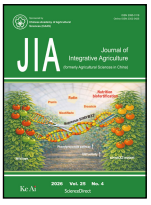
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RESEARCH ARTICLE

Nitrogen redistribution during the grain-filling stage and its correlation with senescence and *TaATG8* expression in leaves of winter wheat

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Highlights

- The rate of nitrogen redistribution serves as a measure of nitrogen transport capacity.
- Nitrogen application hinders nitrogen transport during the initial phase of grain filling, but enhances it in the intermediate and later stages.
- Concurrent with the peak expression of autophagy genes, the transportation of potential amino acids is likewise heightened.

Abstract

Nitrogen is a key nutrient for wheat (*Triticum aestivum* L.) growth and yield, particularly during the grain-filling stage, where most nitrogen is redistributed from vegetative organs to the grain, significantly influencing yield. However, it remains unclear during which period the nitrogen translocation from the vegetative phase to grain maturation occurs and how it correlates with flag leaf senescence. In this study, a field experiment was conducted using the winter wheat cultivar 'Xinong 511' under two nitrogen fertilizer treatments: regular nitrogen supply (240 kg ha^{-1} (N_{240})) and no nitrogen supply (0 kg ha^{-1} (N_0)). The results revealed that nitrogen accumulation in wheat flag leaves peaked at 7–14 days, with a nitrogen content 4.55%, after which nitrogen was redistributed to the grains. Nitrogen content in flag leaves decreased by 56% during 21–35 days, while that in the grains increased by 51%. The plant analysis development value (relative chlorophyll content), photosynthetic rate, free amino acid concentration, and soluble protein content in flag leaves peaked at 7–14 days, indicating nitrogen transportation from the flag leaves to the grains. Nitrogen application significantly increased the nitrogen remobilization rate in flag leaves by 20% compared with that of N_0, reduced reactive oxygen species accumulation by 21%, and delayed flag leaf senescence. Under nitrogen deficiency, autophagy was induced earlier, with a 5–7-fold increase in the expression of autophagy-related genes (*TaATG8*), suggesting that regulation of the autophagy pathway and enhancement of autophagy activity can optimize nitrogen fertilization. Our study demonstrates that the remobilization of nitrogen from vegetative parts to grains initiates leaf senescence and is closely correlated with the expression of autophagy-related genes.

Keywords: winter wheat, senescence, autophagy, nitrogen redistribution, grain-filling stage

1. Introduction

Wheat (*Triticum aestivum* L.) is an important staple crop, feeding 50% of the population globally (Zaveri and Lobell 2019). Nitrogen is essential for wheat production (Bly and Woodard 2003; Fageria and Baligar 2005), playing a critical role in plant growth, biomass accumulation, and grain yield (Lawlor and Lemaire 2001). Nitrogen deficiency reduces tiller and grain numbers and grain weight (Huang *et al.* 2013; Balkcom and Burmester 2015; Wang *et al.* 2016; Terrile *et al.* 2017). Therefore, there is a need for research on improving nitrogen use efficiency in wheat.

Increasing photosynthetic rates, particularly in flag leaves, is crucial for grain filling and yield (Stamp and Herzog 1976; Gregersen *et al.* 2013). Flag leaves contribute the most photoassimilates during grain filling (Sharma *et al.* 2003). During the grain-filling stage, nitrogen is remobilized from

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senescing leaves to the grain (Sorin *et al.* 2016; Zakari *et al.* 2019). In cereal crops, nitrogen remobilized from leaves accounts for 50–90% of seed nitrogen, and grain yield is positively correlated with the amount of nitrogen remobilization (Masclaux-Daubresse *et al.* 2010). Nitrogen deficiency increases leaf senescence, reducing biomass accumulation and grain yield (Shah *et al.* 2017). However, the regulatory mechanisms involved in nitrogen remobilization from leaves remain unclear.

Leaf senescence occurs coordinately during grain filling, and premature or delayed senescence can affect assimilation or nutrient remobilization (Lim *et al.* 2007; Srivalli *et al.* 2009). During senescence, autophagy remobilizes nutrients from source organs to sink organs, thereby enhancing productivity (Breeze *et al.* 2009; Gregersen *et al.* 2013; Avila-Ospina *et al.* 2014). Autophagy is closely correlated with *ATG* genes, with *ATG8* playing a critical role (Li and Vierstra 2012). In *Arabidopsis*, *ATG8* overexpression promotes autophagy and increases nitrogen remobilization (Chen *et al.* 2019). Under hypoxic conditions, wheat roots depend on autophagy to remove reactive oxygen species (ROS) and maintain cell survival (Lin *et al.* 2021). ROS is closely associated with autophagy (Pérez *et al.* 2012), and H_2O_2 regulates leaf senescence (Jajic *et al.* 2015). During leaf senescence, changes in free amino acids and soluble protein content are closely correlated with nitrogen remobilization. As leaves senesce, the levels of free amino acids and soluble proteins gradually decrease, indicating nitrogen translocation from leaves to grains.

During the grain filling, nitrogen from vegetative organs is remobilized to the grain. Autophagy is thought to play an important role in nitrogen remobilization and may influence the expression of *TaATG8* genes. However, no studies have explored this correlation. Therefore, this study aimed to investigate the timing of nitrogen remobilization and its association with *TaATG8* expression.

2. Materials and methods

2.1. Test materials and growth conditions

We carried out a 2-year study (2020–2022) during which Xinong 511 wheat variety was planted in an experimental field at the Northwest A&F University, China (34°16′56.24′′N, 108°4′27.95′′E). The experiments were arranged in a randomized complete block design with two nitrogen levels, N_0 (0 kg ha^{-1}) and N_{240} (240 kg ha^{-1}), and were replicated thrice. The study had six plots measuring 37.5 m^2 each. The N_0 treatment comprised calcium superphosphate (44%) and potassium chloride (60%) applied at a constant rate of 120 kg ha^{-1}. The N_{240} treatment comprised urea (46%), calcium superphosphate (44%), and potassium chloride (60%) applied at 240, 120, and 120 kg ha^{-1}, respectively. Briefly, 50% of the nitrogen fertilizer was applied as a basal application, and 50% was top-dressed at the jointing stage. All phosphorus and potassium fertilizers were basally applied. The inherent soil fertility was analyzed before sowing, and the results are presented in Appendix A.

