

Mitochondrial uncoupling protein contributes to the regulation of carbon and nitrogen metabolism and seed yield under low nitrogen stress in Arabidopsis

Xinyan Qiao

Lanzhou University

Tao Yu

Lanzhou University

Mengjiao Ruan

Lanzhou University

Chaiyan Cui

Lanzhou University

Cuiyun Chen

Cold and Arid Regions Environmental and Engineering Research Institute: Northwest Institute of Eco-Environment and Resources

Yuanzhi Zhu

Lanzhou University

Fanglin Li

Lanzhou University

Shengwang Wang

Lanzhou University

Xiaofan Na

Lanzhou University

Xiaomin Wang

Lanzhou University

Yurong Bi (✉ yrbi@lzu.edu.cn)

Lanzhou University <https://orcid.org/0000-0003-2412-3673>

Research Article

Keywords: Alternative oxidase, Carbon metabolism, Low nitrogen, Nitrogen assimilation, Uncoupling protein

Posted Date: May 5th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-437717/v1>

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Abstract

Nitrogen (N) availability is a critical factor for plant development and yield, and it closely correlates to carbon (C) metabolism. Uncoupling protein (UCP) and alternative oxidase (AOX) in mitochondria exhibit a strong correlation with N and C metabolism. Here, we investigated the respective functions of UCP and AOX by using *ucp1*, *aox1a* and *aox1a/ucp1* mutants under low-N condition. Low-N markedly increased *AOX1a* and *UCP1* expression, alternative pathway capacity and UCP activity. Eight-day-old *aox1a/ucp1* seedlings were more sensitive to low-N condition than Col-0 and single mutants, *i.e.*, the lower primary root length and higher anthocyanin accumulation. The net photosynthetic rate (A) and electron transport rate (ETR) under low-N stress were markedly decreased in *ucp1* and *aox1a/ucp1* compared to those in Col-0, but not in *aox1a*. The leaf area, total C and N content in shoots showed a similar profile as A and ETR. Nitrate acquisition rate was accelerated in *aox1a/ucp1*, but its transport activity from root to shoot was decreased, which resulted in the low nitrate content and nitrate reductase activity in shoots upon low-N condition. Additionally, the low C and N content in *ucp1* and *aox1a/ucp1* seeds led to the decreased seed yield compared to Col-0 upon low-N stress. RNA-seq analysis revealed that a large number of genes involved in photosynthesis, C/N metabolism were markedly down-regulated in *aox1a/ucp1* compared to Col-0 under low-N stress. These results highlight the more key role of *UCP1* than *AOX1a* in modulating C and N metabolism and seed yield in Arabidopsis adaptation to low-N stress.

Introduction

The availability of nitrogen (N) in soil is essential for plant growth and defense [1–3]. However, plants often encounter limited N sources under nature environmental conditions, which leads to chlorophyll degradation, decreased photosynthesis and protein synthesis [4, 5]. Moreover, application of N fertilizers results in contamination of groundwater and production of nitrogenous greenhouse gases [6, 7]. Ammonium (NH_4^+) and Nitrate (NO_3^-) are two major forms of useable N for plants. NH_4^+ can be directly assimilated into amino acids by glutamine synthetase and glutamate synthase [8]. However, NO_3^- is first reduced to nitrite (NO_2^-) by nitrate reductase (NR) in cytoplasm using NAD(P)H as the electron donor; then NO_2^- is reduced to NH_4^+ by nitrite reductase (NiR). The reduction of NO_2^- and assimilation of NH_4^+ occur in plastids/chloroplasts and the reduced ferredoxin is used as the reductant [8]. Inhibition of the reductant output via the malate-oxaloacetic acid (Mal-OAA) shuttle from chloroplasts decreases the NO_3^- assimilation rate [9, 10]. Therefore, plants need to balance the photosynthetic energy partition between carbon (C) and N assimilation at different development stages.

N assimilation is in closely related to C metabolism. The balance of N and C in plants is essential for the optimal growth and development [5]. NO_3^- availability affects transcriptions of a variety of genes involved in C metabolism, while C depletion decreases protein biosynthesis and alters N metabolism [11, 12]. It is well accepted that low N inevitably results in low photosynthetic capacity and yield in plants [5]. Photosynthesis and respiration provide not only the reducing power but also C skeletons (2-oxoglutarate, citrate, and isocitrate) for N assimilation [9, 13]. Many reports have confirmed that photosynthesis and

respiration rates highly correlate to the intracellular N level [5, 9, 10]. Glycolytic pathway and the tricarboxylic acid cycle could be up-regulated in plant adaptation to low N condition [11, 14, 15]. Additionally, the capacity of alternative electron transport pathways (aETPs) in the respiratory chain also exhibits a strong correlation with the intracellular NO_3^- level [9, 10, 16]. aETPs are essential for plant adaptation to various stresses [17–19]. Particularly, they play key roles in maintaining a high photosynthetic capacity by dissipating the excess reductant from chloroplasts [10, 20]. However, it is not well understood how aETPs coordinate and contribute to optimizing the C metabolism and N assimilation under low-N stress in plants.

