and control plants. Similarly, carotenoid metabolism remained almost unaffected, displaying punctual reductions in neurosporene and lutein content in MG and BR1 fruits, respectively. Tocopherols were more abundant in SIORE1S02-knockdown than in control fruits, which is in accordance to the transcript levels of SIGGDR, SlVTE6, HOMOGENTISATE PHYTYL TRANSFERASE (SlVTE2), 2,3-DIMETHYL-5-PHYTYLQUINOL METHYLTRANSFERASE (SlVTE3(1)), TOCOPHEROL CYCLASE (SlVTE1) and TOCOPHEROL C-METHYL TRANSFERASE (SlVTE4).
Therefore, \textit{SIORE1SO2}-knockdown plants showed organ-specific regulation of isoprenoid metabolism.
The leaves are the major photosynthetic organs and the net carbon fixed during the photosynthetic period is critical for plants’ fitness and sink organ development (Wu et al., 2012). Thus, source-sink relationship is tightly linked to leaf lifespan and, consequently, affected by the fine control of senescence. In this regard, a straightforward approach to increase yield is to extend the photosynthetic function phase. This has been achieved in grasses by silencing NAC TF encoding genes. Although an increase in grain yield was observed due to extended grain filling time, seed quality was compromised by reduced nutrient remobilization (Waters et al., 2009; Liang et al., 2014). Yet, the impact of delayed senescence remains poorly explored in non-monocarpic species that do not undergo whole-plant senescence after the reproductive phase (Thomas, 2012).

To gain insight on how fleshy fruit development and yield can be affected by altering leaf senescence, the senescence master regulator ORE1 was targeted in S. lycopersicum. Three putative orthologs were identified in the tomato genome, namely SlORE1S02, SlORE1S03 and SlORE1S06. As all were induced by senescence-inducing treatments, regulated by SlmiR164, able to physically interact with SIGLKS and capable to rescue Atore1 mutant, SlORE1S02, the phylogenetically closest one to AtORE1, was chosen for knockdown by RNA interference. The reduction in the amount of SlORE1S02 transcripts affected the metabolism of source leaves, both of young and mature plants. The increase in chlorophyll content and the expression of SIGLK1 TF, together with the increment in carbon assimilation and the reduced SISAG12 mRNA levels in leaves of mature plants, hint that senescence is delayed in transgenic plants. This was corroborated by the dark induction senescence assay on detached leaves. The aforementioned phenotype in leaves from the SlORE1S02-deficient plants might be explained, at least in part, because GLK TFs are known to coordinate the expression of genes related to chlorophyll synthesis (e.g. PROTOCHLOROPHYLLIDE OXIDOREDUCTASE) and the photosynthetic
machinery (e.g. light harvesting complex proteins) leading to proper chloroplast differentiation and maintenance (Waters et al., 2009; Nguyen et al., 2014). This is in agreement with the increased chloroplast number in mesophyll cells of mature leaves. Additionally, higher levels of SICHLG transcript, whose encoded protein is a key enzyme for chlorophyll biosynthesis and also regulates the synthesis of chlorophyll-binding proteins (Shalygo et al., 2009), contributes to the extended photosynthetic active period in transgenic plants.

Tocopherol metabolism was also affected in leaves of SIORE1S02-knockdown plants. As chlorophyll, tocopherols are isoprenoid-derived compounds that play a main role in protecting photosynthetic machinery by either quenching oxygen singlet ($^1$O$_2$) or inhibiting the progression of lipid peroxidation (Havaux et al., 2005). It has been demonstrated that the recycling of chlorophyll degradation-derived phytol is the main source of tocopherol side chain in tomato (Almeida et al., 2015). In agreement with this, the delayed senescence progression proposed above for SIORE1S02-knockdown plants explains the reduction in tocopherol content measured in leaves from mature plants. In this sense, it has been suggested that increasing tocopherol content along senescence contributes to the proper dismantling and recycling of plastid thylakoid lipids (Chrost et al., 1999). Additionally, the boost in chlorophyll turnover, evidenced by the increment in mRNA abundance of SIPPHL1 (Lin et al., 2016), SIVTE5 (Almeida et al., 2016) and SICHLG, driving PDP towards chlorophyll synthesis, further support this statement.

