Two *Nucleoporin98* homologous genes jointly participate in the regulation of starch degradation to repress senescence in *Arabidopsis*

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Abstract

Background: Starch is synthesized during the day for temporary storage in leaves and then degraded during the subsequent night to support plant growth and development. Impairment of starch degradation leads to stunted growth, even senescence and death. The nuclear pore complex is involved in many cellular processes, but its relationship with starch degradation is unclear until now. We previously identified that two *Nucleoporin98* (*Nup98*) genes (*Nup98a* and *Nup98b*) redundantly regulate flowering through *CONSTANS* (*CO*) independent pathway in *Arabidopsis thaliana*. The *nup98a-1 nup98b-1* double mutant also shows severe senescence phenotypes.

Results: We find that *Nucleoporin 98* (*Nup98*) participates in the regulation of sugar metabolism in leaves and in turn involves in senescence regulation in *Arabidopsis*. We show that *Nup98a* and *Nup98b* redundantly function in the different steps of starch degradation and the *nup98a-1 nup98b-1* double mutant accumulates more starch than wild type plants and has a severe early senescence phenotype compared to wild type plants. The expression of marker genes related to starch degradation is impaired in the *nup98a-1 nup98b-1* double mutant, and marker genes of carbon starvation and senescence express earlier and in higher abundance in the *nup98a-1 nup98b-1* double mutant than in wild type plants, suggesting that abnormality of energy metabolism is the main cause of senescence of the *nup98a-1 nup98b-1* double mutant. Addition of sucrose to the growth medium rescues early senescence phenotypes of the *nup98a-1 nup98b-1* mutant.

Conclusions: Our results provide a line of evidence on a novel role of the nuclear pore complex in energy metabolism related to growth and development, in which *Nup98* functions in starch degradation conferring growth regulation in *Arabidopsis*.

Background

The nuclear pore complex (NPC) is the key bridge for communication of macromolecules between the nucleus and cytoplasm and regulates gene expression [1]. NPC is built by at least 30 unique nucleoporins (Nups), which are highly conserved in eukaryotic cells [2, 3]. Nup98 is a mobile and peripheral Phe-Gly domain (FG) nucleoporin and spans from the nucleus to cytoplasmic of the central channel of the NPC [4, 5]. *Arabidopsis thaliana* Nup98a (also known as DRACULA2, DRA2) is also found in different subcellular locations [6]. *Nup98* is involved in regulation of cargo export and import, gene expression, transcriptional memory, and multiple developmental processes in animals and yeast [4-9]. In *Arabidopsis*, *DRA2* regulates the shade avoidance syndrome [6]. In rice, the *Nup98* homolog *APIP12* (*AVRPIZ-T INTERACTING PROTEIN12*) is involved in basal resistance against the pathogen *Magnaporthe oryzae* and targeted by the *Magnaporthe* effector AvrPiz-t [10]. In a recent report in *Arabidopsis*, the protein-interaction between Nup98 and Nup88/MOS7 (MODIFIER OF SNC1,7) is required for plant immunity against the necrotrophic fungal pathogen *Botrytis cinerea* and mitogen-activated protein kinase signalling [11]. We found that *Nup98* contributes to flowering regulation in *Arabidopsis* [12].

Senescence is an important cellular process sensing developmental and environmental cues [13]. Before death, plants remobilize resources in senescing organs and translocate them to sink organs to support their growth and development [14-16]. To date, at least 200 genes have been identified that regulate or participate in senescence process in plants. Starch is synthesized during the day in leaves and then degraded during the subsequent night to support plant growth and development. Impairment of genes functioning in different steps of starch degradation hinders plant growth in different extents [17, 18]. Sugar directly or indirectly regulates senescence: sugar accumulation not only triggers and accelerates, but also delays senescence [19-21]. In fact, in response to sugar, senescence process in plants is in an age- or condition-dependent manner [20, 22, 23]. Within the sugar pathway, three key genes have been identified: glucose sensor *HEXOKINASE 1* (*HXK*1), energy sensor *PROTEIN KINASE10* (*KIN10*) and *KIN11*, and *TOR* (*THE TARGET OF RAPAMYCIN*) [21, 24-27].

Currently, the mechanism of NPC in regulation of starch degradation and senescence in plants is still unknown. However, in animals there are several studies reporting the NPC controls cell senescence through modifying chromosome structure, DNA repair and replication or cell division [28-32]. Impairment of NPCs results in dysfunction of nucleo-cytoplasm transportation [30]. Both *Nup107* [33] and *Tpr* (*TRANSLOCATED PROMOTER REGION*) [34, 35] have been linked to cancer cell proliferation and cellular senescence in aging cells..

In this study, we focused on two *Nup98* homologous genes, *Nup98a* and *Nup98b,* in *Arabidopsis thaliana*. Single mutants of *Arabidopsis*, either *nup98a* or *nup98b*, have no obvious flowering and senescence phenotypes. However, the double mutant, *nup98a-1 nup98b-1*, show significant early-senescence phenotypes. Gene expression analysis demonstrates that *Nup98a* and *Nup98b* participate in starch degradation conferring growth regulation. Further analysis suggeste that the early-senescence in the *nup98a-1 nup98b-1* double mutant may result from a defect in the initial steps of starch degradation and resulte in dysfunction in energy supply. Interestingly, the early senescence phenotype in this double mutant could be rescued by addition of sugar in the growth medium. Our data suggests that *Nup98a* and *Nup98b* might redundantly function in regulation of starch degradation and contribute normal growth and development in *Arabidopsis*.

Results

Nup98 mutation results in early senescence in *Arabidopsis thaliana*

Nup98 is a highly conserved nuclear pore protein in eukaryotes. In *Arabidopsis thaliana*, there are two homologs of the mammalian *Nucleoporin98* in *Arabidopsis*, *Nup98a* (At1g10390) and *Nup98b* (At1g59660), and they share highly conserved amino acid sequences in the Phe-Gly (FG)-repeats and autoproteolytic domain (APD, Supplementary Fig. 1) [3, 6]. *Nup98a* was previously reported as *DRACULA2* (*DRA2*), a regulator of the shade avoidance syndrome (SAS) in *Arabidopsis* [6] and immune responses to a rice fungal pathogen [11]. To investigate *Nup98* functions in plant development, we screened mutants of *nup98a* (SALK_080083, SALK_090744, SALK_023493, SALK_103803, SALK_015016) and *nup98b*

(CS803848 and GABI_288A08) ordered from ABRC and GABI T-DNA mutant center, respectively. Homozygous lines were isolated for the following insertional mutants of SALK_103803, SALK_015016, GABI_288A08, and among them SALK_103803 and GABI_288A08 were the mutants reported by Parry [9]. The T-DNAs in these homozygous mutants are inserted in coding regions (Fig. 1A) and RT-PCR results demonstrated that these mutants were null alleles (Fig. 1B), consistent with the Parry's results [9]. We did not observe any obvious phenotypes in flowering and senescence in either the *nup98a* or *nup98b* single mutants compared to wild type plants under long-day photoperiod conditions (Fig. 1C), as previous studies showed [9]. As Nup98a and Nup98b share high conserved amino acid sequences (Supplementary Fig. 1), we tested the hypothesis that *Nup98a* and *Nup98b* functioned redundantly within the *nup98a nup98b* double mutants, which made by crossing the *nup98a* to *nup98b* single mutants. Strikingly, both double mutants, *nup98a-1 nup98b-1* and *nup98a-2 nup98b-1*, displayed similar early senescence phenotypes compared to wild type plants (Fig. 1C and 1D). The senescence phenotype appeared not only on leaves, but also in the whole plant. Also, the double mutant plants had additional phenotypes, such as smaller inflorescences, flowers and siliques, short stature, and severe sterility compared to wild type plants (Supplementary Fig. 2 and 3). We recently reported that the *nup98a-1 nup98b-1* double mutants has early flowering phenotype [12]. When expressing the *Nup98b* gene in the double mutant, the senescence phenotypes were rescued (Fig. 1E). Our results demonstrate that *Nup98a* and *Nup98b* act redundantly.