Alternative pathway (AP) and uncoupling pathway are two major pathways of aETPs, which are mediated by alternative oxidase (AOX) and the uncoupling protein (UCP), respectively. AOX can directly transport electron from ubiquinone to O_2 , but it did not transfer H^+ across the mitochondrial inner membrane, thus dissipating energy as heat [21]. Comparatively, UCP increases the permeability of H^+ across the mitochondrial inner membrane, which destroys the H^+ gradient and decreases the ATP formation [22]. In Arabidopsis, five AOX genes (*AOX1a*, *AOX1b*, *AOX1c*, *AOX1d*, *AOX2*) and six UCP genes (*UCP1-6*) have been reported [23]. Recently, it was found that the *AOX1a* or *UCP1* mutation can alter foliar N and C assimilation rates in Arabidopsis fed with NO_3^- or NH_4^+ [9]. Moreover, the transcripts of *AOX1a* and *UCP1* are significantly up-regulated by low-N stress [10, 16]. Excessive carbohydrates are preferentially respired by AOX, which suppresses the increase of the C/N ratio upon low-N condition [15, 24]. However, it is indicated that AOX induced by low-N condition does not play important roles on C/N ratio regulation [16]. Sweetlove et al. [20] reported that the *UCP1* mutation markedly decreases the AOX protein content, but does not affect other aETPs in *ucp1* plants. Moreover, UCP1 is essential for the efficient photosynthesis mainly through maintaining photorespiratory rate in Arabidopsis [20]. However, it is still unknown whether UCP1 participates in regulating C/N ratio upon low-N condition at the whole plant level; and the respective contribution and coordination of UCP1 and AOX in Arabidopsis adaptation to low-N stress remain unclear.

Although above promising results have been reported, the authors rarely analyzed the effects of *AOX1a* and *UCP1* mutation on the C/N ratio and yield of seeds as well as their respective roles in plant adaptation to low-N condition. The aim of this study is to clarify the functions of *AOX1a* and *UCP1* in Arabidopsis tolerance to low-N stress by using the *aox1a*, *ucp1* and *aox1a/ucp1* mutants. In particular, we focused on the effects of *AOX1a* and *UCP1* mutation on C and N assimilation processes, photosynthetic capacity, C/N ratio, and seed yield.

Materials And Methods

Plant materials and growth conditions

Arabidopsis wild type (Col-0) and the T-DNA insertion lines of AOX family genes [*aox1a* (Salk_084897), *aox1c* (CS804611, CS877307), *aox1d* (CS390166), *aox2* (CS766955)] and UCP family genes [*ucp1* (CS821384), *ucp2* (Salk_037074), *ucp3* (Salk_006106), *ucp4* (CS373159), *ucp6* (Salk_111403C)] were

purchased from the Arabidopsis Biological Resource Center (ABRC). *aox1b* point mutants [*aox1b* (1-2-5), *aox1b* (14-9-7)] were provided by Dr. Aigen Fu at Northwest University, China. The *aox1a/ucp1* double mutant was generated by crossing *aox1a* and *ucp1*. The *aox1a* mutant was identified by using two primer pairs (Salk_084897-RP and LBb1; Salk_084897-RP and Salk_084897-LP). The *ucp1* mutant was identified by using two primer pairs (CS821384-RP and LB1; CS821384-RP and CS821384-LP).

For the experiments at seedling stage (0-10 d), the seeds were vernalized at 4°C for 48 h, then germinated and cultured in half-strength solid MS (1/2 MS) medium (pH 5.8). For the experiments at the vegetative stage and the reproductive stage, the seedlings were cultured with the hydroponic method [25]. After vernalized at 4°C for 48 h, the seeds were germinated and grew in the germination solution for 10 d. Then the seedlings were transferred to the growth solution. All the solutions were replaced every 2 d. The seedlings were cultured in a growth chamber with 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity, 60-70% humidity and a 16 h/8 h light/dark cycle at $22 \pm 2^\circ\text{C}$.

Generation of *proAOX1a: GUS* and *proUCP1: GUS* transgenic lines

The promoter sequences of *AtAOX1a* and *AtUCP1* were amplified by using the primer pairs (*proAOX1a*-FP and *proAOX1a*-RP; *proUCP1*-FP and *proUCP1*-RP), respectively. Then, they were cloned into the expression vector pBIB-GUS by using the Gateway technology. *Agrobacterium*-mediated transformation was used to deliver the recombinant plasmids into Col-0 by floral-dipping method. The T₀ seeds were screened for positive lines by using the herbicide Basta.

Low nitrogen treatment

For the low nitrogen experiment at the seedling stage (0-10 d), NH_4NO_3 and KNO_3 were removed from the 1/2 MS medium, then KNO_3 as the only nitrogen source was added at the concentrations of 0, 0.1 mM, and 1 mM, which were denoted as 0 N, 0.1 N, and 1 N medium, respectively, in this study. The depleted K^+ was supplied with KCl. The seeds were germinated and grew in 0 N, 0.1 N, or 1 N medium for treatments.

For the low nitrogen experiment of the hydroponically grown plants, NH_4NO_3 and $\text{Ca}(\text{NO}_3)_2$ were removed from the growth solution, and KNO_3 was added at concentrations of 1mM (low) or 5 mM (moderate), which were denoted as 1 N or 5 N medium, respectively. The depleted Ca^{2+} was supplied with CaCl_2 . The 14-day-old seedlings were used for experiments.

The extraction of DNA and RNA and qRT-PCR analysis

Genomic DNA was extracted from Arabidopsis leaves on the basis of Tan et al. [26]. The extraction of total RNA and subsequent cDNA synthesis were performed by using the Plant RNA Extraction Kit (Thermo Fisher) and the Prime Script RT Reagent Kit with gDNA Eraser Kit (TRANS), respectively. qRT-PCR were carried out by using Transtart Green qPCR Super Mix Kit (TRANS). The primers are described in Table. S1. The qRT-PCR was programmed as 94°C for 2 min; 94°C for 30 s; 58°C for 15 s; 72°C for 20 s with 40

cycles according to the ABI PRISM7500 real-time PCR system (China). The gene expressions were normalized to *ACTIN2* (AT3G18780) and analyzed by using the $2^{-\Delta\Delta Ct}$ method.