2.2. Free amino acids, total soluble protein, total nitrogen, and yield determination

Free amino acids and total soluble protein contents were measured using the ninhydrin and Coomassie brilliant blue Bradford methods (G250; Thermo Fisher Scientific, Waltham, MA, USA), respectively. Seven days after wheat anthesis, 10 well-developed flag leaves were sampled every week. At the anthesis and maturity stages, whole plants were sampled and separated by organ. Samples were initially fixed in an oven at 105°C for 30 min and dried at 70°C for 48 h until constant weight. Total nitrogen content was determined using a semi-micro Kjeldahl method and a nitrogen analyzer (SmartChem 200, Mandel Scientific Company, Guelph, ON, Canada). At wheat maturity, the number of spikes per plot was recorded, and 20 spikes were randomly selected to determine the number of grains per spike. Wheat was sampled from 3 m² of each plot, threshed, and air-dried to approximately 12.5% grain moisture content for the determination of grain yield.

2.3. Hydrogen peroxide and superoxide anion content determination

The Beijing Solarbio test box (Solarbio, Beijing, China) was used to measure the absorbance of the samples at 530 nm and calculate the $O_2^{-}\cdot$ content in the tissues (Wang *et al.* 1990). The Beijing Solarbio test box (Solarbio) was used to measure the absorbance of the samples at 415 nm and calculate tissue H_2O_2 content (Sima *et al.* 2011).

2.4. RNA extraction and real-time polymerase chain reaction

Total RNA was extracted using TRIzol reagent (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized from 3 μg of total RNA treated with DNase I (TaKaRa) using M-MLV reverse transcriptase (TaKaRa). Real-time polymerase chain reaction (qPCR) was performed using the double-stranded DNA-specific dye SYBR Green (TaKaRa) and monitored in real-time using a 7500-qPCR System (Applied Biosystems, Waltham, MA, USA). The wheat ACTIN gene (*LOC_Os03g50885*) was used as an internal reference. Three biological replicates were analyzed for each sample. The primers used for qPCR are listed in Appendix B.

2.5. Determination of SPAD and photosynthetic rate of flag leaves

A LI-6400 XT photosynthetic meter (Applied Biosystems, CA, USA) was used to measure the net photosynthetic rate of flag leaves (P_n, μmol m^{-2} s^{-1} from 9:00–11:00 in sunny weather every 7 days after and during wheat flowering. For measurement, red and blue light sources were used in the chamber of the photosynthetic apparatus (Applied Biosystems), and the photosynthetically active radiation intensity was uniformly adjusted to 1,000 μmol m^{-2} s^{-1}. After the full expansion of flag leaves, five flag leaves were selected and assigned to each treatment. SPAD were measured at flowering and during grain filling using the SPAD-502Plus system (Thermo Fisher Scientific). Measurements were taken in the middle of each flag leaf.

2.6. *TaATGs* bioinformatics analysis

ATG subfamily sequences for wheat, rice (*Oryza sativa* L.), *Arabidopsis thaliana* (L.) Heynh., and soybean (G*lycine max* (L.) Merr.) were obtained from previous studies and used to elucidate the evolutionary relationships between ATG members of different plant species. Protein sequence data were obtained from the Ensembl Plants database (http://plants.ensembl.org/index.html), and a phylogenetic tree was constructed using MEGA X software (https://www.megasoftware.net/show_eua) using the maximum likelihood method. ClustalW analysis of the aligned sequences revealed that the best amino acid substitution model was WAG+I+G, which was used for phylogenetic tree construction. The iTOL web tool (https://itol.embl.de/) was used to visually modify the phylogenetic tree.

2.7. Statistical analysis

Statistical differences between groups were analyzed using Student's *t*-test and Duncan's test, and significance levels were determined at $P<0.001$, $P<0.01$, and $P<0.05$.

3. Results

3.1. Changes in ROS levels, photosynthetic rate, and chlorophyll content in wheat flag leaves at different stages after anthesis

As wheat leaves senesced, ROS, including H_2O_2 and $O_2^{-}\cdot$, gradually accumulated. The H_2O_2 and $O_2^{-}\cdot$ levels in wheat flag leaves were measured at 7, 14, 21, 28, and 35 days after flowering. The results revealed that ROS levels increased over time in the flag leaves of N_{240} and N_0 treatments (Fig. 1-A and D). Moreover, ROS levels were consistently higher in the N_0 treatment compared with those in the N_{240} treatment, indicating that nitrogen starvation has a similar effect to that of increased senescence, suggesting that nitrogen starvation increases the premature senescence of flag leaves.

Photosynthetic rate and chlorophyll content were higher in the N_{240} treatment compared to the N_0 treatment (Fig. 2-A and F). In all treatments, both parameters peaked at 14 days after anthesis (DAA). They then gradually declined, with the rate of decline increasing linearly over time, indicating that photosynthesis is progressively reduced with time, and nitrogen application delays flag leaf senescence by maintaining photosynthesis.

3.2. Changes in total nitrogen, soluble protein, and free amino acids in flag leaves of wheat at different stages after anthesis

As flag leaves senesced, the total nitrogen, soluble protein, and free amino acid contents gradually decreased (Fig. 3-A and F). Additionally, all indicators in the N_0 treatment were lower than those in N_{240}, indicating that nitrogen deficiency increased nitrogen breakdown and translocation in the flag leaves. As wheat development progressed, a higher proportion of nitrogen was retained in the flag leaves during 7–14 days, while a higher proportion of nitrogen was found in the grain during 21–35 days. Nitrogen application significantly increased the proportion of nitrogen content in the grains. The N_{240} treatment significantly enhanced nitrogen accumulation and grain yield in wheat, though nitrogen use efficiency correspondingly decreased. Nitrogen application (N_{240}) significantly increased the nitrogen allocation ratio in various organs (leaves, stems+sheaths, and rachis+glumes), with the most notable increase observed in leaf nitrogen allocation. Furthermore, nitrogen application enhanced the nitrogen translocation from these organs to the grains, promoting the accumulation of nitrogen in the grain Appendices C and D.