The delayed senescence progression was also reflected in both leaf and fruit sugar composition. During the vegetative phase, before the onset of flower anthesis, while the fifth leaf from control young plants were the main source of assimilates for sink organs, in SIORE1S02-deficient plants, these leaves retained higher levels of fixed carbon mainly as starch, as older leaves retained high photosynthetic activity. As fruits began to develop, the starch was remobilised to sucrose and reallocated to these organs. The boosted carbon exportation in SIORE1S02-knockdown plants increased yield as the result of the increment in
fruit number. Interestingly, the fruits developed slightly faster than the control genotype as
the number of days to reach the breaker stage was reduced (Supplemental Fig. S9A).
Additionally, BR1 and BR6, but not MG fruits were enriched in total soluble protein content,
which might indicate that SIORE1S02-knockdown affect protein degradation during
chloroplast-chromoplast transition (Supplemental Fig. S9B).
Regarding fruit nutritional quality, SIGGDR mRNA accumulated to higher levels in transgenic
fruits during ripening increasing PDP availability for tocopherol synthesis. Moreover, in
SIORE1S02-knockdown genotype, the tocopherol biosynthesis-related genes were upregulated
along fruit ripening, contributing to the increment of this nutraceutical.
Altogether, the metabolic alterations indicate that SIORE1S02 deficiency delays ageing by
means of extending chloroplast maintenance and carbon assimilation. Consequently,
photoassimilate exportation towards fruits is enhanced, ultimately, improving harvest index
and the content of tocopherol antioxidant and soluble sugars in ripe fruits. Thus, the data
obtained suggests that the manipulation of leaf senescence is a promising strategy to improve
fleshy fruit yield and metabolism.

Materials and Methods

Plant material, growth conditions and sampling
In vitro treatments for senescence induction were performed with wild type Solanum
lycopersicum (cv Micro Tom, MT) harboring the wild-type allele SIGLK2. Tomato seeds were
surface sterilized, sown and cultivated in vitro as described by Lira et al. (2014). Four-week-old
plants had senescence induced either by application of ethylene (gaseous to a final
concentration of 5 M.L⁻¹), jasmonic acid (gaseous to a final concentration of 30 µM), salicylic
acid (liquid solution to a final concentration of 30 µM), salt (liquid NaCl solution to a final
concentration of 300 mM) or kept in darkness. Leaf samples were collected 30 min, 1 h, 3 h, 6 h, 48 h after treatment.

For **SlORE1S** fruit transcriptional profiling, tomato plants were grown in 1-L pots in a greenhouse under automatic irrigation in an average mean temperature of 25°C, 11.5/13 (winter/summer) light hours and 250-350 μmol m⁻² s⁻¹ of incident photo-irradiance. The pericarp from the top portion of the fruit was sampled at immature green 3 (IG3), immature green 5 (IG5), mature green (MG), breaker (BR), breaker + 3 (BR3) and breaker + 5 (BR5) stages were harvested approximately at 10, 22, 37 (displaying jelly placenta) and, approximately, 42 (when the first signals of carotenoid accumulation are visualized), 45 and 48 days after anthesis, respectively. All samples were frozen in liquid N₂, powdered and stored at -80°C. The transgenic lines overexpressing **AtMiR164a** (miR_2 and miR_3) were generated via a 176 bp fragment encompassing the **AtMiR164a** precursor (MIR164aAt2g47585) that was amplified from genomic DNA of *Arabidopsis thaliana* ecotype Columbia (Col-0). The PCR product was sub-cloned into TOPO TA (Invitrogen, http://www.lifetechnologies.com) and sequenced. The confirmed **AtMiR164a** precursor was digested using the XbaI and Pst restriction enzymes, and subsequently cloned into binary vector under the control of the CaMV 35S promoter. Once cleaved, the mature **AtMiR164a** has the exact sequence of the mature SlmiR164 (Accessions for miRBase; http://www.mirbase.org/; AtMiR164: MI0000197; SlmiR164: MI0027570). The genetic transformation was made in *Slijk2 Solanum lycopersicum* (cv Micro Tom) background according to Pino et al. (2010). T2 plants were grown as above described and non-senescent (NS), early senescing (ES) and late senescing (LS) leaves were collected. Leaves stages were set according to the untransformed genotype yellowish phenotype.

For subcellular localization and BiFC assays, *Nicotiana tabacum* plants were cultivated for 4 weeks in 1-L pots as described above.

To evaluate the capability of **SlORE1S02**, **SlORE1S03** and **SlORE1S06** to recover Atore1 mutant phenotype, this mutant was independently transformed with the three tomato gene coding
sequences under the control of the constitutive CaMV 35S promoter. A. thaliana plants were
grown and transformed according to Balazadeh et al. (2010). Plants from three independent
lines of T2 generation were used for dark-induced senescence assay. A pool of 10 detached
leaves from three-month-old plants for each genotype were kept in darkness for seven days.