Analysis of anthocyanin content

Anthocyanin content was determined as described previously by Vandebussche et al.[27]. 0.1 g of fresh tissues were put into a tube containing 600 μ l of extract buffer [methanol/HCl (v/v)=99/1] for 24 h at 4°C in dark. 400 μ l of chloroform and 400 μ l of H₂O were added in sequence and then centrifuged at 10,000 *g* for 10 min. The absorbance at 530 nm was recorded.

Measurement of nitrate content

The nitrate content was determined according to Cataldo et al. [28]. 0.3 g of fresh tissues were ground with ddH₂O. After treated for 30 min at 100°C, the homogenate was centrifuged at 13,000 *g* for 15 min. The supernatant was mixed with 5% salicylic acid. After 20 min, 2 M NaOH were added. The absorbance at 410 nm was recorded.

Measurement of respiratory rates

The 14-day-old hydroponically grown seedlings were grown under control (11 N) or low nitrogen (1 N) conditions for 16 d. The shoots were collected and used for isolation of mitochondria [29] and the analysis of alternative pathway capacity (V_{alt}) and UCP activity [30]. The O₂ consumption was detected in a Chlorolab II liquid-phase oxygen electrode (Hansatech, UK) in 1.5 ml of reaction buffer [10 mM TES (pH 7.5), 0.3 M mannitol, 5 mM KH₂PO₄, 2 mM MgSO₄, 10 mM NaCl and 0.1% BSA]. For the measurement of V_{alt} , 5 mM pyruvate, 10 mM succinate, 10 mM malate, 0.15 mM ADP, 1 mM NADH, 10 mM glutamic acid, 2 mM KCN were added. KCN was used to inhibit the cytochrome *c* oxidase activity. For UCP activity analysis, 10 mM malate, 0.15 mM ADP, 10 mM glutamic acid, 10 μ M linoleic acid (LA), oligomycin (2.5 μ g/mg protein), 2 mM GDP (added or not) were added. LA and GDP are the activator and inhibitor of UCP, respectively.

Determination of chlorophyll (Chl) contents

The Chl was extracted with the solution [80% acetone/80% ethanol (v/v) = 1/1, 31]. Chlorophyll content was calculated based with the following formulas.

$$\text{Chl a (mg/L)} = 12.63 \times \text{OD}_{664.5} - 2.52 \times \text{OD}_{647};$$

$$\text{Chl b (mg/L)} = 20.47 \times \text{OD}_{664.5} - 4.73 \times \text{OD}_{647}.$$

Measurement of photosynthetic fluorescence parameters

Photosynthetic fluorescence parameters were analyzed using the LI-6400XT photosynthesis system (LI-COR). The leaf temperature was maintained at 22°C during measurements. Photosynthesis was

determined under ambient growth condition by using 300 PAR light intensity and 400 ppm CO₂. The photosynthetic light-response curve was determined at 0-1200 PAR light intensity and 400 ppm CO₂.

Measurement of C and N contents

The contents of C and N were analyzed by using a C/N element analyzer (Elementar vario EL cub). The dry samples were powdered and used for analysis.

GUS staining assay

GUS staining assay was performed as described by Pelagio-Flores et al. [32]. Briefly, the *proAOX1a::GUS* and *proUCP1::GUS* transgenic lines were incubated in the staining buffer containing 0.1 M sodium phosphate (pH 7.0), 1 mM X-Gluc, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], 10 mM EDTA, and 0.1 % Triton X-100 for 3 h at 37°C. After destained with 75% ethanol, samples were photographed with Nikon camera or Olympus stereoscopic microscope.

Determination of nitrate reductase (NR) and nitrite reductase (NiR) activity

The NR activity was analyzed as described by Du et al. [33]. The fresh tissues were homogenized with the buffer [0.1 M HEPES-KOH (pH 7.5), 3 % polyvinylpolypyrrolidone, 1 mM EDTA and 7 mM cysteine], and centrifuged at 10,000 *g* for 10 min. The supernatant was used for NR activity analysis.

The activity of NiR was determined according to Datta and Sharma [34]. Leaves were homogenized with the buffer [50 mM phosphate buffer (pH 8.8), 3% BSA, 1 mM EDTA and 25 mM cysteine]. Homogenate was centrifuged at 13,000 *g* for 15 min at 4°C. The supernatant was used directly for NiR activity measurement.

Determination of nitrate uptake and transport

The uptake and transport of nitrate were analyzed by using ¹⁵N-KNO₃ (Sigma-Aldrich 57654-83-8) according to Gandi et al. [9]. 14-day-old seedlings were treated for 10 d on low-N (1 N) condition and then 24 h on 0 N condition, then transferred to the growth medium containing 10 mM ¹⁵N-KNO₃ (CK) or 1 mM ¹⁵N-KNO₃ (1 N) for 6 h. After 6 h of labelling, samples were washed and oven-dried, and then ground to fine powder. Total ¹⁵N enrichment was measured using a gas stable isotopic ratio mass spectrometer (IRMS).