To investigate the senescence phenotype is specific to the *nup98a-1 nup98b-1* mutant, we selected another three nucleoporin mutants, *nup96-1*, *nup160-1*, and *nup107-1*, which show flowering phenotypes in our previous report [36], to analyze the effect of other nuclear pore components on senescence. To our surprise, there is no early senescence phenotypes observed in these mutants (Supplementary Fig. 4), suggesting that some of nucleoporins may not be involved in the regulation of senescence and that *Nup98* likely has specific functions on senescence regulation.

Nup98 gene involves in multiple pathways of senescence initiation

Because the early senescence observed in the *nup98a-1 nup98b-1* double mutant could be a secondary effect of altered development, we further explored the role of *Nup98a* and *Nup98b* in senescence regulation. To date, at least 200 genes have been identified to participate in senescence regulation in plants [37]. We summarized main literatures as shown in Supplementary Fig. 5, which shows that various endogenous and environmental cues, such as hormones, sugar, light and photoperiod, and stresses, trigger plant senescence in multiple cross-talking patterns. To identify the potential link to early senescence, we measured mRNA abundance of important senescence-associated genes in the *nup98a-1 nup98b-1* double mutant plants (Supplementary Fig. 5) at ZT0 (Zeitgeber 0, the time when light turned on) and ZT16 (the time when light turned off) in plants grown under long day conditions by real time quantitative PCR (RT-qPCR). As shown in Fig. 2, the transcript abundance of many genes tested in the *nup98a-1 nup98b-1* mutant was obviously different from that in wild type plants. In the first category, *WRKY53* (*WRKY DNA-BINDING PROTEIN53*), *SAG13* (*SENESCENCE-ASSOCIATED GENE13*), *WRKY6*, *NAC1* (*NAM, ATAF, and CUC*) and *NAC2* displayed higher transcript abundances at both ZT0 and ZT16 (Fig. 2A),

suggesting that genes in stress pathway (*WRKY53*, *WRKY6*, and *NAC1*) and SA pathway (*WRKY53*, *WRKY6*, and *NAC2*) were related to the senescence phenotypes of the *nup98a-1 nup98b-1* double mutant. In the second category, *SAG12*, *NAP* (*NAC DOMAIN CONTAINING PROTEIN*), *SAG2* and *CAT1* (*CATALASE1*), also genes in stress and SA pathways, were only increased at either ZT0 or ZT16 (Fig. 2B). In contrast, in the third category, *SAUR36* (*SMALL AUXIN UPREGULATED36*), *WRKY70*, *ARP4* (*ACTIN-RELATED PROTEIN4*), *SEN1* (*SPLICING ENDONUCLEASE1*), and *COI1* (*CORONATINE INSENSITIVE1*) were decreased in abundance at either ZT0 and/or ZT16 (Fig. 2C), indicating that auxin (*SAUR36*) and jasmonate (*COI1*) signalling may be negatively related to the *nup98* senescence phenotypes. The abundance of *AGL15* (*AGAMOUS-LIKE15*), *EBP1* (*ERBB-3 BINDING PROTEIN1*), *RPS6a* (*RIBOSOMAL PROTEIN6a*), and *NPR1* (*NONEXPRESSOR OF PR-GENES1*) had opposite changes at ZT0 and ZT16 (Fig. 2D), suggesting that the function of these genes on *Nup98* senescence regulation may be dependent on circadian clock.

This expression profile suggests that there are at least three characteristics of senescence processes in the *nup98* double mutant. Firstly, several pathways, mainly stress and SA pathways, were involved in regulation of senescence processes in the *nup98a-1 nup98b-1* double mutant. Secondly, different genes functioned in their own special modes, positively or negatively at different phases (morning or afternoon phases). Thirdly, some of genes, such as *WRKY53*, *WRKY6*, *NAP1*, and *SAG2*, may play roles in multiple pathways. The results indicate that the senescence phenotypes of the *nup98* double mutant resulted from multiple pathways. Many genes showed circadian expression patterns, consistent with our previous report that *Nup98* genes participate in regulation circadian clock [12].

Starch metabolism is impaired in the *nup98a-1 nup98b-1* double mutant

During photosynthesis, starch is synthesized and stored in chloroplast, and then degraded at night. A number of genes are involved in starch degradation in plants (Supplementary Fig. 6) [17, 18]. Firstly, we checked starch homeostasis in leaves of the *nup98a-1 nup98b-1* double mutant plants grown under 12 h light/12 h dark conditions (at ZT0, dawn, and ZT12, dusk) (Fig. 3). Leaves of all plants accumulated starch at dusk, however, double mutant plants had much more starch than wild type plants as determined by both iodine staining (Fig. 3A) and starch quantitative assay (Fig. 3B). The more intense signals in older, 21 and 28-day, leaves in the double mutant may be a consequence of accumulating starch over time. Therefore, the double mutant displayed much more starch not only at dawn but also at dusk than wild type plants, and this phenotype was much clearer in 14 and 21-day old seedlings (Fig. 3A and 3B). Together, these results suggest that starch metabolism is impaired in the *nup98a-1 nup98b-1* double mutant.

We measured the transcript abundance of genes involved in starch metabolism [17] by RT-qPCR (Fig. 4) and found that many genes had significantly lower abundance in the double mutant at least at one time point compared to wild type plants. These genes encode enzymes for the degrading process of starch not only in the early steps in chloroplasts [17], such as *GWD1* (*α-GLUCAN WATER, DIKINASE1*; or *STARCH EXCESS1*, *SEX1*), *β-BAM1* (*β-AMYLASE1*), *BAM3*, *BAM5*, *BAM6*, *BAM7*, *BAM8*, *SEX4*, and *LSF1* (*LIKE SEX*

FOUR1), but also in the later steps, such as *LDA* (*LIMIT-DEXTRINASE*), *AMY1* (*α-AMYLASE*) and *AMY2*, *DPE1* (*DISPROPORTIONING ENZYME*) and *DPE2*, *PHS1* (*α-GLUCAN PHOSPHORYLASE*) and *PHS2*. Time-dependent low-expression of these genes suggest that they are under the control of circadian clock, since *Nup98* is involved in circadian regulation in *Arabidopsis* [12]. These genes function at different steps of starch degradation [17]. GWD1 is α-glucan water dikinase, which phosphorylates glucosyl residues of amylopectin at the C-6 position. BAMs are a family of *β*-amylase breaking down the *α*-1,4-linked glucose chains. LSF1 and SEX4 release the phosphate bound at C-6 and C-3 of glucosyl residues. Both ISA (ISOAMYLASE 3) and LDA (LIMIT-DEXTRINASE) hydrolyze *α*-1,6 branch points but have different substrates. AMYs act on *α*-1,4-linkages releasing linear *α*-1,4-linked oligosaccharides and branching *α*-1,4- and *α*-1,6-linked oligosaccharides. DPEs (DISPROPORTIONATING ENZYMEs) transfer glucose/α-1,4-linked glucan moiety from a donor glucan to an acceptor, releasing the non-reducing end of glucose/glucan moiety. PHSs (α–GLUCAN PHOSPHORYLASEs) act on the non-reducing end of *α*-1,4-linked glucose. Lower abundance of these genes resulted in a starch-excess phenotype in the *nup98a-1 nup98b-1* double mutant (Fig. 3) as these gene mutants [17]. The mRNA abundance of some other genes involved in starch metabolism, including *GWD2*, *GWD3*, *ISA3* (*ISOAMYLASE3*) and *AMY3*, was not significantly affected, suggesting the effect of *Nup98a/b* on starch metabolism was limited to some specific enzymes.

We also measured the mRNA abundance of genes related to photosynthesis and sugar metabolism by RT-qPCR in the *nup98a-1 nup98b-1* double mutant and wild type plants (Supplementary Fig. 7). In terms of photosynthesis related genes, the decrease of mRNA abundance of *LHCA* (*PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE A*) and *LHCB* (*PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE B*) was observed in the *nup98a-1 nup98b-1* double mutant at different time points, e.g., *LHCA1/2* and *LHCB1.1* at ZT0 and *LHCA1* and *LHCB1.4* genes at ZT16. We also observed the decrease of mRNA abundances of *KIN10* and *KIN11*, which is expected since both of genes are sugar signaling genes, in the double mutant compared to wild type plants. Both genes are involved in delaying plant senescence [21, 26, 27], therefore the reduced mRNA abundance may be associated with earlier senescence (Supplementary Fig. 5). We also observed slightly-increased mRNA abundance of *HXK1* at dusk (ZT16), that may contribute to earlier senescence via the cytokinin signaling pathway [24]. Unexpectedly, mRNA abundance of *TPS1,* a senescence activator [20], was reduced in the *nup98a-1 nup98b-1* double mutant compared to wild type plants, suggesting that T6P (trehalose-6-phosphate) was not related to senescence of the *nup98a-1 nup98b-1* double mutant. The results indicate that starch synthesis and sugar signalling are impaired in the double mutant.