Nitrogen application increased the nitrogen content of flag leaves, which is the nitrogen source during grain filling. Compared with that in the N_0 treatment, the nitrogen redistribution rate in the N_{240} treatment was reduced in the early stages of grain filling and increased in the later stages, which ultimately delayed flag leaf senescence (Fig. 4-A and B). The active oxygen accumulation rate in the flag leaf was higher than the total nitrogen redistribution rate at the early stage of grain filling (Fig. 4-C and F). However, at the late stage of grain filling, the total nitrogen redistribution rate in the flag leaf was higher than the active oxygen accumulation rate, indicating that nitrogen redistribution in flag leaves affects the ROS accumulation and slows down flag leaf senescence.

3.3. Identification of wheat autophagy genes

The role of autophagy in crop performance under nitrogen deficiency has become a focal point of research. Autophagy plays a crucial role in nitrogen remobilization at multiple levels. Therefore, we analyzed the sequence characteristics of five ATG8 members in *T. aestivum* L. The sequences

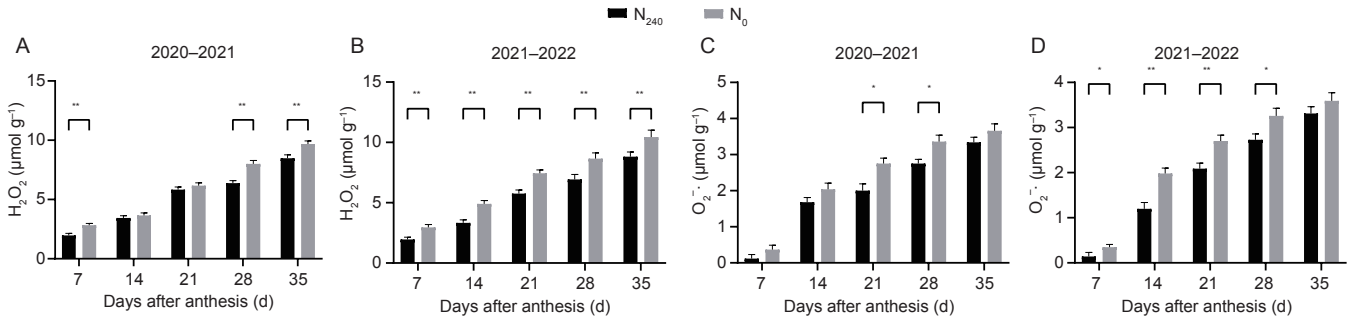


Fig. 1 Changes of reactive oxygen species (ROS) after anthesis hydrogen peroxide content (A and B) and superoxide anion content (C and D). N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. Bars mean SD (n=3). * and ** indicate significant differences at $P<0.05$ and $P<0.01$, respectively.

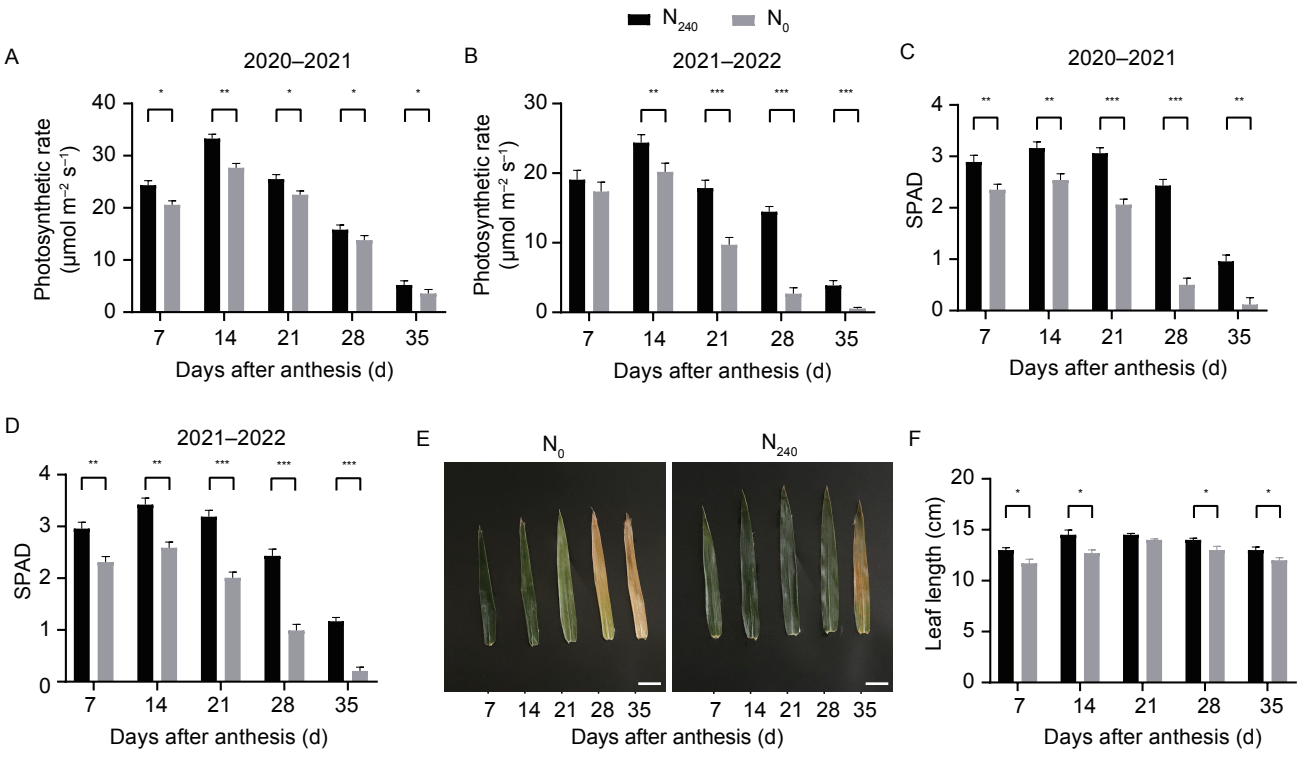


Fig. 2 Flag leaf senescence and its photosynthetic rate changes after anthesis. A and B, photosynthetic rate. C and D, SPAD. E, post-anthesis senescence process of flag leaves. Scale bar=2 cm. F, post-anthesis change in flag leaf length. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. Bars mean SD (n=3). *, **, and *** indicate significant differences at P<0.05, P<0.01, and P<0.001, respectively.

are presented in Table 1. The results revealed that their amino acid sequences were all 119 amino acids in long, with molecular weights ranging from 13,568.59–13,616.79 Da. The isoelectric point (pI) values ranged from 7.78 to 9.07, indicating that these proteins may exhibit different charge properties in various cellular environments. By calculating the grand average of hydropathicity index, we found that there were differences in hydrophilicity and hydrophobicity among the *ATG8* members. Subcellular localization revealed that Ta2A_ATG8a, Ta2B_ATG8c, Ta2D_ATG8d, and Ta2D_ATG8e were primarily localized in the cytoplasm, mitochondria, and nucleus, while Ta2A_ATG8b was mainly localized in the cytoplasm and mitochondria. Among them, Ta2A_ATG8a, Ta2B_ATG8c, and Ta2D_ATG8e characteristics were identical, indicating that their DNA sequences are highly similar.