SIORE1S02-knockdown lines (L1, L3 and L6) were generated by constitutively expressing an
intron-spliced hairpin RNA (RNAi) construct of 186 bp targeted to SIORE1S02
(Solyc02g088180). The fragment was cloned into pK7GWIWG2(I) (Karimi et al, 2002) and
introduced in wild type Solanum lycopersicum (cv Micro Tom) background, which harbours the
wild-type SIGLK2 allele, following the protocol described in Pino et al. (2010). Gas exchange
and chlorophyll fluorescence parameters were measured in the fifth leaf of 70-day-old plants
(YPL) and in the sixth leaf of 120-day-old plants (MPL) between 10 and 12 h and were
subsequently harvested for biochemical analyses and transcriptional profiling. T1 plants were
grown as above described. Pericarp samples from fruits at MG, BR1 and BR6 stages were
harvested. For tocopherol content determination, leaf and pericarp samples were dried by
lyophilization before extraction. For yield evaluation, an independent set of plants without any
destructive sampling was cultivated and all ripe fruits were sampled and weighted until the
plants reach 160-day-old when destructive harvest took place. At harvest time, aerial part was
weighted and harvest index calculated according to HI = (total ripe fruit mass)\*100/(aerial part
mass + total ripe fruit mass). Brix was measured in ripe fruits with a refractometer DR201-95
(Kruss).

**Phylogenetic analysis**

For the phylogenetic analysis, all NAC transcriptions factors of S. lycopersicum and A. thaliana
(Supplemental Table S1) were retrieved from the Plant Transcription Factors Database
(http://planttfdb.cbi.pku.edu.cn/). The retrieved protein sequences were aligned by Clustal in
MEGA 6.0 software (Tamura et al. 2013) using default parameters. The tree reconstruction
from the obtained alignment was performed using PHYML 3.0 algorithm (Guindon et al. 2010) hosted at http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html with the following parameters: LG substitution model, four substitutions rate categories, tree optimization by topology and branch length and, improvement by subtree pruning and regrafting. From the dataset, the proportion of invariable sites, equilibrium of frequencies and gamma shape parameter were estimated. Branches were supported by SH-like support.

qPCR and miRNA analysis

RNA extraction, complementary DNA (cDNA) synthesis, primer design and qPCR assays were performed as described by Quadrana et al. (2013). Primer sequences used are detailed in Supplemental Table S5. Stem loop pulse reverse transcriptase were performed as described previously in Varkonyi-Gasic et al., (2007) and modified 5’RACE were performed as described in Morea et al. (2016). qPCR reactions were carried out in a QuantStudio 6 Flex Real-Time PCR system (Applied Biosystems) using 2X Power SYBR Green Master Mix reagent (Life Technologies) in a 14 µL final volume. Absolute fluorescence data were analysed using the LinRegPCR software package (Ruijter et al. 2009) in order to obtain quantitation cycle (Cq) values and calculate PCR efficiency. Expression values were normalised against the geometric mean of two reference genes, TIP41 and EXPRESSED, according to Quadrana et al. (2013). A permutation test lacking sample distribution assumptions (Pfaffl et al. 2002) was applied to detect statistical differences ($P < 0.05$) in expression ratios using the algorithms in the fgStatistics software package version 17/05/2012 (Di Rienzo 2009).

Subcellular localization and BiFC assay

The full-length open reading frames encoding AtORE1 (AT5G39610), AtGLK2 (AT5G44190), SlGLKs and SlORE1s were amplified using specific primers listed in Supplemental Table S5 and cloned into pCR™ 8/GW/TOPO TA Cloning vector (Invitrogen). For the subcellular localization
experiment, each recombination cassette was amplified using the universal M13 pair of primers and then recombined into the binary vector pK7FWG2 (Karimi et al., 2002) using LR clonase. For BiFC assay, each entry vector was recombined into the binary vector pDEST-VYCE(R)GW or pDEST-VYNE(R)GW (Gehl et al., 2009), which carry the C-terminal and N-terminal fragment of Venus YFP, respectively, using LR Clonase. All BiFC fusion proteins were tagged at the C-terminus. The binary vectors were introduced in Agrobacterium tumefaciens strain GV3101. For subcellular localization, cultures were resuspended in infiltration buffer (50 mM MES pH 5.6, 2 mM sodium phosphate buffer pH 7, 0.5% glucose and 200 µM acetylsyringone (Sigma-Aldrich)) to a final OD600 of 0.5, incubated for 3 h in the dark at room temperature, and infiltrated into leaves of 4-week-old Nicotiana tabacum plants. For BiFC experiments, leaves were co-infiltrated with a mix of both cultures at 0.5 OD600. To avoid SIGLKS degradation, 100 µM of the proteasome inhibitor MG132 (Sigma-Aldrich #C2211) was added to the solution before infiltrating. After 48 or 72 h for localization and BiFC experiments, respectively, the transformed tissues were observed in a confocal laser microscope (Zeiss LSM 780-NLO). DAPI staining was performed by infiltrating 10 µg/mL of water dissolved DAPI (Life Technologies #D1306) 20 min before the confocal microscope observation. GFP signal was captured over a 508-553 nm range after excitation at 488nm, while DAPI fluorescence was excited at 405 nm and captured over a 415-501 nm range.