Exogenous sugar rescues the early senescence in the *nup98a-1 nup98b-1* double mutant

Based on our results, we interpreted that the carbon or energy supply was impaired in *nup98a-1 nup98b-1* double mutant plants. We tested the idea by supplying exogenous carbon in the form of sucrose in growing medium to see if the early senescence phenotype in the *nup98a-1 nup98b-1* double mutant plants could be rescued. Our results showed that sucrose and MS basal nutrients could support the double mutant plants growing well even though they were weak compared to wild type plants (Fig. 5).

Both plants completed their life cycles on medium containing agarose supplement with sucrose and basal nutrients. Then, we allowed double mutant and control plants to grow in MS medium until inflorescence emergence and then transferred them to soil. As expected, the double mutant grew well as wild type plants did on MS medium before transplanting (Supplementary Fig. 8). However, after transferring to soil, senescence symptoms on mutant plants' leaves quickly appeared at day 6, and the mutant plants wilted at day 30 (Supplementary Fig. 8). If seeds were sown on agarose medium plus either MS basal nutrients or sucrose, both wild type plants and the nup98 double mutant would not survive as plants growing on agarose medium without any supplements (Supplementary Fig. 9).

To rule out the potential effect of soil on senescence phenotypes observed above, we carried out another experiment to test if exogenous macro and micronutrients would complement the phenotypes observed in the mutant by continuously growing plants on medium at different strengths of sucrose and macro- and micro-nutrients (Supplementary Fig. 9). To our surprise, not only did sucrose suppress the early senescence phenotype in the double mutant but also macro- and micro-nutrients in the presence of sucrose. The lower strength basal nutrients (½ MS) enhanced the lower sucrose (1.5%) effect on suppressing senescence, suggesting that both energy supply and basal nutrients metabolism are impaired in the *nup98a-1 nup98b-1* double mutant.

Starvation and senescence marker genes express highly in the *nup98a-1 nup98b-1* double mutant

Results above imply that the *nup98a-1 nup98b-1* double mutant may suffer from sugar starvation and senescence. Next, we asked if markers genes of starvation or senescence expressed highly in the *nup98a-1 nup98b-1* double mutant. *DORMANCY-ASSOCIATED PROTEIN-LIKE 1* (*DRM1/DYL1*, At1g28330) and *DARK INDUCIBLE 6* (*DIN6*, At3g47340) [38, 39] are two well-studied sugar starvation marker genes, whereas *SAG12* (At5g45890) and *WRKY53* (At4g23810) are well-characterized senescence markers [40-42]. Autophagy is an important event occurring during sugar starvation and senescence [38, 43, 44], and *AUTOPHAGY8a* (*ATG8a*, At4g21980) and *ATG8e* (At2g45170) are two typical molecular indicators for autophagy in plants [45]. Therefore, we investigated expression changes of these genes in the *nup98a-1 nup98b-1* double mutant compared to that in wild type plants, and the results showed that they had different changes in a time- and developmental-dependent mode (Fig. 6). In the double mutant, *DRM1* had significantly higher expression at ZT0, but lower at ZT12 from very early stage (day 5 after germination) (Fig. 6A). *DIN* is a light-repressed and dark-induced gene [46], and its high level of expression at ZT0 in the *nup98a-1 nup98b-1* double mutant became obvious at day 15, but at ZT12 higher abundancy appeared earlier from day 10 (Fig. 6A). Compared to wild type plants, the senescence marker *WARKY53* in the *nup98a-1 nup98b-1* double mutant expressed higher at the early stage when *DRM1* expression was in disorder (day 5) (Fig. 6B). *SAG12* is a developmentally-controlled indicator for the later stage of senescence [47, 48]. We found that there was no much difference of *SAG12* expression in the early stage (day 5) between the *nup98a-1 nup98b-1* double mutant and wild type plants. However, *SAG12* had a higher expression level in the *nup98a-1 nup98b-1* double mutant at both ZT0 and ZT12 from day 10 (Fig. 6B). In the meanwhile, the two markers of autophagy, *ATG8a* and *ATG8e*, also had higher abundancy of mRNA in most of samples of the double mutant from day 10. Token together, our

results showed that the *nup98a-1 nup98b-1* double mutant appeared the sign of energy starvation, at least at molecular level, in early developmental stage when plants did not display visible senescence phenotypes. And these expression changes had circadian and developmental characters. A previous report shows that different sugars (such as sucrose, glucose, and fructose) have different effects on the regulation of senescence [39]. Therefore, these earlier and higher expression levels of these marker genes were consistent with the senescence phenotype of the *nup98a-1 nup98b-1* double mutant.

Nup98 proteins mainly localize to both the nuclear membrane and nucleoplasm

Nup98 is one of the mobile and peripheral FG (Phe-Gly domain) nucleoporins and locates at both the nuclear and cytoplasmic sides of the NPC central channel [4, 5]. *Arabidopsis* Nup98a (also known as DRA2) is also found distributing in different subcellular compartments [6]. We constructed transgenic *Arabidopsis* plants expressing *35S::GFP:Nup98a* and *35S::GFP:Nup98b* and analyzed the subcellular localization of both translation fusion proteins. Not surprisingly, both proteins were distributed in the cytoplasm, the nucleoplasm and at the nuclear periphery (Fig. 7). We also observed no significant difference in the subcellular distribution of Nup98a and Nup98b and this is consistent with our observations of their genetic redundancy. In conclusion, our combined results demonstrate that Nup98b proteins localize at both the nucleus and cytoplasm as Nup98a proteins [6] and their homologs in other organisms do [4, 5].

Discussion

Senescence is a process integral to plant growth and development during the plant life cycle, eventually leading to cell and tissue disintegration and death. In such a physiological process, various basal nutrients are redistributed from senescing organs, such as leaves, to reproductive organs as seeds [13]. However, premature senescence could lead to organ failure or even whole plant death [49]. Fine tuning senescence could benefit plants by avoiding the deleterious effect of abiotic stresses and thereby lead to an optimal reproductive outcome. A number of factors, including hormones, developmental age, abiotic stress and light, participate in regulation of plant senescence [21, 22, 50-59]. While these factors play important and clear roles in plant senescence, the role of sugar is unclear as different research groups have published contradictory results [19-21, 60]. The NPC is an important gatekeeper for both macromolecular transportation between the nucleus and cytoplasm and gene transcription, and therefore plays an important role in different developmental processes in plants [28-32]. Our study provides some additional insights into the plant senescence research as we found that the NPC participated in senescence regulation. Our investigation confirmed that *Nup98* genes involved in starch degradation, conferring senescence initiation in *Arabidopsis*.

Obviously, *Nup98a* and *Nup98b* function redundantly in senescence regulation in *Arabidopsis*, because the *nup98* single mutants have no obvious senescence phenotypes (Fig. 1), even though a previous report showing longer hypocotyls in the *nup98a* mutant [6]. Similar protein sequences (Fig. S1) and subcellular-localization between the Nup98a and Nup98b proteins (Fig. 7) support this hypothesis. Such a

senescence phenotype is likely specific to mutation of *Nup98* genes, because other nucleoporin mutants detected have no similar phenotypes (Fig. S4). However, it is important to check more mutants of other nucleoporins for elucidating the specificity of *Nup98* functions on senescence regulation. Molecular data support that the *Nup98* genes regulate senescence through multiple pathways, including ethylene, salicylic acid, ABA, cytokinin, and stress pathways, because the marker genes in these pathways, such as *SAG12*, *NAP1*, *WRAY53*, *WRKY6*, *WRKY70*, *NAC1*, *NAC2* and *HXK1*, have significant changes in gene expression (Fig. 2). Additionally, these genes may play a role in a temporal (circadian) manner as their significant expression changes are only at a specific time, at either dawn or dusk (Fig.2). We recently reported that the expression level of *ELF3* gene and other clock genes significantly reduced in the *nup98a-1 nup98b-1* double mutant compared to that in the wild type plants [12]. Therefore, circadian clock may be involved in the senescence network regulated by the *Nup98* genes.