RNA-Seq analysis

The 14-day-old hydroponically grown seedlings were treated under control (11 N) or low nitrogen (1 N) conditions for 16 d. The shoots were collected and used for the high-throughput sequencing in Bai Mike Company (<http://www.biocloud.net/>). Gene expression levels were estimated by fragments per kilobase of transcript per million fragments (FPKM) mapped. Differential expression analysis of two groups was performed using the DESeq2 [35]. The *p* values were adjusted using the Benjamini and Hochberg's

approach for controlling the false discovery rate (FDR). Genes with an adjusted $p < 0.01$ found by DESeq2 were assigned as differentially expressed. The $FDR < 0.01$ and $|\log_2(FC)| \geq 2$ were set as the threshold for significantly differentially expressed genes (DEGs). The KOBAS software was used to test the statistical enrichment of DEGs in KEGG pathways [36].

Statistical analysis

The data were expressed as means \pm SD ($n \geq 3$). All statistical analysis were conducted via one-way ANOVA and SPSS17.0. In the figures, the lowercase letters indicate the significant difference at $P < 0.05$.

Results

Double mutant *aox1a/ucp1* has compromised tolerance to low nitrogen stress

It has been reported that *ALTERNATIVE OXIDASE 1a* (*AOX1a*) and *UNCOUPLING PROTEIN 1* (*UCP1*) respond to low nitrogen (N) stress at the transcriptional level in plants [16]. To further investigate the function of *AOX* and *UCP* genes upon low-N condition, the physiological responses of *AOX* mutants [*aox1a* (Salk_084897), *aox1c-1* (CS804611), *aox1c-2* (CS877307), *aox1d* (CS390166) and *aox2* (CS766955)] and *UCP* mutants [*ucp1* (CS821384), *ucp2* (Salk_080118C), *ucp3* (Salk_006106), *ucp4* (CS373159), *ucp6* (Salk_111403C)] were analyzed under low-N condition. Fig. S1 and S2 showed that low N (0.1 N) resulted in the increased primary root length, accumulation of anthocyanin, but decreased fresh weight in Col-0 and the mutants. However, there were no significant differences among them. qRT-PCR results showed that low N significantly induced the expressions of *AOX1a*, *AOX1c* and *AOX2*, which were increased by 9.3-fold, 5.6-fold and 17.0-fold, respectively; while *AOX1b* and *AOX1d* expressions were decreased (Fig. S1d). Similarly, the transcripts of *UCP1*, *UCP2* and *UCP3* were increased by 1.8-fold, 2.0-fold and 1.3-fold, respectively; but *UCP4*, *UCP5* and *UCP6* transcripts were decreased (Fig. S2d). The highest expression of *AOX1a* in the *AOX* family and *UCP1* in the *UCP* family suggests their essential function in Arabidopsis tolerance to low N.

To explore the function and relationship of *AOX1a* and *UCP1* in Arabidopsis tolerance to N deficiency, the *aox1a* and *ucp1* were crossed to generate the double mutant *aox1a/ucp1*. PCR analysis confirmed the screened homozygous lines (Fig. S3). Phenotypic analysis of 8-day-old single and cluster seedlings showed that there was no difference among Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* on 1/2 MS and 1 N conditions (Fig. 1a). However, under 0.1 N and 0 N conditions, the primary root length of *aox1a/ucp1* seedlings was shorter than that of Col-0 seedlings (Fig. 1a, d), and the difference reached the maximum at day 8 (Fig. S4). Under 0.1 N condition for 8 d, the primary root length of *aox1a/ucp1* was reduced by 25.4% compared to Col-0 (Fig. 1d). Moreover, the anthocyanin content and the purple rate of *aox1a/ucp1* cotyledons were higher than that of Col-0 and single mutants under 0.1 N condition (Fig. 1b, c, e). The cotyledon purple rate of Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* seedlings was 60.2%, 58.4%, 61.1%, and 82.8%, respectively (Fig. 1e). Thus, *AOX1a* and *UCP1* might be collaboratively involved in the adaptation of Arabidopsis seedlings to low-N stress.

UCP1 plays more important roles in regulating photosynthesis than AOX1a upon low-N condition

To further investigate the mechanism of AOX1a and UCP1 involvement in Arabidopsis adaption to low-N stress, we analyzed the rosette leaf area, shoot biomass and photosynthetic capacity of Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* seedlings. In order to accurately control the N concentration in the medium, seedlings were grown hydroponically. Results showed that the rosette leaf area, fresh weight (FW) and dry weight (DW) of *ucp1* and *aox1a/ucp1* seedlings were markedly lower than that of Col-0 under moderate-N (5 N) and low-N (1 N) conditions, while *aox1a* had no significant difference compared to Col-0 (Fig. 2). Under low-N condition, the rosette leaf area, FW and DW in *ucp1* were reduced by 24.4%, 29.0% and 27.7%, respectively, and by 44.6%, 26.3%, and 27.3% in *aox1a/ucp1*, respectively, compared to Col-0 (Fig. 2b-d). These results indicate that loss of UCP1 function results in more significant inhibition of growth than loss of AOX1a function under low-N condition.

The total chlorophyll content (chl_t) in *ucp1* and *aox1a/ucp1* plants were decreased by 7.4% and 9.2%, respectively, in comparison with that in Col-0 upon low-N condition. However, in *aox1a*, it did not change (Fig. 3a). Under the control condition, the net photosynthetic rate (A) and electron transfer rate (ETR) in *ucp1* were significantly lower than that in Col-0. However in *aox1a* and *aox1a/ucp1* plants, A and ETR were not different from that in Col-0 (Fig. 3b, c). Under low-N condition, A and ETR in *aox1a* was similar to that in Col-0. In contrast, they were significantly lower in *ucp1* and *aox1a/ucp1* than that in Col-0. A and ETR were reduced by 27.4% and 31.0%, respectively, in *ucp1*, and by 29.8% and 26.5%, respectively, in *aox1a/ucp1* (Fig. 3b, c). The analysis result of light response curve showed that the light saturation point in *ucp1* was markedly lower than that in Col-0 when the light intensity exceeded 200 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (PAR) under the control condition (Fig. 3d). Under low-N condition, the light saturation point in both *ucp1* and *aox1a/ucp1* plants were significantly lower than that in Col-0 and *aox1a*, but there was no difference between *ucp1* and *aox1a/ucp1* (Fig. 3e). These results indicate that *aox1a/ucp1* and *ucp1* plants have low photosynthetic capacity under low-N stress. UCP1 plays more important roles in regulating photosynthesis than AOX1a does in Arabidopsis tolerance to low-N stress.