Ta2A_ATG8a, Ta2B_ATG8c, and Ta2D_ATG8e had identical amino acid sequences. There was one sequence difference between Ta2A_ATG8b and Ta2B_ATG8d, and there were 16 sequence differences between the first three and the latter two of these five members (Fig. 5). Thus, it can be concluded that Ta2A_ATG8a, Ta2B_ATG8c, and Ta2D_ATG8e originated from the same replication event, whereas Ta2A_ATG8b and Ta2B_ATG8d are derived from a different event.

A phylogenetic tree of the autophagy gene family of the flag leaf of wheat was constructed using the maximum likelihood method and visualized using the iTOL web tool and the results are presented in Fig. 6. Phylogenetic tree data were derived from the ATG8 protein sequences of four different species: *A. thaliana* (9), rice (6), soybean (10), and wheat (5), for 30 ATGs. The ATG8 subfamily in wheat consists of eight members, designated as TaATG8a–h. ATG8 is the most abundant subfamily in the wheat autophagy gene family, indicating its significance and diversity. This abundance suggests that the ATG8 function may require extensive gene expression support to achieve its critical role in wheat autophagy.

We used the spatiotemporal expression profile data of wheat in the expVIP database (wheat-expression.com) to screen the heatmap display of ATG family genes. The results are presented in Fig. 7-A. The horizontal axis of Fig. 7-A presents the sample period, and the vertical axis presents the gene name. Furthermore, Fig. 7-A presents the mean values, and expression levels are arranged from high to low. The expression levels of *TaATG8a–e* (TaATG8 subfamily) ranked the highest among the top of all autophagy genes, and those of *TaATG8f–h* were ranked at the medium level. Consistent with the results of the evolutionary analysis, we found that the *TaATG8* genes play a key role in autophagy. Its adjacency to the *AtATG8s*, *OsATG8s*, and *TaATG9s* genes in the phylogenetic tree suggests that they may have similar mechanisms of action.

Transcriptome data after flag leaf senescence from the expVIP database are presented in Fig. 7-B, and transcriptome data from the present study (taken 21 DAA (Fig. 7-C)) were screened for ATG8 subfamily genes and visualized using a heatmap. Among these genes, *Ta2A_ATG8b* had the highest

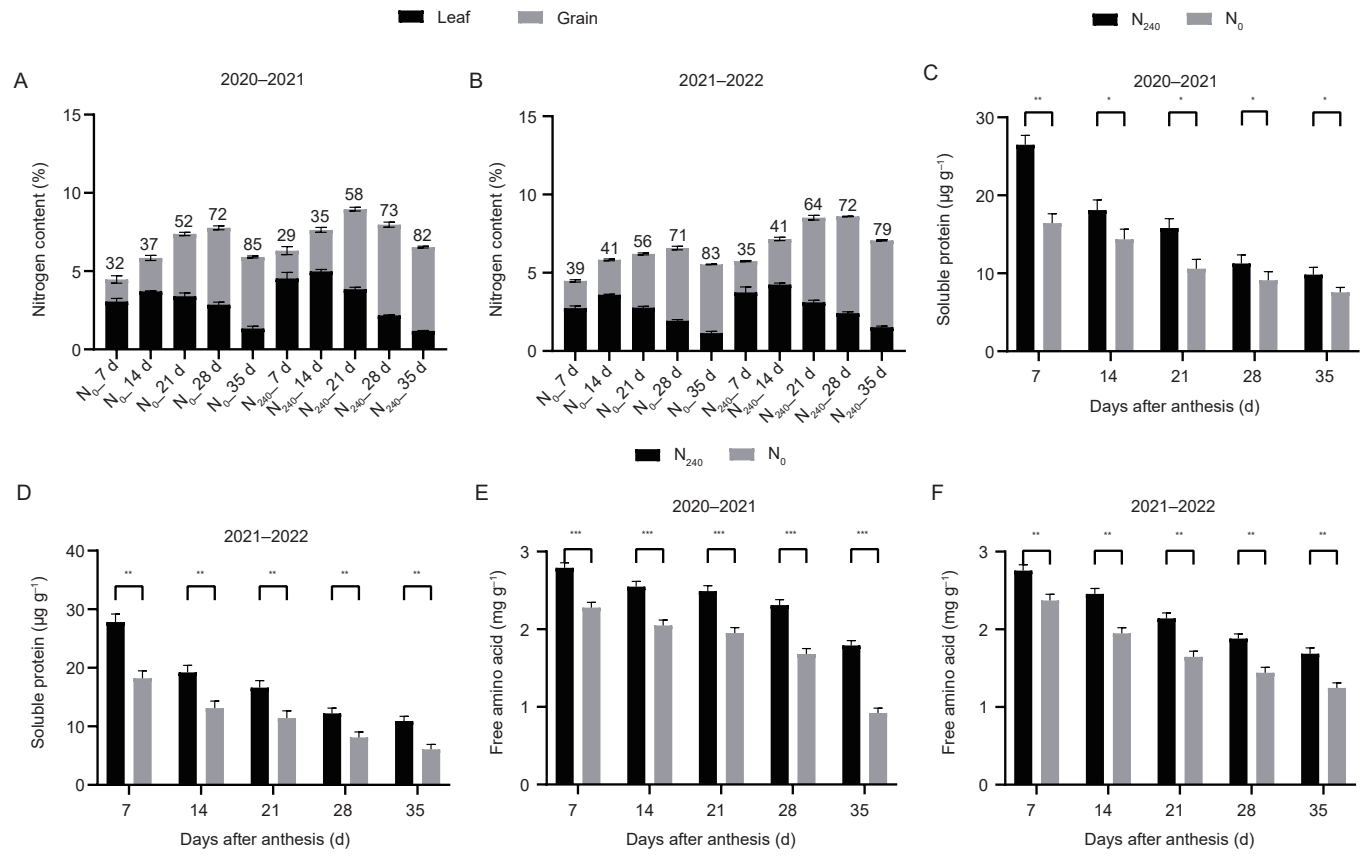


Fig. 3 Changes in nitrogen and protein content in wheat organs. A and B, changes in total nitrogen content of flag leaves and grains after anthesis; the numbers above represent 100×Grain total nitrogen/(Grain total nitrogen+Flag leaf total nitrogen). C and D, changes in soluble protein content after anthesis. E and F, changes in free amino acid content after anthesis. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. Bars mean SD (n=3). *, **, and *** indicate significant differences at P<0.05, P<0.01, and P<0.001, respectively.