Sugar is confirmed as an important signal in the control of plant growth and development [61]. Many important genes in sugar signaling pathways (*TOR*, *KIN10*, *KIN11*, *TPS1*, *SnRK1*) (Fig. S7) and carbon starvation genes (*DRM1* and *DIN6*) (Fig. 6) were all mis-regulated in *nup98a-1 nup98b-1* double mutant plants. Based on the presented data, sugar availability is likely hindered in the *nup98a-1 nup98b-1* double mutant, suggesting that this might be the main reason for the initiation of senescence in this double mutant (Fig. 5).

In plants, sugar is derived from photosynthesis and stored firstly as starch. Starch accumulates in the chloroplast during the day and is degraded at night [17, 18]. Our evidence showed the *nup98a-1 nup98b-1* double mutant accumulated much higher levels of starch, which was unlikely due to higher photosynthesis efficiency (Fig. S7) but impaired starch degradation (Fig.3). This was evidenced by 1) lower expression levels of genes related to photosynthesis (*LHCA1*, *LHCA2*, *LHCB1.1* and *LHCB1.4*), which would lead to reducing starch synthesis in the double mutant (Fig. S7); 2) lower expression of starch degradation genes (Fig. 4) embracing many steps of starch degradation (Fig. S6) [17], that would lead to starch accumulation in the *nup98a-1 nup98b-1* double mutant. Impairment of starch degradation in the *nup98a-1 nup98b-1* double mutant directly lead firstly to sugar starvation, subsequently leaf senescence, and finally whole plant death.

Beyond this, *Nup98a* and *Nup98b* may also have functions in nutrient metabolism as the concentration of basal nutrients in the growth medium had a significant impact on the growth and senescence in *nup98a-1 nup98b-1* double mutants (Fig. 5). In this case, components (sugar or MS) in growth medium should be taken into consideration, especially for senescence study in future.

Previous studies show that the circadian clock regulates starch metabolism in plants [62, 63]. *ELF3* positively regulates starch accumulation, and degradation of starch was significantly slower in *elf3* mutant plants than in the corresponding wild type plants [64]. Therefore, *ELF3* and other clock evening genes, such as *ELF4* and *LUX ARRHYTHMO*, also affect leaf senescence [65, 66]. Many genes studied here showed time-dependent difference in gene expression (Fig. 2, 4 and 6). We previously showed that the expression of many clock genes significantly changed in the *nup98a-1 nup98b-1* double mutant [12].

Token together, clock genes might participate in signalling pathway of *Nup98a/Nup98b* regulating starch degradation and senescence in *Arabidopsis*.

We just reported that the *nup98a-1 nup98b-1 ft-10* triple mutant displays the late flowering character as the *ft-10* mutant but maintains early senescence phenotypes as the *nup98a-1 nup98b-1* double mutant [12], suggesting that *Nup98a* and *Nup98b* genes are involved in regulation of flowering and senescence processes in unrelated or independent pathways.

It is obvious that the function of *Nup98* in senescence regulation is indirect. Mutation of *Nup98* genes may lead sequentially to dysfunction of circadian clock, hinderance of starch degradation, sugar starvation, leaf senescence, and plant death. In the future, it would be interested to determine how *Nup98* controls the function of genes related to starch degradation mediated by circadian clock.

It is should be pointed that even though *Nup98a* and *Nup98b* function redundantly in regulation of flowering and senescence, they may also play specific functions in other developmental processes, because the *nup98a* single mutant (*dra2*) displays shade avoidance [6].

Conclusion

NPC has multiple functions in plant development. To the best of our knowledge, the present study is the first report of NPC regulating senescence in plants. This result is promising and implies a novel function of NPC in bridging the gap between starch metabolism and senescence control, that is, *Nup98a* and *Nup98b* overlappingly control starch degradation conferring senescence regulation in *Arabidopsis*.

Methods

Plant materials and growth conditions

Seeds of the T-DNA insertion mutants of *nup98a* (SALK_080083, SALK_090744, SALK_023493, SALK_103803, and SALK_015016) and *nup98b* (CS803848 and GABI_288A08) were ordered from ABRC and GABI T-DNA mutant center, respectively. Homozygous screening was according to the protocol provided by SALK (http://signal.salk.edu/). All mutants were identified by Dr. Long Xiao. *Arabidopsis thaliana* Columbia wild type plants and its derived mutants were grown under long day (16 h/8 h, light/dark, except where indicated in the text) conditions, with 100 μmol m^{-2} s^{-1} lighting provided by fluorescent lamps. Plants were grown on soil in pots with a diameter of 10 cm or on medium in petri dishes containing different strengths of sucrose and MS (Murashige & Skoog) basal medium (Sigma, #M5524), which contains macronutrients and micronutrients of the original classic formulation (NH_4NO_3, 1650.0 mg/L; KNO_3, 1900.0 mg/L; $CaCl_2$, 332.2 mg/L; $MgSO_4{\cdot}7H_2O$, 180.7 mg/L; KH_2PO_4, 170.0 mg/L; $FeSO_4$-$7H_2O$, 27.8 mg/L; H_3BO_3, 6.2 mg/L; $MnSO_4{\cdot}H_2O$, 16.9 mg/L; $CoCl_2{\cdot}6H_2O$, 0.025 mg/L; $ZnSO_4{\cdot}7H_2O$, 8.6 mg/L; $Na_2MoO_4{\cdot}2H_2O$, 0.25 mg/L; KI, 0.83 mg/L; $CuSO_4{\cdot}6H_2O$, 0.025 mg/L;

$Na_2EDTA{\cdot}2H_2O$, 37.26 mg/L; Glycine (free base), 2 mg/L; myo-Inositol, 100 mg/L; Nicotinic acid (free acid), 0.5 mg/L; Pyridoxine·HCl, 0.5 mg/L; Thiamine·HCl, 0.1 mg/L) [67].

Gene and promoter cloning, plasmid construction

Standard GATEWAY (Invitrogen) methods were employed for gene cloning and plasmid construction. Most vectors are developed by our lab [68]. The full-length of *Nup98a* and *Nup98b* open reading frames was PCR-amplified with specific primers (Table S1), and then cloned into Fu30 [68] containing N-terminal GFP marker. The gene entry vectors (Fu30-*GFP:Nup98a* or Fu30-*GFP:Nup98b*), the *35S* promoter entry vector (Fu76-*35S*) and the binary vector (Fu39-2) [68] were applied to LR reaction (Invitrogen). The resulting Fu39-2-*35S:GFP:Nup98a* and Fu39-2-*35S:GFP:Nup98b* binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90RK, and then transformed into *A. thaliana* using the floral dipping method [69]. Homozygous transgenic plants were used for phenotypic and molecular characterization.

Semi-quantitative PCR, quantitative real time RT-PCR, and subcellular localization

The whole seedlings were harvested at ZT0 (the time point of light on) and ZT16 (the time point of light off) at day 14 after germination. RNA preparation, cDNA synthesis and both quantitative real-time and semi-quantitative RT-PCRs were carried out following Xiao et al. [70], except for the use of *At4g34270* as a reference gene in triplicate [71, 72]. All gene accession numbers and relevant primer sequences are listed in Table S1. GFP fluorescent signals were visualized and captured by confocal microscopy, while propidium iodide (PI) is employed for cell wall staining [68].

Starch staining and quantification analysis

Starch in leaves of 14- 21-, and 28-day-old wild type plants and mutant *Arabidopsis* plants was stained with iodine [73]. Plants were grown under a 12-h-light/12-h-dark photoperiod, harvested at ZT0 (the time point of light on) and ZT12 (the time point of light off), decolorized with hot 80% (v/v) ethanol, and stained with iodine-potassium iodide solution. Representative plants were shown. Starch quantitative analysis was carried out according to the instruction of Starch Determination Kit (Solarbio, Beijing, Cat#BC0700) on Spectrophotometer (RAYLEIGH, VIS-7220N; Beijing Beifen-Ruili Analytical Instruments (Group) Co., Ltd) with standard curve method, which was generated with different concentration of glucose (0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.003125, 0.00156 mg/mL).