Mutation of AOX1a and UCP1 decreases C and N contents in the shoots of *aox1a/ucp1*

Above results confirmed that mutation of *AOX1a* and *UCP1* affects the photosynthetic capacity of *aox1a/ucp1*. We subsequently analyzed the changes of C and N contents under low-N stress. Upon low-N and moderate-N conditions, the percentage of C in Col-0, *aox1a*, *ucp1*, and *aox1a/ucp1* shoots was decreased. Interestingly, there was a marked difference only between *ucp1* and Col-0 under 1 N condition (Fig. 4a). Low N similarly decreased the percentage of N in all plants (Fig. 4b). The total C and N contents in *ucp1* and *aox1a/ucp1* shoots were significantly lower than that in Col-0 upon low-N condition (Fig. 4c, d), which were reduced by 21.6% and 26.1%, respectively, in *ucp1* and by 24.2% and 23.11%, respectively, in *aox1a/ucp1* under 5 N condition. Under 1 N condition, the total C and N contents were reduced by 29.6% and 28.6%, respectively, in *ucp1* and by 27.0% and 27.9%, respectively, in *aox1a/ucp1*. However, in *aox1a*, the total C and N contents were similar to that in Col-0 under all N conditions. Thus, the photosynthetic C fixation and N assimilation capacity in *ucp1* and *aox1a/ucp1* were lower than that in

Col-0 and *axo1a* plants under low-N condition, and this explained the decreased biomass in *ucp1* and *axo1a/ucp1* plants.

Expression patterns of AOX1a and UCP1 and changes of respiratory rates upon low-N condition

To investigate the expression patterns of *AOX1a* and *UCP1* upon low-N condition, the *proAOX1a:GUS* and *proUCP1:GUS* transgenic lines were generated. GUS staining results showed that 0.1 N treatment induced marked increase of *AOX1a* and *UCP1* expression in 6-day-old seedlings. *AOX1a* was expressed in the whole seedling, whereas *UCP1* was mainly expressed in cotyledons and the maturation zone of primary roots (Fig. 5a-d). Under low-N condition, the *AOX1a* expression was higher than *UCP1* in rosette leaves (Fig. 5e-h). At the reproductive stage, low N also increased the expression of *AOX1a* and *UCP1* in flowers and stamens (Fig. 5i-p). Specifically, the expression of *AOX1a* was mainly increased in the anthers of stamens (Fig. 5n), while *UCP1* was mainly expressed in the filaments of stamens (Fig. 5p).

qRT-PCR results reported that low N induced the expression of *AOX1a* and *UCP1* at almost every developmental stage. When Arabidopsis seeds were germinated and grown for 6 d in 0.1 N medium, the expression of *AOX1a* and *UCP1* were enhanced by 9.3-fold and 1.8-fold, respectively (Fig. 5q). In rosette leaves, the response of *AOX1a* expression to low-N condition was higher than that of *UCP1* (Fig. 5r). However, in flowers and siliques, the *AOX1a* expression increased about 1.5-fold and 2.1-fold upon low-N condition, respectively, while the *UCP1* expression was increased by 1.7-fold and 2.3-fold (Fig. 5s, t).

To clarify the relationship between *AOX1a* and *UCP1* as well as the effect of low N on the alternative pathway capacity (V_{alt}) and UCP pathway activity (V_{ucp}), the respiratory rates were analyzed. Fig. S5 showed that low N significantly increased V_{alt} and V_{ucp} in Col-0. Under high-N (control) condition, V_{alt} in *ucp1* was decreased by 84.3% and V_{ucp} in *axo1a* was decreased by 42.1% compared to Col-0. Interestingly, the mutation of *UCP1* in *ucp1* stimulated the V_{alt} by 63.5% upon low-N condition (Fig. S5a). Similarly, V_{ucp} was also increased in *axo1a* and Col-0 upon low-N condition. Thus, there should be the close relationship between *AOX1a* and *UCP1* under normal growth condition.

Mutation of AOX1a and UCP1 affects N metabolism upon low-N condition

To investigate the effects of *AOX1a* and *UCP1* mutations on N metabolism in Arabidopsis in response to low N, the differences of the uptake, assimilation and transport of N were analyzed in Col-0, *axo1a*, *ucp1*, *axo1a/ucp1* plants. The results showed that low N decreased the NO_3^- content and NR activity, but increased NiR activity in Col-0, *axo1a*, *ucp1* and *axo1a/ucp1* shoots (Fig. 6a-c). Compared to Col-0 under 1 N condition, the NO_3^- content, NR and NiR activities were reduced by 11.5%, 9.5%, 15.0%, respectively, in *ucp1* and by 34.0%, 21.8%, 13.3%, respectively, in *axo1a/ucp1*; however, there was no significant difference between *axo1a* and Col-0.