expression across all three datasets, followed by *Ta2A_ATG8a*. *Ta2A_ATG8c–e* exhibited intermediate expression levels, while *Ta2A_ATG8f–h* had the lowest. Furthermore, expression levels in the N_0 treatment were higher than those in the N_{240} treatment, indicating a heightened autophagic response to nitrogen starvation at the gene expression level.

3.4. Expression of *TaATG8* genes at different stages after flowering and the correlation between indicators

The qPCR analysis of the highly expressed *TaATG8a–e* gene revealed similar expression patterns within this group (Fig. 8-A–E), with an almost identical response to nitrogen starvation. *TaATG8a–e* expressions in the N_{240}-treatment group increased from 21–28 DAA, whereas N_0 inhibited this process, suggesting that nitrogen may be involved in autophagy regulation and may regulate *TaATG8*s expression during flag leaf senescence. From 28–35 DAA, the expression level remained stable in N_{240}; however, it increased sharply in N_0, indicating that *TaATG8s* had a strong response to nitrogen starvation during the senescence stage of wheat flag leaves, suggesting that, under nitrogen deficiency, plants regulate their energy metabolism and maintain the nitrogen content required for growth by increasing autophagy. The correlation matrix (Fig. 8-F) revealed that the expression patterns of *TaATG8*s were highly correlated (mostly >0.99). The lowest correlation was between *Ta2B_ATG8d* and *Ta2B_ATG8c* (0.947), both of which were positively correlated with ROS and negatively correlated with nitrogen content and photosynthesis indicators, indicating that these two genes are involved in the oxidative stress response during aging and regulate the absorption and utilization of nitrogen and photosynthesis.

The results revealed that from 14 days after wheat anthesis, the total nitrogen content, free amino acids, and soluble protein content in flag leaves decreased (Fig. 3). This decline in total nitrogen and soluble protein was accompanied by a sharp decrease in photosynthetic rate and SPAD values (Fig. 2), indicating the onset of leaf senescence. According to the Pearson correlation matrix analysis (Fig. 8), there were correlations among various physiological indices, including total nitrogen content, free amino acids, soluble protein, ROS, photosynthetic rate, and chlorophyll content. ROS indicators were generally negatively correlated with photosynthetic rate and nitrogen content. The negative correlation between soluble protein, free amino acids, chlorophyll content, and the two ROS indicators was strong, whereas the negative correlation between total nitrogen content and ROS was weak. There was a positive correlation between photosynthesis and nitrogen content. The correlation coefficient between SPAD and total nitrogen was 0.96, and free amino acids were