Statistical analysis

Each experiment has at least three biological replicates and similar results were gotten. For photographs, we selected one representative plant to draw figures. For statistical analysis, all data were analyzed and determined using SPSS software package (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to the control). Error bars indicate ± SD of the mean.

Abbreviations

NPC, nuclear pore complex, Nup, nucleoporin; CO, CONSTANS; ABA, abscisic acid; ABI, ABA INSENSITIVE; ACS, aminocyclopropane-1-carboxylate (ACC) synthase; AGL15, AGAMOUS-LIKE 15; AHK, Arabidopsis histidine kinase; AHP, Arabidopsis thaliana histidine phosphotransfer proteins; AMY3, alpha-amylase-like 3; APD, autoproteolytic domain; ARF, auxin response factor; ARR, Arabidopsis response regulator; C2H4, ethylene ; ATG8, autophagy8; BAM, beta-amylase; CAT, catalase; CK, cytokinin; COI1, coronatine insensitive1; CWINV, cell wall invertase; DIN6, dark inducible6; DRM1/DYL1, dormancy-associated protein-like1; DPE, disproportionating enzyme; EBP1, erbb-3 binding protein; EEL, enhanced em level; EIN, ETHYLENE INSENSITIVE; ELF, EARLY FLOWERING; ETR, ETHYLENE RESPONSIVE; G6P, 6-phosphoric acid glucose; GLK, GOLDEN 2-LIKE TRANSCRIPTION FACTOR; GWD1, α-glucan water, dikinase; HXK1, hexokinase1; ISA3, iso-amylase3; KIN, PROTEIN KINASE; LDA, limit-dextrinase; LHCA, PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE A; LHCB, PHOTOSYSTEM I LIGHT HARVESTING COMPLEX GENE B; LSF1, like sex four1; N, nitrogen; NAC, NAM, ATAF, AND CUC; NAP, NAC-LIKE PROTEIN; NPR1, NONEXPRESSER OF PR GENES 1; NYC, NONYELLOW COLORING; ORE, ORESARA; PHS, α-glucan phosphorylase; PHYB, PHYTOCHROME B; PIF, PHYTOCHROME INTERACTING FACTOR; PYR/PYL/RCAR, PYRABACTIN RESISTANCE/PYR1-LIKE ORREGULATORY COMPONENT OF ABA RECEPTOR; RPS6A, RIBOSOMAL PROTEIN S6A; SAG, SENESCENCE-ASSOCIATED GENE; SAS, SHADE AVOIDANCE SYNDROME, SAUR, SMALL AUXIN UPREGULATED; SAUR36, SMALL AUXIN UPREGULATED; SEX4, STARCH EXCESS 4; SGR, STAYGREEN; T6P, trehalose-6-phosphate; TOR, TARGET OF RAPAMYCIN; TPS1, trehalose-6-phosphate synthase1; WRKY, WRKY DNA-BINDING PROTEIN;

Declarations

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Author Contributions

Conceptualization, Y.-F.F. and X.Z.; Methodology, L.X. and Y.-F.F.; Investigation, L.X., F.C., S.J., Z.C., P.H, Y.M., C.L., and L.L.; Formal analysis, L.X., F.C., X.W., and Q.C.; Visualization, L.X. and Y.-F.F.; Writing – Original Draft, Y.-F.F., and Q.C. Writing –Review & Editing, I.S. and Y.-F.F.; Funding Acquisition, Y.-F.F. and X.Z.; Project administration, Y.-F.F. and X.Z.; Resources, X.Z, Y.M., X.W., and Q.C; Supervision, Y.-F.F. All Authors read and approved the manuscript.

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the experimental design of the study, data collection, analysis and interpretation, and in writing the manuscript.

Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent to publish

All Authors read and approved the manuscript.

Plant specimens

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Competing Interests

The authors declare no competing interests.

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Additional File Information

Additional file 1: Supplementary Table 1. Primers used in this study.

Additional file 2: Figure S1. Protein domains in Nup98a and Nup98b of *Arabidopsis thaliana*.

Additional file 3: Figure S2. The *nup98a1 nup98b1* double mutant showed pleiotropic defeat in various organs.

Additional file 4: Figure S3. The *nup98a1 nup98b1* double mutant showed senescence and sterility phenotype.

Additional file 5: Figure S4. Senescence phenotypes were specific to th

Additional file 6: Figure S5. Molecular network of senescence initiation in plants.

Additional file 7: Figure S6. The simple pathway of starch degradation in chloroplast.

Additional file 8: Figure S7. The expression analysis of genes related to photosynthesis and sugar metabolism in the *nup98a1 nup98b1* double mutant.

Additional file 9: Figure S8. Exogenous sucrose reduces early senescence in the *nup98a1 nup98b1* double mutant plants.

Additional file 10: Figure S9. Phenotype analysis of the *nup98a1 nup98b1* double mutant compared to WT grown on different mediums.

Figures

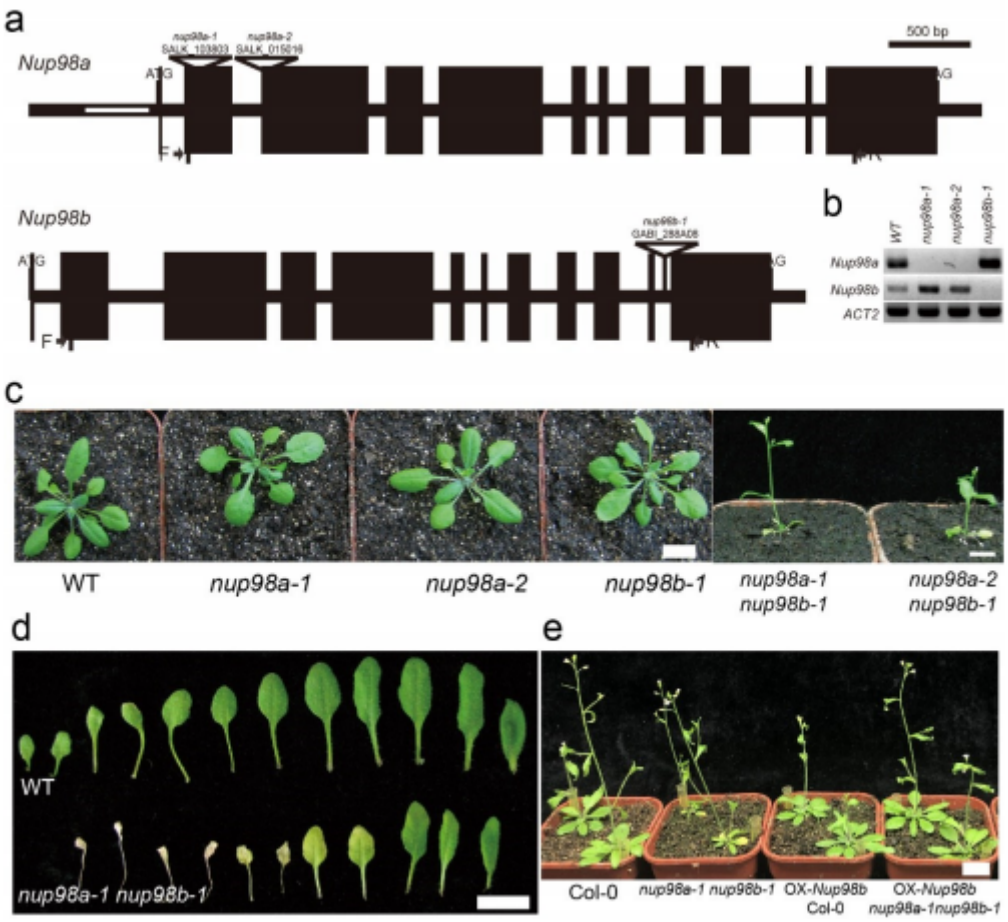

Figure 1

Mutation of Nup98 leads to an early senescence phenotype. A, T-DNA insertion alleles in Nup98a and Nup98b. Black bars indicate exons, thin black lines indicate introns or UTR, open line in the 5'-UTR indicates an intron in 5'-UTR, triangles shows T-DNA insertions and the mutant name and T-DNA identifier above the symbol. B, RT-PCR confirmed the single mutants of nup98a-1, nup98a-2, and nup98b-1. The position of primers of F and R were indicated in A (black arrows). ACT2 was used as a control. C, Single mutants of nup98a-1, nup98a-2, and nup98b-1 displayed similar phenotypes to wild type plants however the double mutants of nup98a-1 nup98b-1 and nup98a-2 nup98b-1 showed severe early senescence. The photos were taken on day 20 after germination. D, The leaf senescence phenotype of the nup98a-1 nup98b-1 double mutant rosette leaves harvested at inflorescence emergence. E, Ectopic expression of Nup98b rescued the leaf senescence phenotype of the nup98a-1 nup98b-1 double mutant. All plants grew in soil from germination. The photos were taken on day 30 after germination. An * indicates measurements that were significantly (*P < 0.05; **P <0.01) different from the control. Error bars indicate ± SD of the mean.