To analyze the uptake rate of NO_3^- and the transport from root to shoot, Arabidopsis seedlings were starved under 0 N condition for 24 h, and then transferred to the growth solution containing 10 mM ^{15}N -

KNO₃ (control) or 1 mM ¹⁵N-KNO₃ (1 N) for 6 h. Results showed that low N decreased the uptake rate of NO₃⁻ in Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* roots (Fig. 6d). However, compared to Col-0, the uptake rate of NO₃⁻ in *aox1a*, *ucp1* and *aox1a/ucp1* were elevated by 15.4%, 16.7% and 21.6%, respectively, under the control condition, and by 6.9%, 12.0% and 21.2%, respectively, under 1 N treatment (Fig. 6d). In contrast, the transport activity of NO₃⁻ from root to shoot in *aox1a*, *ucp1* and *aox1a/ucp1* was significantly decreased compared to Col-0 under both control and low-N conditions (Fig. 6e). Based on these results, although mutations of *AOX1a* and *UCP1* resulted in acceleration of NO₃⁻ uptake under low-N stress, the low assimilation rate and transport activity of N in *aox1a/ucp1* seedlings impaired the N metabolism.

Mutation of AOX1a and UCP1 decreases the seed yield and the C/N content in *aox1a/ucp1* under low-N stress

N deficiency inevitably limits the reproductive growth of plants. Our results showed that low N significantly reduced the silique number and seed yield of Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* plants (Fig. 7a, b). Loss of function of *AOX1a* and *UCP1* resulted in more decrease of silique number and seed yield in *aox1a/ucp1* (by 22.3% and 23.6%, respectively) than that in Col-0. A similar trend was also found in *ucp1* mutant. However, there was no difference between *aox1a* and Col-0 (Fig. 7a, b). Figure 7c, d showed that the percentage of N was decreased in seeds of four genotypes upon low-N condition. However, the percentage of C in seeds had no significant changes compared to the control. Further result confirmed that the *AOX1a* mutation alone in *aox1a* did not affect the total C and N contents in seeds, but in *ucp1* and *aox1a/ucp1* seeds, the total C content was reduced by 21.1% and 21.8%, respectively, and the total N content was reduced by 27.4% and 34.1%, respectively (Fig. 7e, f). Thus, the decrease of C and N distribution in seeds accounted for the low seed yield in both *ucp1* and *aox1a/ucp1* plants.

Distinct transcript profiles in Col-0, *aox1a*, *ucp1* and *aox1a/ucp1* plants under low-N stress

The different physiological response of Col-0, *aox1a*, *ucp1*, and *aox1a/ucp1* seedlings to low-N stress might be caused by differential expressions of downstream genes involved in different metabolic pathways. To test the hypothesis and elucidate the molecular metabolism of *UCP1* and *AOX1a* in Arabidopsis tolerance to low N, we compared gene expression profiles in Col-0, *aox1a*, *ucp1*, and *aox1a/ucp1* shoots by using RNA sequencing. Compared to Col-0, transcriptomes of *aox1a*, *ucp1*, and *aox1a/ucp1* had marked changes (Fig. S6). With thresholds of log₂(FC) ≥ 2 and FDR < 0.01, there were 1605 up-regulated genes and 1559 down-regulated genes in Col-0 upon low-N condition. The up- and down-regulated genes were 1656 and 1153, respectively, in *aox1a*, 687 and 1378, respectively, in *ucp1*, and 2321 and 1796, respectively, in *aox1a/ucp1* (Fig. S6a-d). KEGG functional enrichment analysis indicated that the DEGs in Col-0 were mainly enriched in ribosome, plant hormone signal transduction, biosynthesis of amino acids, and plant-pathogen interaction. DEGs were mainly enriched in ribosome, biosynthesis of amino acids and carbon metabolism in *aox1a*; in plant hormone signal transduction, plant-pathogen interaction and photosynthesis in *ucp1*; and in carbon metabolism, biosynthesis of amino

acids, and photosynthesis in *aox1a/ucp1* (Fig. S6e-f). Thus, mutation of *UCP1* and *AOX1a* in *aox1a/ucp1* markedly disturbed the photosynthesis and carbon metabolism pathways upon low-N condition.

To further investigate the mechanism of decreased resistance to low N in *aox1a/ucp1*, DEGs in Col-0 and *aox1a/ucp1* were analyzed. Venn diagram showed that 1233 and 923 genes were co up- and down-regulated, respectively, in Col-0 and *aox1a/ucp1* (Fig. 8a). KEGG results showed that DEGs in both Col-0 and *aox1a/ucp1* were mainly enriched in ribosome, biosynthesis of amino acids, photosynthesis and N metabolism. DEGs in Col-0 only (1961 genes) were mainly enriched in ribosome, plant hormone signal transduction, plant-pathogen interaction. DEGs only in *aox1a/ucp1* (1008 genes) were mainly enriched in C metabolism, amino acid synthesis, C fixation in photosynthesis organisms, and glycolysis (Fig. 8b-d). The numbers of DEGs involved in the N metabolism, C metabolism, photosynthesis, por and chl metabolism and ribosome were 4, 44, 11, 6, 12, respectively, in *aox1a/ucp1*, but were 2, 3, 1, 2, 57, respectively, in Col-0 (Fig. 8e, f). Figure 8g further showed that most DEGs in both Col-0 and *aox1a/ucp1* in the aforementioned metabolism pathways were generally down-regulated. However, the decrease was more significant in *aox1a/ucp1* than in Col-0 under low-N stress (Fig. 8g). qRT-PCR was performed to validate some of the DEGs in Col-0 and *aox1a/ucp1*. Among the six genes examined, they all showed similar expression patterns as the RNA-seq results (Fig. 8h). Thus, the decreased expressions of genes related to C metabolism, photosynthesis, N metabolism in *aox1a/ucp1* could be one of the reasons for the low photosynthetic capacity and C/N level in seeds.