The controls VYNE-Cnx6 and VYCE-Cnx6 constructs were kindly provided by Prof. Jörg Kudla, Institute of Plant Biology and Biotechnology, Münster, Germany. CNX6 is a protein that belongs to the complex of molybdopterin synthase involved in MoCo biosynthesis. As reported in Gehl et al. (2009), when VYNE-Cnx6 and VYCE-Cnx6 fusion proteins (approximately 40 KDa), are transiently co-expressed in N. benthamiana leaves by agroinfiltration, they localize not only in the cytoplasm but also in the nucleus. This is due to their small size that allows the diffusion through the nuclear pores, which exclusion size is 60 KDa. Thus, Cnx6 auto-
Dimerization was used as positive control, while VYNE-Cnx6/SIGLK2-VYCE and VYCE-Cnx6/SIORE1S02-VYNE interactions were used as negative controls.

**Leaf gas exchange and fluorescence measurements**

Gas exchange and chlorophyll fluorescence parameters were measured using a portable open gas-exchange system incorporating infra-red CO₂ and water vapor analysers (LI-6400XT system; LI-COR) equipped with an integrated modulated chlorophyll fluorometer (LI-6400-40; LI-COR). Reference CO₂ concentration was held at 400 µmol mol⁻¹ and leaf temperature at 28°C for all measurements. Air humidity inside the leaf chamber was equivalent to values measured inside the greenhouse (approximately 75%). Carbon assimilation rate (A), leaf stomatal conductance (gₛ), transpiration (E) and fluorescence parameters were measured at 800 µmol PPFD m⁻² s⁻¹. For the values of minimal (Fo) and maximal (Fm) fluorescence and leaf dark respiration, leaves were dark-adapted for 30 min before a saturating pulse of light. The parameters derived from chlorophyll fluorescence, including dark-adapted PSII maximum quantum efficiency (Fv/Fm), proportion of open PSII centers (photochemical quenching, qP), PSII operating efficiency (Φₚₛᵢᵢ), non-photochemical quenching (NPQ) and electron transport rate (ETR) were calculated according to Maxwell and Johnson (2000).

**Chloroplast number**

Chloroplasts in leaf mesophyll cells were counted following the methodology described in Pyke (2011). Briefly, leaf pieces were fixed in 3.5% (v/v) glutaraldehyde solution for 1 h, then calcium ions were chelated by transferring the leaf samples to 0.1 M NaEDTA solution and keeping them for 4 h at 60°C and then overnight at 4°C. The pieces were transferred to a microscope slide and macerated with a blunt scalpel for tissue desintegration, and visualised by light microscopy.
Transmission electron microscopy

Leaf segments were fixed at 4 °C in Karnovsky's solution [2.5% (v/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.2] for 24 h. After washing in buffer, the samples were post-fixed in buffered 1% (w/v) osmium tetroxide, washed, dehydrated in a graded series of acetone, and embedded in Spurr resin. The resin was polymerized at 60°C. Ultrathin sections were stained with saturated uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and observed using a Zeiss EM 900 transmission electron microscope.

Total soluble proteins, starch and soluble sugars quantification

Total soluble proteins extraction and quantification were performed as described in Jones et al. (1989). Starch and soluble sugars extraction were performed as described in Lira et al. (2014). Soluble sugar quantification was performed according to Mainardi et al. (2006). Briefly, 1 mL of the extract was evaporated under vacuum in a SpeedVac system. The residue was resuspended with 1 mL of ultrapure water and filtered through 0.22 um membrane. Soluble sugars (i.e., glucose, fructose, sucrose) were analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD; Dionex, Sunnyvale, CA, USA), using a Carbopac PA1 (250 x 4 mm, 5 um particle size, Dionex) in an isocratic run with 18 mM NaOH as mobile phase. The content of each sugar was calculated with the standard curves made with pure glucose, fructose and sucrose (Sigma-Aldrich, Co).