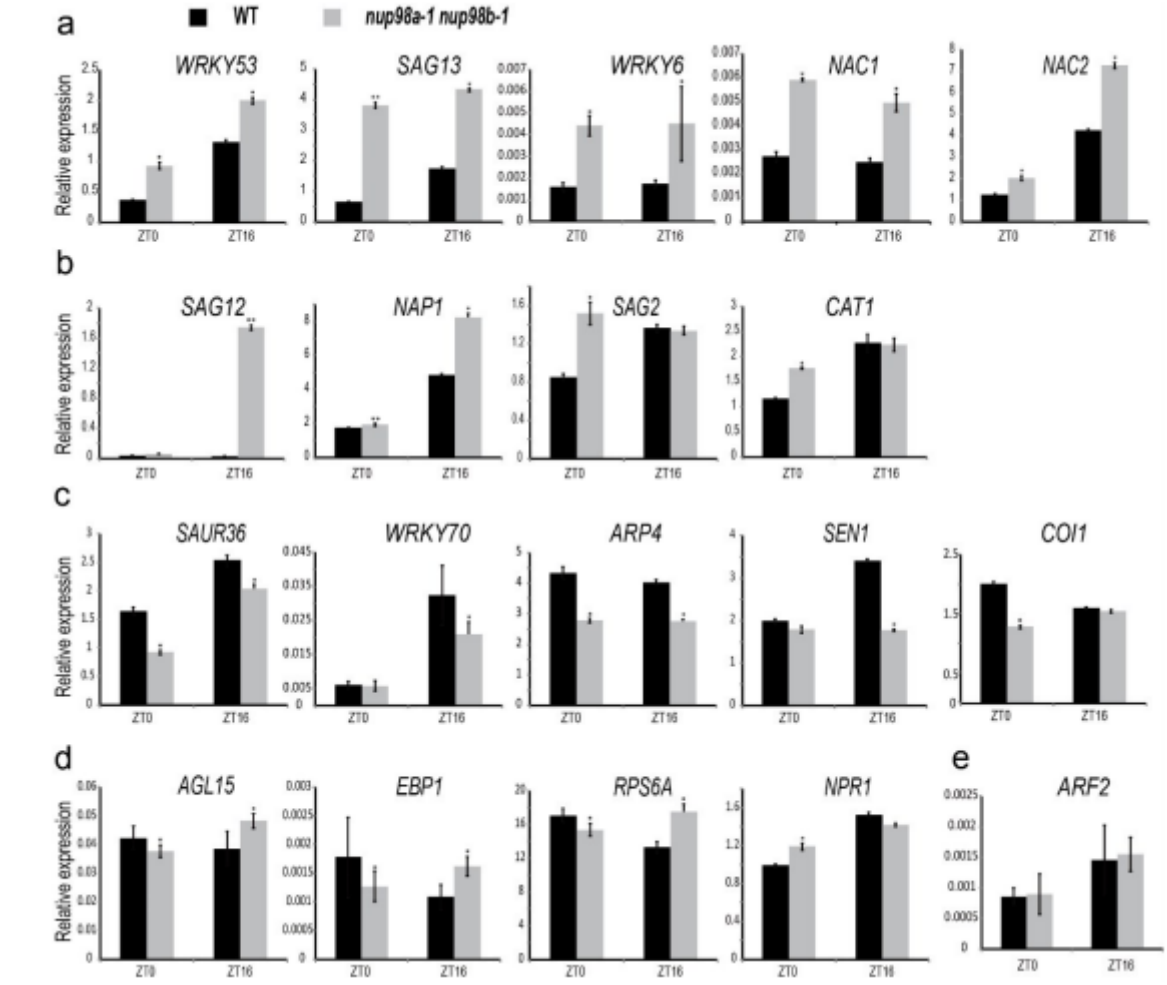

Figure 2

The expression of genes related to senescence are altered in the nup98a-1 nup98b-1 double mutant. A, Genes up-regulated both at dawn (ZT0) and dusk (ZT16). B, Genes up-regulated at dawn (ZT0) or dusk (ZT16). C, Genes dawn-regulated at dawn (ZT0) and/or dusk (ZT16). D, Genes up-regulated or dawn-regulated at dawn (ZT0) and dusk (ZT16), respectively. E, Genes with no significant changes both at dawn (ZT0) and dusk (ZT16). The nup98a-1 nup98b-1 double mutant and wild type plants were grown in petri dishes in long day conditions for 14 days, and then harvested at ZT0 and ZT16 for gene expression analysis. All RT-PCR measurements were repeated at least three times, in triplicate. All RT-PCR gene expression measurements were normalized to the control TIP41 (At4g34270) and expressed as a relative expression value. Student's t test was used to statistically analyze the data. An * indicates measurements that were significantly (*P < 0.05; **P <0.01) different from the control. Error bars indicate ± SD of the mean.

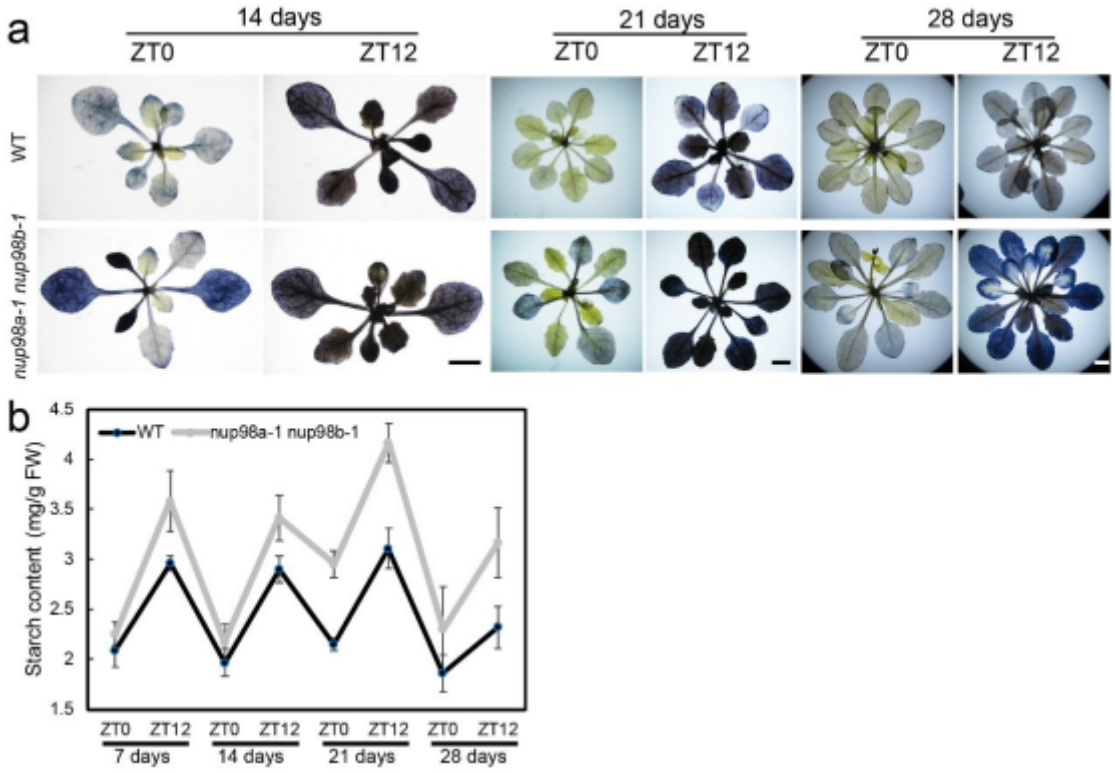

Figure 3

The nup98a-1 nup98b-1 double mutant has abnormal starch degradation. Arabidopsis seeds were sown on 1/2 MS medium and placed at 4°C for 3 days, then transferred to 12 h light/12 h dark conditions. Plants were grown for an additional 7, 14, 21, or 28 days and were harvested at ZT0 and ZT12 for starch staining by iodine-potassium iodide (A) and starch quantitative assay by using Starch Determination Kit (Solarbio, Beijing, Cat#BC0700) on Spectrophotometer (B).