Discussion

Nitrogen (N) assimilation is essential for leaf metabolism and seed yield. In plants, carbon (C) metabolism are closely related to the N levels and its assimilation capacity [5, 37–38]. The energy generated by light reaction not only supply C fixation, but also N assimilation [8, 9]. Therefore, plants must balance the energy distribution between C and N metabolism. Many reports have indicated that the alternative pathway and the uncoupling protein (UCP) pathway play key roles in decreasing photooxidation damage and maintaining photosynthesis rate by dissipating excessive reductants in chloroplasts under stresses [10, 20]. Moreover in plants, the dysfunction of *AOX1a* or *UCP1* disturbs the N assimilation and C metabolism [9, 10]. In the study, we investigated the functions of *AOX1a* and *UCP1* involved in regulating the balance of C and N metabolism in Arabidopsis adaptation to low-N stress.

Dysfunction of *AOX1a* and *UCP1* decreases the tolerance of Arabidopsis to low-N stress at the seedling stage

Alternative pathway is extensively involved in plant tolerance to environmental stresses, *i.e.*, cold, salt, drought, low-N and so on [10, 16, 30, 39]. qRT-PCR results showed that low-N significantly induces the expressions of *AOX1a*, *AOX1c* and *AOX2*, but decreases the expressions of *AOX1b* and *AOX1d*. It has been reported that the expression of *AOX1a* is tightly related to the N level in plants [10, 16] and is involved in regulating N metabolism and the intensity of mitochondrial stress signal pathway [40]. However, the primary root length and fresh weight of seedlings of *AOX* mutants exhibit no significant

difference to Col-0 upon low-N condition. Watanabe et al. [16] reported that low N can stimulate *AOX1a* expression, however, dysfunction of *AOX1a* did not affect the biomass of Arabidopsis. Thus, there should be additional compensation mechanisms when the alternative pathway is affected in plant adaptation to low N.

The functions of AOX and UCP are closely coordinated, as demonstrated in plant heat generation, cell energy balance and adversity defenses [22, 30, 41]. The effect of UCP1 dysfunction on N metabolism is more significant than *AOX1a* deficiency [16, 18]. qRT-PCR showed that low N significantly increases *UCP1*, *UCP2* and *UCP3* expression. However, the phenotypes of 8-day-old *UCP* mutants are also similar to that of Col-0 under low-N condition. Double mutation of *AOX1a* and *UCP1* in *aox1a/ucp1* results in reduced primary root length and accumulation of anthocyanin compared to Col-0 and the single mutants. It is well known that increase of the root/shoot ratio, the accumulation of anthocyanin and the recycling of N from old leaves to young leaves are common features during plant tolerance to low-N condition [4, 42]. *pap1*, a mutant defective in anthocyanin biosynthesis, shows decreased tolerance to low-N stress [43]. Our results suggest that *aox1a/ucp1* has lower tolerance to low N than Col-0 and the single mutants. The high anthocyanin level and purple ratio of cotyledons could facilitate *aox1a/ucp1* seedlings adaptation to low-N stress. In the process, *AOX1a* and *UCP1* collaboratively function in the adaptation of Arabidopsis seedlings to low-N stress.

Mutation of UCP1 leads to low photosynthetic capacity as well as C and N content in *aox1a/ucp1* under low-N stress

It has been reported that loss of *UCP1* function leads to photorespiration limitation and reduction of C assimilation rate under high light condition, but not loss of *AOX1A* function [9, 20]. Under the control condition (high N), A and ETR in *ucp1* are markedly decreased compared to that in Col-0. Furthermore, the light saturation point in *ucp1* is significantly lower than that in Col-0, but not in *aox1a* and *aox1a/ucp1*. Interestingly, *UCP1* mutation results in low AOX protein content under normal growth condition, but does not affect other mETPs [20]. The present result further showed that *UCP1* mutation significantly decreases V_{alt} under high-N condition. Similarly, *AOX1a* mutation also significantly decreases the activity of the UCP pathway. Under low-N stress, higher V_{alt} is induced in *ucp1* seedlings. These results indicate that there is a close correlation between the alternative pathway and the UCP pathway in Arabidopsis.

The photosynthetic capacity in plants is positively related to N levels in leaves [5, 44]. The N availability in plant affects leaf growth and photosynthetic area through influencing protein synthesis [37]. Under moderate and low-N conditions, the total rosette leaf area of *ucp1* and *aox1a/ucp1* is lower than that of Col-0. N mainly exists in photosynthetic enzymes and chlorophyll in plant shoots, so its level will affect the function and the number of chloroplasts [45, 46]. The chlorophyll content, A and ETR in *ucp1* and *aox1a/ucp1* are significantly reduced under low-N condition. However, *AOX1a* mutation does not affect the photosynthesis capacity and leaf area. Moreover, there is a similar trend in above parameters between *ucp1* and *aox1a/ucp1* under low-N stress, indicating that the dysfunction of *UCP1* is responsible for the low photosynthetic capacity and biomass in *aox1a/ucp1* under low-N condition.