Tocopherol and pigment quantification

Tocopherols were extracted from approximately 25 mg dry weight as described in Almeida et al. (2015). The samples were adjusted to 4 mL final volume. Aliquots of 3 mL were dried under N₂ and dissolved in 200 µL of mobile phase composed of hexane/tert-butyl methyl ether (90:10). Chromatography was carried out on a Hewlett-Packard series 1100 HPLC system coupled with a fluorescence detector (Agilent Technologies series 1200) on a normal-phase
column (LiChrosphere® 100 Diol Si; 250 mm x 4.0 mm, 5 µm; Agilent Technologies, Germany) at room temperature, with the mobile phase running isocratically at 1 mL min⁻¹. Eluted compounds were detected by excitation at 295 nm and fluorescence was quantified at 330 nm.

Chlorophyll extraction was carried out as described in Porra et al. (1989). One mL of dimethylformamide (DMF) was added to 100 mg or 300 mg of fresh weight for leaf or fruit samples, respectively. After sonication for five min and further centrifugation of 9000 g for 10 min, the supernatant was collected. The procedure was repeated until total removal of tissue green colour. Spectrophotometer measurements were performed at 664 and 647 nm.

Carotenoid extraction was modified from Sérino et al. (2009). Aliquots of 200 mg of fresh weight were sequentially extracted with a solution of saturated NaCl, dichloromethane and hexane:diethyl ether (1:1). After centrifugation, the supernatant was collected and the last step was repeated three more times. Samples were dried by vacuum and dissolved in 200 µL of acetonitrile. Chromatography was carried out on Agilent Technologies series 1100 HPLC system on a normal-phase column Phenomenex (Luna C18; 250 x 4.6 mm; 5 µm particle diameter) at room temperature with a flow rate of 1 mL min⁻¹. The mobile phase was a gradient of ethyl acetate (A) and acetonitrile:water 9:1 (B): 0-4 min with 20% A/80% B; 4-30 min with 65% A/35% B; 30-35 min with 65% A/35% B; 35-40 min with 20% A/80% B. Eluted compounds were detected between 340-700 nm and quantified at 450 nm.

Data analyses

Differences in parameters were analyzed in Infostat software version 17/06/2015 (Di Rienzo et al. 2011). When the data set showed homoscedasticity, Student’s t-test ($P < 0.05$) was performed to compare transgenic lines against the control genotype. In the absence of homoscedasticity, a non-parametric comparison was performed by applying the Mann-Whitney test ($P < 0.05$). All values represent the mean of at least three biological replicates.
parameter was considered to be affected by SIORE1S02 silencing if at least two out of the three transgenic lines differed significantly from the untransformed genotype.

Supplemental Data

Supplemental Figure S1. Phylogenetic representation of the NAC subfamily of Solanum lycopersicum and Arabidopsis thaliana.

Supplemental Figure S2. Alignment of SIORE1s, AtORE1 and AtORS1.

Supplemental Figure S3. Chlorophyll and senescence marker transcript levels after 48 hours of senescence-inducing treatments.

Supplementary Figure S4. Nuclear subcellular localization of SIGLks and SIORE1s proteins.

Supplemental Figure S5. SIORE1S02 knockdown effectiveness. SIORE1S02 mRNA level in young plant leaves (YPL), mature plant leaves (MPL), mature green fruits (MG), breaker +1 fruits (BR1) and breaker +6 fruits (BR6) of untransformed and SIORE1S02 knockdown plants.

Supplemental Figure S6. SIORE1S02 knockdown effect over SIORE1S03 and SIORE1S06 transcript abundance.

Supplemental Figure S7. Chloroplast ultrastructure of MPL leaves of SIORE1S02-knockdown and MT control genotype.

Supplemental Figure S8. SIORE1S02-knockdown effects on fruit morphology and size.

Supplemental Figure S9. Effect of SIORE1S02 knockdown on fruit development and metabolism.

Supplemental Table S1. NAC sequences accessions used for phylogenetic reconstruction.

Supplemental Table S2. Relative transcript values of genes addressed in leaves of OEmiR164a lines.

Supplemental Table S3. Carotenoid, tocopherol and chlorophyll content in SIORE1S02-knockdown lines.

Supplemental Table S4. Relative transcript values of genes addressed in leaves and along fruit ripening of SIORE1S02-knockdown lines.