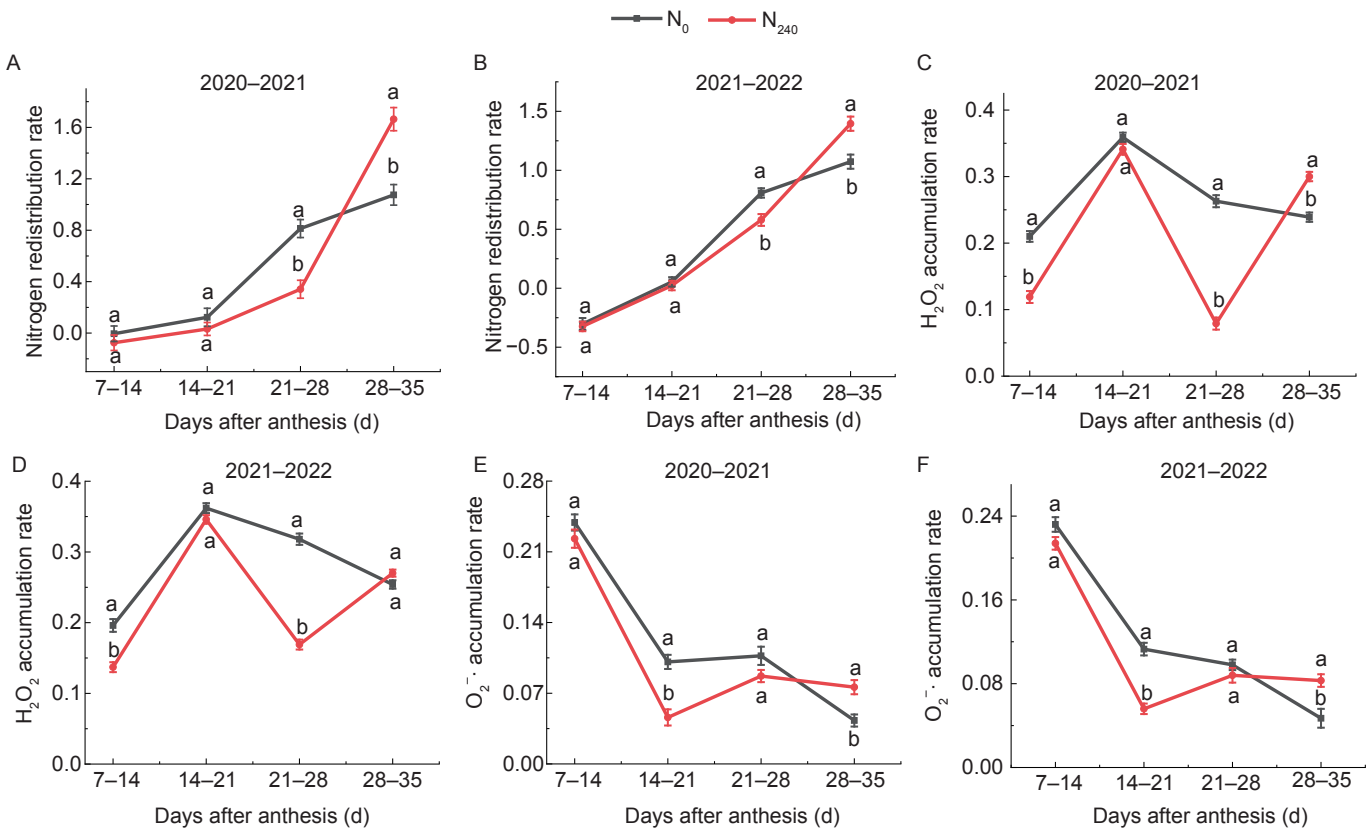


Fig. 4 Rates of nitrogen redistribution and reactive oxygen species (ROS) accumulation. A and B, changes in nitrogen redistribution rate after flowering. C and D, changes in hydrogen peroxide (H_2O_2) accumulation rate after flowering. E and F, changes in superoxide anion ($O_2^{-}\cdot$) accumulation rate after flowering. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. Bars mean SD (n=3).

Table 1 Sequence characteristics of wheat ATG8s members[1)]

Name	ID	Len	pI	MW (Da)	GRAVY	Subcellular localization
Ta2A_ATG8a	TraesCS2A02G224000.1	119	7.78	13,568.59	–0.427	Cytoplasmic mitochondrial nuclear
Ta2A_ATG8b	TraesCS2A02G468300.1	119	9.07	13,603.79	–0.328	Cytoplasmic mitochondrial
Ta2B_ATG8c	TraesCS2B02G274200.1	119	7.78	13,568.59	–0.427	Cytoplasmic mitochondrial nuclear
Ta2B_ATG8d	TraesCS2B02G491200.1	119	9.07	13,616.79	–0.351	Cytoplasmic mitochondrial nuclear
Ta2D_ATG8e	TraesCS2D02G229900.1	119	7.78	13,568.59	–0.427	Cytoplasmic mitochondrial nuclear

[1)] Len, length, referring to the length of a protein or nucleic acid sequence, which is the number of amino acids or nucleotides in the sequence; pI, isoelectric point, the pH value at which the net charge of the protein molecule is zero; MW, molecular weight, usually referring to the molecular weight of a protein; GRAVY, grand average of hydropathy, representing the overall hydrophobicity of the protein, a positive value indicates a more hydrophobic nature, while a negative value indicates a more hydrophilic nature.



Fig. 5 Sequence alignment of TaATG8a–e.

significantly positively correlated with chlorophyll. Among the major categories, the correlation between H_2O_2 and $O_2^{-}\cdot$ was the highest, while correlations among photosynthesis indices and nitrogen content were high.

3.5. Correlation among nitrogen level, physiological indicators, and yield

Nitrogen application reduced ROS production and extended the photosynthetic period, significantly delaying leaf senescence and promoting grain yield. Nitrogen application positively regulated photosynthesis, prolonging the active period, and enhancing nitrogen redistribution in the flag leaves during the mid-to-late grain filling stage. This resulted in increased total nitrogen, free amino acid, and soluble protein content in the N_{240} treatment, which contributed to higher yield. However, autophagy-related genes (*TaATG8s*)

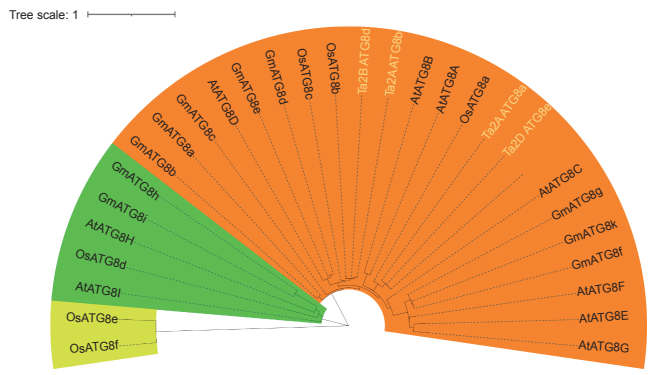


Fig. 6 Maximum likelihood trees of the ATG8 family in four different species: *Arabidopsis*, rice, soybean, and wheat.

were upregulated during senescence, promoting nitrogen remobilization from aging tissues. In the N_0 treatment, although nitrogen redistribution occurred earlier, the lower photosynthetic activity limited source strength, reducing redistribution intensity. Consequently, soluble protein and total nitrogen content were lower in the N_0 group, negatively impacting yield. Overall, nitrogen influences wheat yield by balancing the positive effects on photosynthesis and nitrogen redistribution with the negative impact of autophagy and ROS (Fig. 9).

4. Discussion

4.1. Role of *TaATG8* genes in nitrogen remobilization

According to the *TaATG8* genes expression pattern analysis in this study, at 35 days after flowering, *TaATG8a–e* expression level in the N_0 treatment group was higher than that in the N_{240} treatment group (Fig. 8), indicating that autophagy-related genes may play a key role in the transfer of nitrogen compounds from senescent leaves to developing grain. Previous studies reported that autophagy is activated during the aging of *Arabidopsis* leaves, and *ATG8* is involved in the formation of autophagosomes (Wang and Schippers 2019). Additionally, the increase in autophagy activity promotes leaf senescence and cell death (Avila-Ospina *et al.* 2014), which suggests that *TaATG8a–e* may play an important regulatory role in wheat leaf senescence. However, future studies are needed to investigate the nitrogen redistribution mechanism in aging plants and the regulatory mechanism of nitrogen availability on autophagy gene expression.