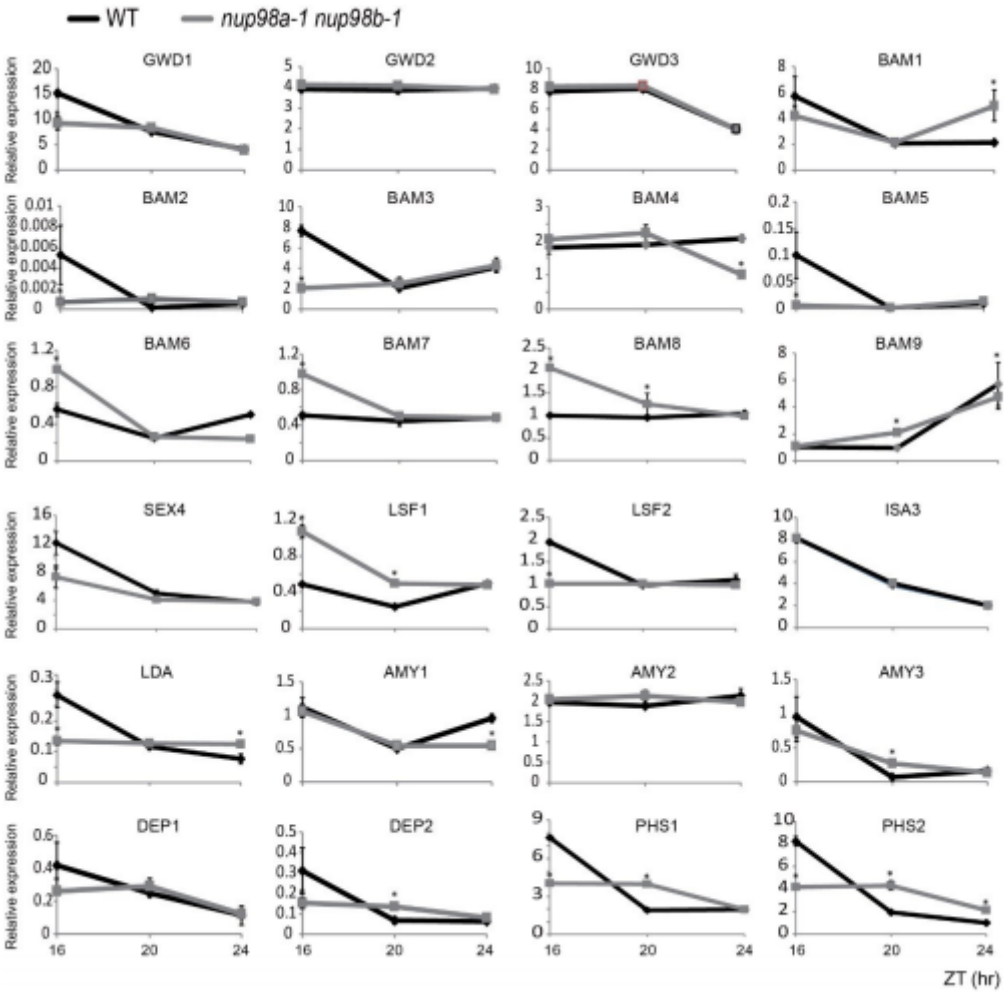

Figure 4

The expression of genes related to starch metabolism are altered in the nup98a-1 nup98b-1 double mutant. The seeds of mutants and wild type plants were sown on MS medium. After low temperature treatment for 3 days, plants were grown under long day conditions. Samples were harvested at ZT16, 20 and 24 during the dark phase. All RT-PCR measurements were repeated at least three times, in triplicate. All RT-PCR gene expression measurements were normalized to the control TIP41 (At4g34270) and expressed as a relative expression value. Student's t test was used to statistically analyze the data. An * indicates measurements that were significantly (*P < 0.05) different from the control. Error bars indicate ± SD of the mean.

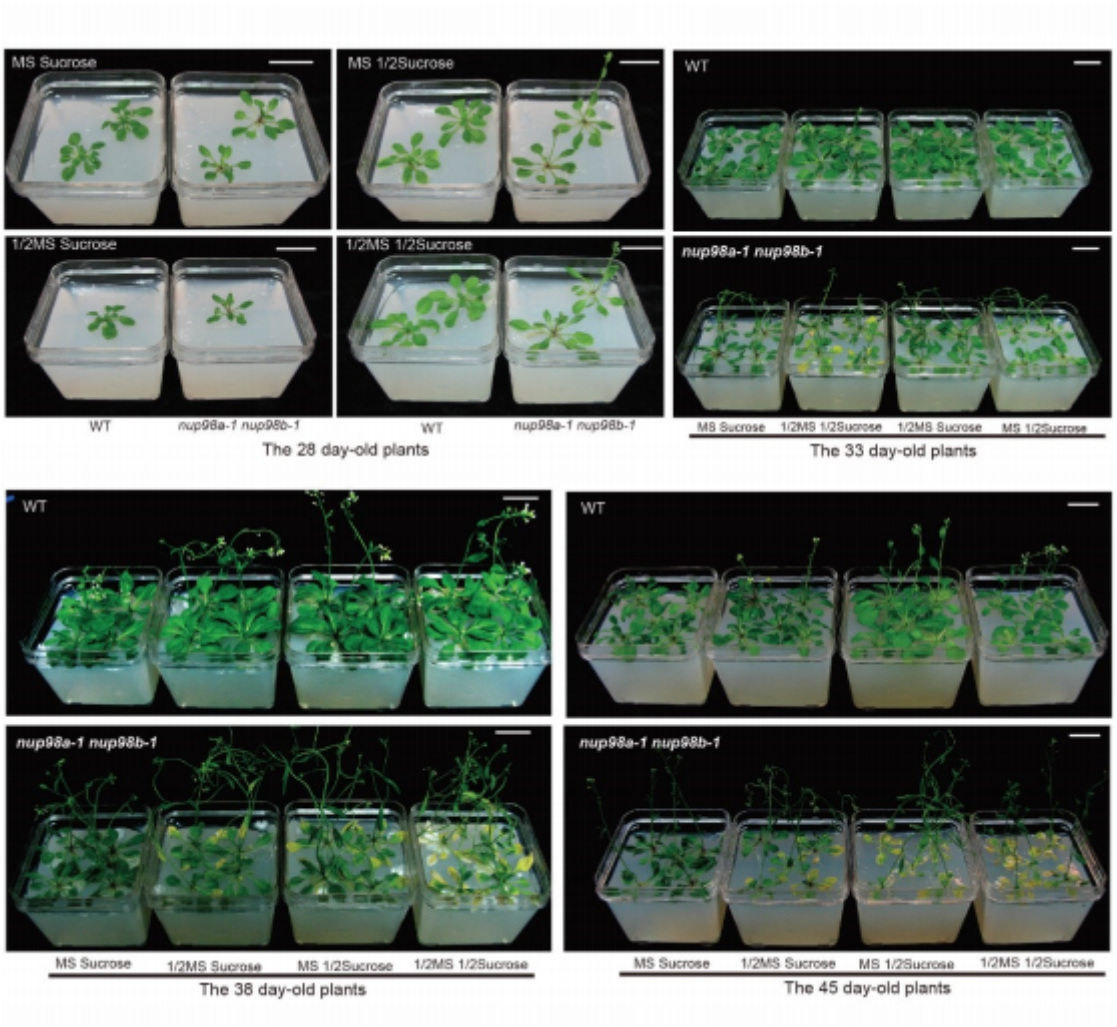

Figure 5

Increased nutrients and sucrose delays senescence in nup98a-1 nup98b-1 double mutants. The seeds of mutants and wild type plants were sown on medium with different strengths of MS (Murashige & Skoog) nutrients and 3% sucrose.1/2MS indicates 1/2 strength of MS. 1/2Sucrose indicates 1.5% sucrose. After stratification, plants were grown under long day conditions. Both nutrients and sucrose delayed senescence phenotypes in the nup98a-1 nup98b-1 double mutant plants. Bars = 5 cm.