Supplemental Table S5. Primers used in the experiments.
**Supplemental Figure S1.** Phylogenetic representation of the NAC subfamily of *Solanum lycopersicum* and *Arabidopsis thaliana*. Phylogenetic reconstruction obtained from the alignment of all NAC transcription factors of *A. thaliana* and *S. lycopersicum* obtained from the Plant Transcription Factor Database. Three putative orthologs (in blue) were chosen, namely SIORE1S02, SIORE1S03 and SIORE1S06 accordingly to the chromosome position. Marked with green or red circles are the functionally characterised proteins from *A. thaliana* or *S. lycopersicum*, respectively.

**Supplemental Figure S2.** Alignment of SIORE1s, AtORE1 and AtORS1. The subdomains A-E of the NAC domain are underlined. Shading threshold = 100%.

**Supplemental Figure S3.** Chlorophyll and senescence marker transcript levels after 48 hours of senescence-inducing treatments. Images of the leaves after 48 hours of senescence-inducing treatments. Heat map indicating the relative transcript ratio of *SlGLK1* (Solyc07g053630) and *SlSAG12* (Solyc02g076910) normalized against values from samples harvested 0h and 48h after ethylene treatment, respectively. Values represent the mean from at least three biological replicates. Black colour = not detected. Statistically significant differences compared to the corresponding controls (*P* < 0.05) are coloured accordingly to the scale. Chlorophyll content in leaf samples. Values represent the mean ± SE from at least three biological replicates. Statistically significant differences compared to not-treated control are marked with asterisks (*P* < 0.05).

**Supplementary Figure S4.** Nuclear subcellular localization of SIGLKs and SIORE1s proteins. GFP fusion proteins were transiently expressed in *Nicotiana tabacum* leaves by infiltration with *Agrobacterium tumefaciens*. GFP, DAPI nuclear marker, bright-field and merged signals are indicated above the panels. Bars, 20 μm.

**Supplemental Figure S5.** *SIORE1S02* knockdown effectiveness. *SIORE1S02* mRNA level in young plant leaves (YPL), mature plant leaves (MPL), mature green fruits (MG), breaker +1 fruits (BR1) and breaker +6 fruits (BR6) of untransformed and *SIORE1S02* knockdown plants. Value mean ± SE of at least three biological replicates normalised against the respective sample from MT control genotype. Asterisks denote statistically significant differences between MT control and the transgenic lines (*P* < 0.05).

**Supplemental Figure S6.** *SIORE1S02* knockdown effect over *SIORE1S03* and *SIORE1S06* transcript abundance. Top: *SIORE1S03* and *SIORE1S06* mRNA level in young plant leaves (YPL) and mature plant leaves (MPL) from control genotype (MT) and *SIORE1S02*-knockdown plants. Value mean ± SE of at least three biological replicates normalised against the respective sample from MT control genotype. Asterisks denote statistically significant differences between MT control and the transgenic lines (*P* < 0.05). Bottom: detail of the alignment of the
coding sequence of SlOREs, the blue boxes highlight the region of SlORE1S02 targeted by the
RNAi construct. Each coloured arrow pair indicates the primers used for qPCR analysis.
Threshold for shading = 100%.

Supplemental Figure S7. Chloroplast ultrastructure of MPL leaves of SlORE1S02-knockdown
and MT control genotype. PG, plastoglobulus; G, granum.

Supplemental Figure S8. SlORE1S02-knockdown effects on fruit morphology and size.
Morphology, diameter and weight of breaker+6 fruits from MT and SlORE1S02-knockdown
genotypes. Values represent the mean ± SE from at least forty four biological replicates.
Asterisks denote statistically significant differences to the MT control genotype (P < 0.05). Bar
= 0.7 cm.

Supplemental Figure S9. Effect of SlORE1S02 knockdown on fruit development and
metabolism. A, Number of days from anthesis to reach breaker stage. Value mean ± SE of at
least ten biological replicates. Asterisk denote statistically significant differences compared to
the corresponding sample from MT control (P < 0.05). B, Total soluble protein (TSP) of mature
green fruits (MG), breaker +1 fruits (BR1) and breaker +6 fruits (BR6) from MT control
genotype and SlORE1S02-knockdown plants. Value mean ± SE of at least three biological
replicates. Asterisk denote statistically significant values compared to the corresponding
sample from MT control (P < 0.05).

Supplemental Table S1. NAC sequences accessions used for phylogenetic reconstruction.

Supplemental Table S2. Relative transcript values of genes addressed in leaves of OEmiR164a
lines.

Supplemental Table S3. Carotenoid, tocopherol and chlorophyll content in SlORE1S02-
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Supplemental Table S5. Primers used in the experiments.