4.2. Correlation between nitrogen and leaf senescence

Nitrogen deficiency can accelerate the aging of leaves and increase nitrogen cycling and reuse, while higher or optimal nitrogen concentrations can promote leaf growth and maintain a green state (Diaz *et al.* 2005). Under normal circumstances, ROS are a key components in the response mechanism of plants to external stress (Signorelli *et al.* 2008; Görlach *et al.* 2015). The accumulation of ROS is influenced

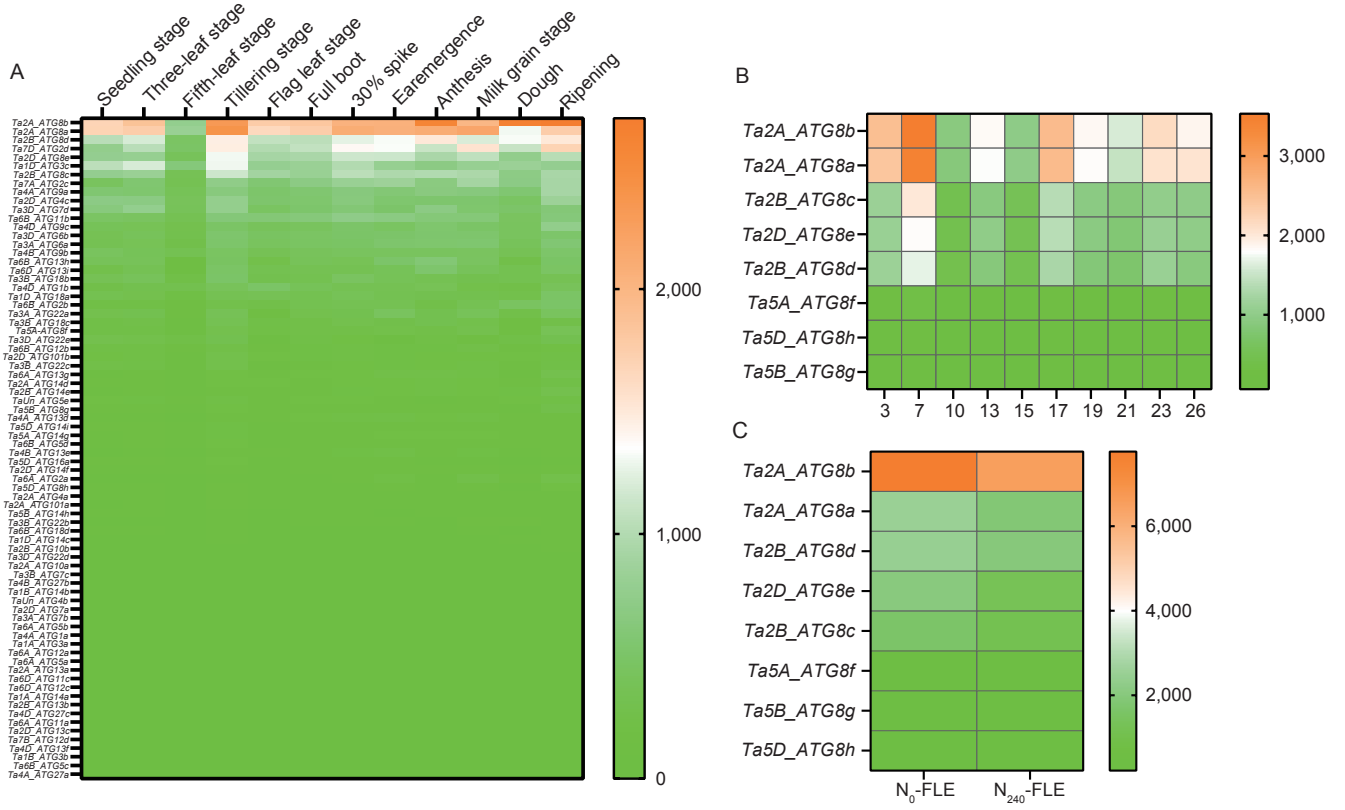


Fig. 7 Expression profiles of wheat *ATG* genes. A, expression of wheat *ATG*s in flag leaves over time. B, expression of wheat *ATG8*s during flag leaf senescence; the horizontal axis represents days after anthesis. C, expression of wheat *ATG8*s at 21 days after anthesis under different nitrogen application levels. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. FLE, flag leaf.