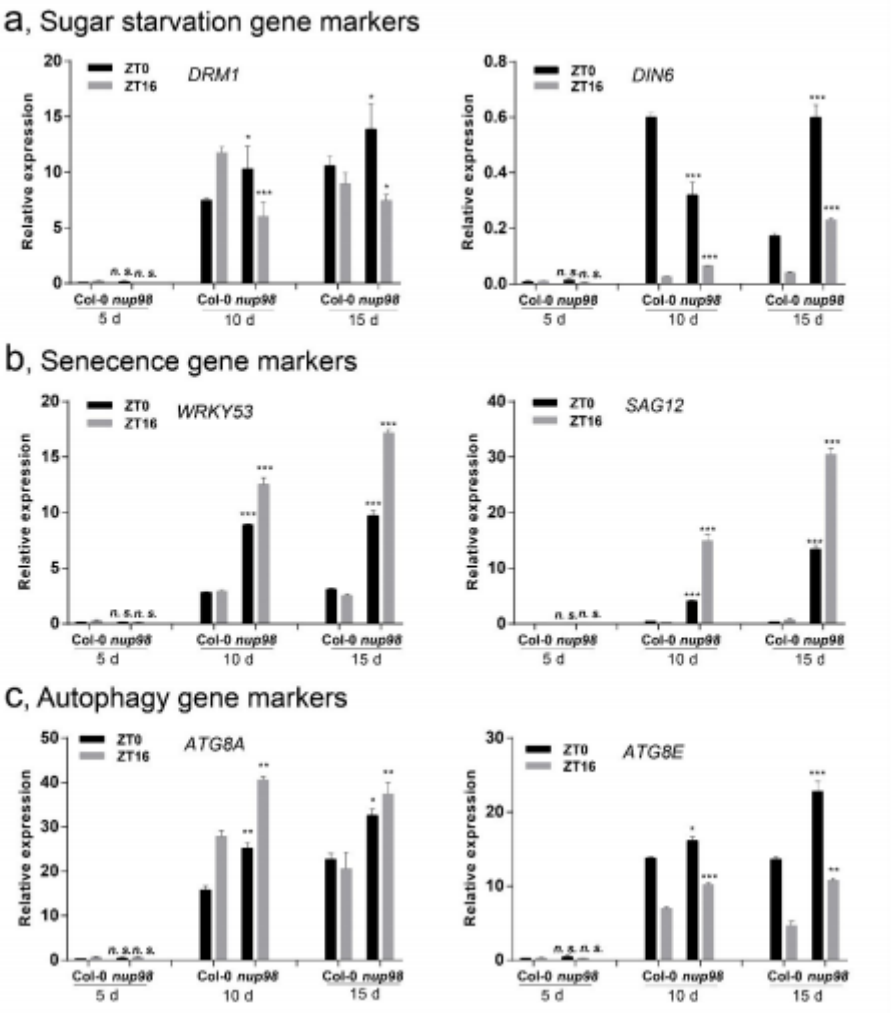

Figure 6

Marker genes of starvation and senescence mis-express in the nup98a-1 nup98b-1 double mutant. A, Sugar starvation genes. B, Senescence genes. C, Autophagy genes. The seeds of the mutant and wild type plants were sown on MS medium. After low temperature treatment for 3 days, the seedlings were grown for 7 days under long day conditions, then seedlings were transplanted in soil. From that, samples were harvested in day 5, 10, and 15 at both ZT0 and ZT16. All qPCR measurements were repeated at least three times, normalized to the reference gene TIP41 (At4g34270) and expressed as a relative expression value. Student's t test was used to statistically analyze the data. Error bars indicate ± SD of the mean. Asterisk indicates significant difference between the nup98 double mutant and wild type plants (one asterisk, $p < 0.05$; double asterisks, $p < 0.01$; triple asterisks, $p < 0.001$. n.s., No significant difference).

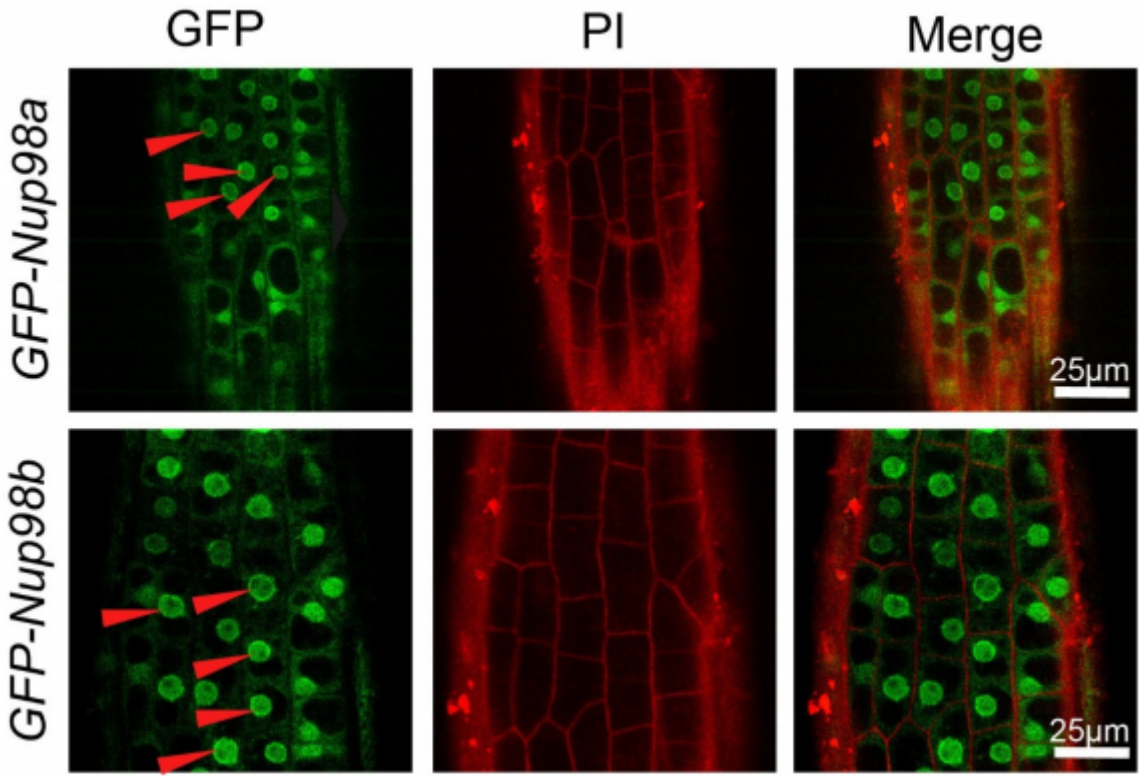

Figure 7

Nup98a and Nup98b proteins are localized to the nuclear membrane and in the nucleoplasm. The green fluorescent protein (GFP) was fused to either Nup98a or Nup98b to generate N-terminal translational fusional proteins, GFP-Nup98a and GFP-Nup98b, and were driven by the CaMV 35S promoter after stable transformation into Arabidopsis thaliana plants. Arrow heads indicated that both GFP-Nup98a and GFP-Nup98b enriched near the nuclear periphery when compared to the cytoplasm. PI (propidium iodide) is for cell wall staining.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- OriginalphotoofagelinFig1B.pdf
- Supplementarydata.pdf