Acknowledgments

The authors thank Prof. Dr. Javier Palatnik from IBR, CONICET, Argentina, for providing the
p35S:AtMiR164a construct and Dr. Tania Misuzu Shiga from FCF, USP, Brazil, for the
assistance in soluble sugar quantification.
Table 1. Photosynthetic parameters of SIORE1S02-knockdown lines.

<table>
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<tr>
<th>Genotype</th>
<th>CO₂ assimilation (A) (μmol CO₂/m² s)</th>
<th>Electron transport rate (ETR) (μmol/m² s)</th>
<th>Photochemical quenching (qP)</th>
<th>Non-photochemical quenching (NPQ)</th>
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<tr>
<td>MT</td>
<td>4.31 ± 0.21</td>
<td>65.80 ± 3.16</td>
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<td>1.69 ± 0.21</td>
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<td>MPL</td>
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<td>35.93 ± 3.90</td>
<td>0.18 ± 0.02</td>
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<tr>
<td>L1</td>
<td>4.63 ± 0.64</td>
<td>75.51 ± 7.47</td>
<td>0.30 ± 0.03</td>
<td>1.57 ± 0.12</td>
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<tr>
<td>MPL</td>
<td>5.93 ± 0.84</td>
<td>49.91 ± 4.38</td>
<td><strong>0.24 ± 0.02</strong></td>
<td>2.13 ± 0.04</td>
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<tr>
<td>L3</td>
<td>8.55 ± 0.95</td>
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<td>2.23 ± 0.34</td>
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<tr>
<td>MPL</td>
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<tr>
<td>L6</td>
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<tr>
<td>MPL</td>
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<td>38.68 ± 2.98</td>
<td>0.21 ± 0.01</td>
<td>1.76 ± 0.18</td>
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Values represent mean ± SD of at least three biological replicates. Statistically significant differences to MT control are in bold.
Figure Legends

Figure 1. Phylogenetic representation of the NAC transcription factor subfamily that includes AtORE1 and SlORE1s. Detail of the phylogenetic reconstruction obtained from the alignment of the protein sequence of all NAC transcription factors of *Arabidopsis thaliana* (green) and *Solanum lycopersicum* (red) obtained from the Plant Transcription Factor Database (Supplemental Table 1). Three putative AtORE1 orthologs (in bold), named SlORE1S02, SlORE1S03 and SlORE1S06 according to their chromosome position, were identified.

Figure 2. Transcript profile of *SlORE1s* in response to senescence-inducing treatments. Transcript profile of *SlORE1s* in leaves of 4-week-old *in vitro*-grown plants after senescence-inducing treatments. Values represent the mean ± SE from at least three biological replicates normalized against the zero hour sample. Each treatment relative transcript ratio is expressed as the ratio between treatment value and the corresponding untreated control (*). Statistically significant differences relative to the zero hour treatment are represented with closed symbols (*P* < 0.05).

Figure 3. Transcript profile of *SlORE1s* along fruit development and ripening. IG: immature green stage; MG: mature green stage; BR: breaker stage; BRX: X days after breaker stage. Values represent the mean ± SE from at least three biological replicates normalized to the corresponding IG3 sample. Statistically significant differences are represented with letters (*P* < 0.05).

Figure 4. Regulation of *SlORE1s* by *SlmiR164*. A, Highlight of the alignment of *SlORE1s* and *SlmiR164* showing the putative binding site. The validation by a modified 5' RACE assay identified cleaved transcripts of *SlORE1S03* and *SlORE1S06*. The red arrowhead and numbers on the right indicate the inferred cleavage site and the fraction of positive cloned PCR products ending at the site, respectively. Shading threshold of alignment = 75%. B, Heatmap representing the transcript profile of non-senescent (NS), early senescing (ES) and late senescing (LS) leaves of wild type control (WT) and *OEmiR164a* (lines miR_2 and miR_3) genotype. Values represent mean of at least three biological replicates normalized against the NS WT sample. Statistically significant differences in comparison to NS WT are represented as coloured squares (*P* < 0.05). The relative transcript values are detailed in Supplemental Table S2.

Figure 5. Analysis of SIGLks and *SlORE1s* protein-protein interaction by Bimolecular Fluorescent Complementation (BiFC). VYNE and VYCE fusion proteins were transiently expressed in *N. tabacum* leaves by infiltration with *A. tumefaciens*. VYNE-Cnx6/VYCE-Cnx6 and AtORE1/AtGLK2 (Gehl et al., 2009) were used as technical and biological positive controls, respectively. VYNE-Cnx6/SIGL2-VYCE and VYCE-Cnx6/SIORE1S02-VYNE interactions were used as negative controls. SIGLks/SIORE1s interaction was confirmed as evidenced by the YFP fluorescence detected. YFP, DAPI nuclear marker, bright-field and merged signals are indicated above the panels. Bars = 20 μm, except in VYNE-Cnx6/VYCE-Cnx6 images = 40 μm.