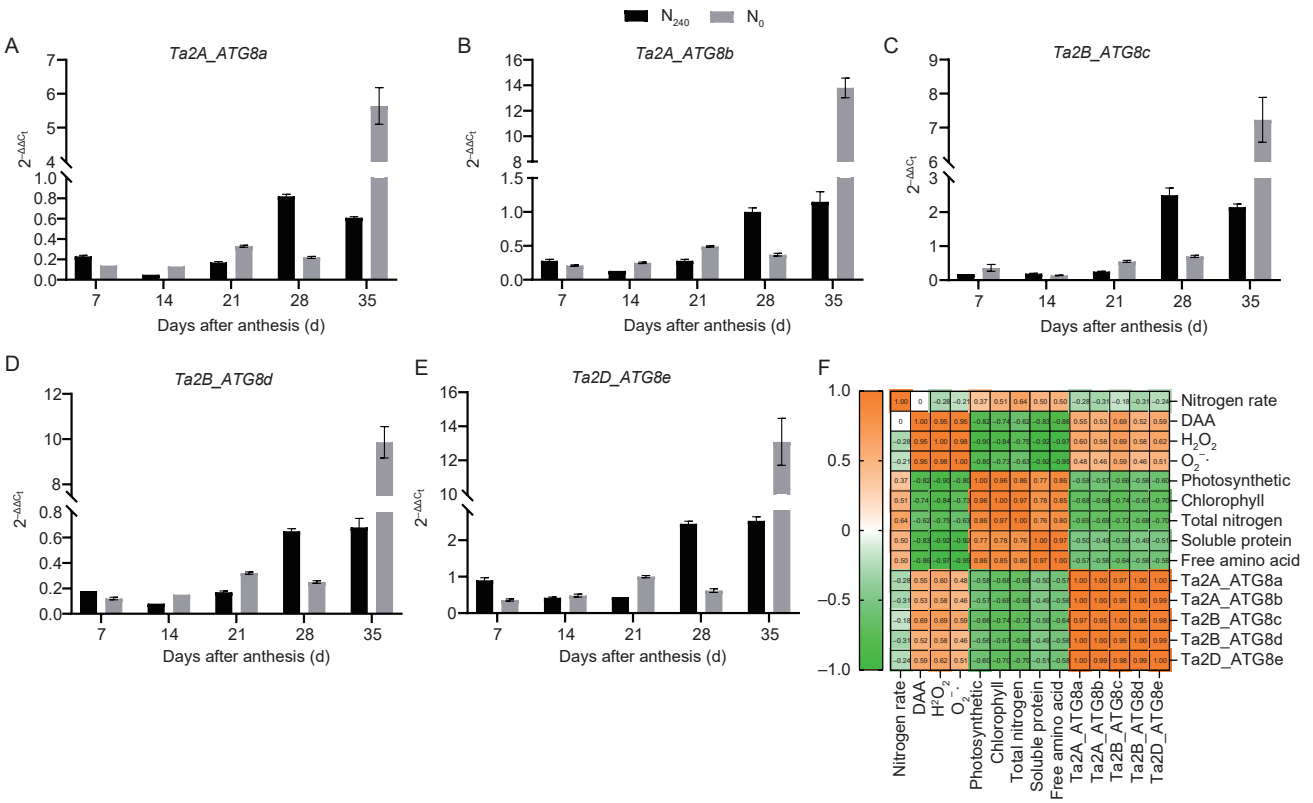


Fig. 8 qPCR results of *TaATG8*s in wheat flag leaves. A–E, expression levels of *Ta_ATG8a–e*. F, Pearson correlation heatmap between expression level and various indicators. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely. DAA, days after anthesis. Bars mean SD (n=3).

by autophagy activity (Signorelli *et al.* 2008). Therefore, there is a need for future studies to investigate the role of ROS in autophagy, and auxin signaling pathways are crucial for understanding the mechanism of leaf senescence.

4.3. Autophagy key role in nitrogen recycling

Autophagy is associated with nitrogen remobilization during flag leaf senescence and grain filling (Masclaux-Daubresse *et al*. 2008) and plays a key role in nutrient cycling, particularly nitrogen fertilization (Guiboileau *et al.* 2013; Pottier *et al.* 2014). Previous studies have revealed that many *ATG*s are involved in nitrogen transport, particularly under nitrogen deficiency (Masclaux-Daubresse and Chardon 2011; Islam *et al.* 2016; Wang *et al.* 2017). In the present study, *TaATG8*s responded considerably to nitrogen starvation during the senescence of wheat flag leaves, suggesting that under nitrogen deficiency, plants regulate energy metabolism and maintain nitrogen levels required for growth by increasing autophagy.

4.4. Correlation between relative chlorophyll degradation and flag leaf senescence

Chlorophyll decomposition is one of the most significant characteristics of senescence, leading to chlorosis (Krautler 2016). The present study revealed that from 14 days after flowering, the chlorophyll content in the N_{240} and N_0 treatment groups gradually decreased, marking the onset of flag leaf senescence. Additionally, the correlation among chlorophyll content, photosynthetic rate, and time follows a quadratic function model, suggesting that time reduces photosynthesis in two dimensions, thereby promoting senescence. Senescence is the final stage of leaf development, and plants that delay senescence typically exhibit stronger photosynthetic capacity, which in turn increases grain yield and nitrogen use efficiency (Kamal *et al.* 2019). In this study, the flag leaves of wheat in the N_{240} and N_0 treatment groups exhibited significant differences in free amino acid, soluble protein, and total nitrogen content after flowering (Figs. 2 and 3), indicating that delaying leaf senescence or extending the green period can effectively prolong the functional period of leaves, enhance source-sink capacity, and thus increase crop yield (Gregersen *et al.* 2013). For instance, delaying leaf senescence can increase the average yield of wheat by 6–28% (Christopher *et al.* 2008), while the average yield of rice can increase by 10% (Mao *et al.* 2017). Leaf senescence in wheat plants is a unique trait. Although previous studies have revealed that the redistribution of nitrogen from leaves to developing seeds accelerates plant senescence and shortens the grain-filling period, this process and its underlying mechanisms have not been fully studied. Suppose the regulation of nitrogen use efficiency affects leaf senescence. In that case, future studies are needed to elucidate the physiological process of leaf senescence in wheat plants and to provide new insights into improving wheat yield and nitrogen use efficiency.

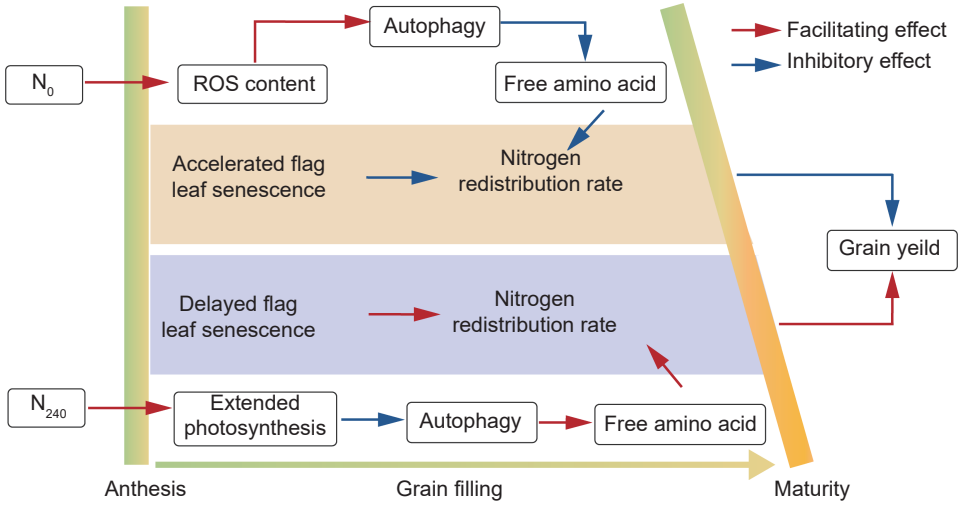


Fig. 9 Schematic diagram of the interactions between various indicators and wheat yield during the grain filling stage, including reactive oxygen species (ROS), autophagy-related genes, photosynthesis, free amino acids, and yield. N_0 and N_{240}, 0 and 240 kg ha^{-1}, respectilvely.

5. Conclusion

Our study demonstrates that nitrogen accumulation in wheat flag leaves peaks at 7–14 DAA, followed by remobilization to the grains. This process is closely coordinated with leaf senescence. Specifically, nitrogen deficiency accelerates senescence and upregulates the expression of autophagy-related genes, particularly *TaATG8s*, at 21–28 DAA. These findings suggest that *TaATG8s* play a crucial regulatory role in nitrogen remobilization and flag leaf senescence, offering a potential target for improving nitrogen use efficiency in wheat.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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