Figure 6. *SlORE1s* partially recover *Atore1* senescence impairment. Detached leaves from three-month-old Col-0, *Atore1* mutant, *Atore1-OE:SlORE1S02, Atore1-OE:SlORE1S03* and *Atore1-OE:SlORE1S06* plants were kept in darkness for seven days to induce senescence. While *Atore1* displayed no signs of senescence, the yellowish colour of the genotypes overexpressing *SlORE1s* hints an ongoing senescence program, yet not to the extent observed in the Col-0 genotype. Bar = 1 cm.
**Figure 7.** SIROS1S02-knockdown affects leaf aging. **A,** Chlorophyll content in leaves. Values represent the mean ± SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control (P < 0.05). YPL: young plant leaves; MPL: mature plant leaves. **B,** Number of chloroplasts per mesophyll cell of MPL. Values represent the mean ± SE from at least 45 cells. Asterisks denote statistically significant differences to MT control (P < 0.05). **C,** S1GLK1 and S1SAG12 transcript ratio in YPL and MPL leaves. Values represent the mean ± SE from at least three biological replicates normalised against the respective sample from MT control. Asterisks denote statistically significant differences to MT control (P < 0.05). The relative transcript values are available in Supplemental Table S4. **D,** SIROS1S02-knockdown effect on dark-induced senescence in tomato leaves. Detached leaflets from 120-day-old plants kept ten days in darkness retain greenness, while MT control leaflets turned yellow. Bar = 5 cm.

**Figure 8.** SIROS1S02-knockdown increases yield and brix. **A,** Harvest index, aerial part weight and ripe fruit number in MT and SIROS1S02-knockdown lines. Values represent the mean ± SE from at least six biological replicates. Asterisks denote statistically significant differences compared to MT control genotype (P < 0.05). **B,** Total soluble solids of ripe fruits measured in brix units. Values represent the mean ± SE from at least twelve biological replicates. Asterisks denote statistically significant differences compared to MT control genotype (P < 0.05).

**Figure 9.** SIROS1S02-knockdown alters sugar profile in leaves and fruits. Content of starch and soluble sugars in leaves and fruits of SIROS1S02-knockdown lines. Values represent the mean ± SE from at least three biological replicates. Asterisks denote statistically significant differences to MT control genotype (P < 0.05). ND: Not detected. YPL, young plant leaves; MPL, mature plant leaves; MG, mature green stage; BR, breaker; BR6: 6 days after BR, respectively.

**Figure 10.** Isoprenoid metabolism. Schematic representation of the interconnection between chlorophyll synthesis and degradation (green), carotenogenesis (orange) and tocopherol biosynthetic (blue) pathways. Dotted lines denote that intermediate steps were omitted. The heatmap represents statistically significant differences in relative transcript and metabolite amounts detected in SIROS1S02-knockdown lines compared to the corresponding organ of MT control genotype (P < 0.05). For simplicity, the mean of the three transgenic line values is represented when, at least, two were statistically significant different than MT control. The absolute metabolite and relative transcript values are detailed in Supplemental Table S3 and S4. Black and grey colours indicate that transcripts were not detected or not addressed, respectively. Enzymes and compounds are named according to the following abbreviations: GGDR, geranylgeranyl diphosphate reductase; PSY, phytoene synthase; PDS, phytoene desaturase; LCYβ, chloroplast-specific β-lycopene cyclase; CYCβ, chromoplast-specific β-lycopene cyclase; GGDP, geranylgeranyl diphosphate; PDP, phytyl diphosphate; PP, phytyl phosphate; CHLG, chlorophyll synthase; PPH, pheophytinase; PPHL1, pheophytinase-like 1; pFCC, primary fluorescent chlorophyll catabolite; VTE5, phytol kinase; VTE6, phytol phosphate kinase; MPBQ, 2-methyl-6-geranylgeranylbenzoquinol; VTE1, tocopherol cyclase; VTE2, homogentisate phytol transferase; VTE3, 2,3-dimethyl-5-phytylquinol methyltransferase; VTE4, tocopherol C-methyl transferase; MEP, methylerthritol 4-phosphate. Adapted from Almeida et al. (2016).


He XJ, Mu RL, Cao WH, Zhang ZG, Zhang JS, Chen SY (2005) AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. The Plant Journal, 44: 903-916.


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