It has been reported that mutation of *AOX1a* and *UCP1* affects the assimilation rate of C and N in Arabidopsis leaves [9]. Under low-N condition, the relative C and N contents in *aox1a*, *ucp1*, *aox1a/ucp1* and Col-0 shoots are decreased. However, the total C and N contents in *ucp1* and *aox1a/ucp1* shoots are lower than that in *aox1a* and Col-0 shoots. Moreover, accumulation of shoot biomass shows a similar trend to the total C and N contents as well as the photosynthetic capacity under low-N condition. Watanabe et al., [16] reported that the balance of C and N in plants might be strictly modulated by pathways other than AP upon low-N condition. Thus, the low photosynthetic capacity in *ucp1* and *aox1a/ucp1* under low-N condition results in decreased total C levels and biomass in shoots; in the process, UCP1 plays a more essential role than AOX1a.

Mutation of UCP1 disturbs the C/N ratio and leads to low seed yield in *aox1a/ucp1* upon low-N condition

The seed yield and pod setting rate in plants are closely related to N level and its assimilation capacity [5, 47, 48]. ¹⁵N tracing results indicate that mutation of *AOX1a* and *UCP1* accelerates the NO₃⁻ acquisition in *aox1a/ucp1* roots under the control and low-N conditions. However, the transport activity of NO₃⁻ from roots to shoots is lower in *aox1a/ucp1* than that in Col-0, which eventually results in a significant decrease of NO₃⁻ level in *aox1a/ucp1* leaves upon low-N condition; moreover, the NR activity in leaves of Col-0, *aox1a*, *ucp1*, and *aox1a/ucp1* is significantly decreased, but the activity decreases the most in *aox1a/ucp1*. NR activity has a significant positive correlation with protein content in leaves, and it can be used as a biochemical indicator to weigh grain yield and protein content [46, 49]. Under low-N stress, the seed yield and silique number were decreased more in *ucp1* and *aox1a/ucp1* than that in Col-0 and *aox1a*. Gandi et al. [9] confirmed that UCP1 plays a more key role than AOX1a in the N and C metabolism under normal and high light conditions in Arabidopsis. Based on our results, we propose that loss of *UCP1* function, not *AOX1a*, leads to the low seed yield in *aox1a/ucp1* upon low-N condition.

The total C and N levels in seeds are positively correlated with the protein and fat contents in seeds, which also determines the quality and yield of seeds [50, 51]. Loss of function of UCP1 and AOX1a in *aox1a/ucp1* did not affect the C content in seeds, but significantly decreased the N content under low-N stress, which resulted in a high C/N ratio in *aox1a/ucp1* seeds. Comparatively, the total C and N content in *ucp1* and *aox1a/ucp1* seeds were significantly lower than those in Col-0 and *aox1a*. Watanabe et al. [16] indicated that AOX induced by low-N condition does not play an important role on the C/N ratio, instead it could be controlled by pathways other than alternative pathway. In short, the low N utilization efficiency in *aox1a/ucp1* disturbs the metabolism of C and N, and eventually results in low seed yield and high C/N ratio under low-N stress; and UCP1 plays a key role for the maintenance of C/N ratio.

RNA-Seq analysis of Col-0 and *aox1a/ucp1* shoots reveals the molecular mechanism for their differences in C and N metabolism as well as seed yield upon low-N condition. KEGG results indicate that low N disturbs ribosomes, photosynthesis, N metabolism, C metabolism, amino acid synthesis, porphyrin (por) and chlorophyll (chl) metabolism in Col-0 and *aox1a/ucp1*. Noticeably, by comparing different DEGs between Col-0 and *aox1a/ucp1*, we found that *aox1a/ucp1* has more DEGs enriched in photosynthesis, N metabolism, C metabolism, and por and chl metabolism than Col-0; and most of their expressions are

down-regulated. Moreover, the expression of common DEGs in photosynthesis and C/N metabolism is more down regulated in *aox1a/ucp1* than that in Col-0 upon low-N condition. Thus, the decreased expressions of genes related to C metabolism, photosynthesis, and N metabolism in *aox1a/ucp1* might be responsible for the low photosynthetic capacity as well as C and N levels in seeds.

Taken together, 8-day-old *aox1a/ucp1* seedlings showed higher sensitivity to low-N stress in comparison with Col-0 and single mutant seedlings, suggesting that *AOX1a* and *UCP1* are both involved in the Arabidopsis tolerance to low N at the seedling stage. The photosynthetic rate and the N assimilation capacity in *ucp1* and *aox1a/ucp1* plants are markedly weakened, leading to decreased leaf area, total C and N content, shoot biomass as well as seed yield compared to Col-0 and *aox1a*. These results indicate *UCP1* plays more essential roles than *AOX1a* in Arabidopsis adaptation to low N at the vegetative and reproductive stages. RNA-seq analysis confirmed that *AOX1a* and *UCP1* mutation leads to more significant down-regulation of genes in photosynthesis and C/N metabolism in *aox1a/ucp1* shoots in comparison with Col-0, which provides powerful evidence for the low seed yield, C and N levels in *aox1a/ucp1*. Noticeably, compared to *AOX1a*, *UCP1* plays a more important role in regulating C/N ratio and yield of seeds in Arabidopsis adaptation to low-N stress.

Declarations

Acknowledgements This work was supported by the National Natural Science Foundation of China [31670244; 31972494], Foundation of Science and Technology of Gansu Province (20JR5RA288), and Fundamental Research Funds for the Central Universities (lzujbky-2020-sp04).

Author contributions XW and YB designed the project; XQ, TY, MR, CC^a, YZ, FL, SW performed the experiments; XQ, MJ, CC^b, XN analyzed the data; XW and TY wrote the paper. All authors have read and approved the manuscript.

Availability of data and materials

Sequencing data is available at NCBI SRA under BioProject ID:PRJNA722574.

Conflict of interest

The authors declare no competing interest.

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Figures

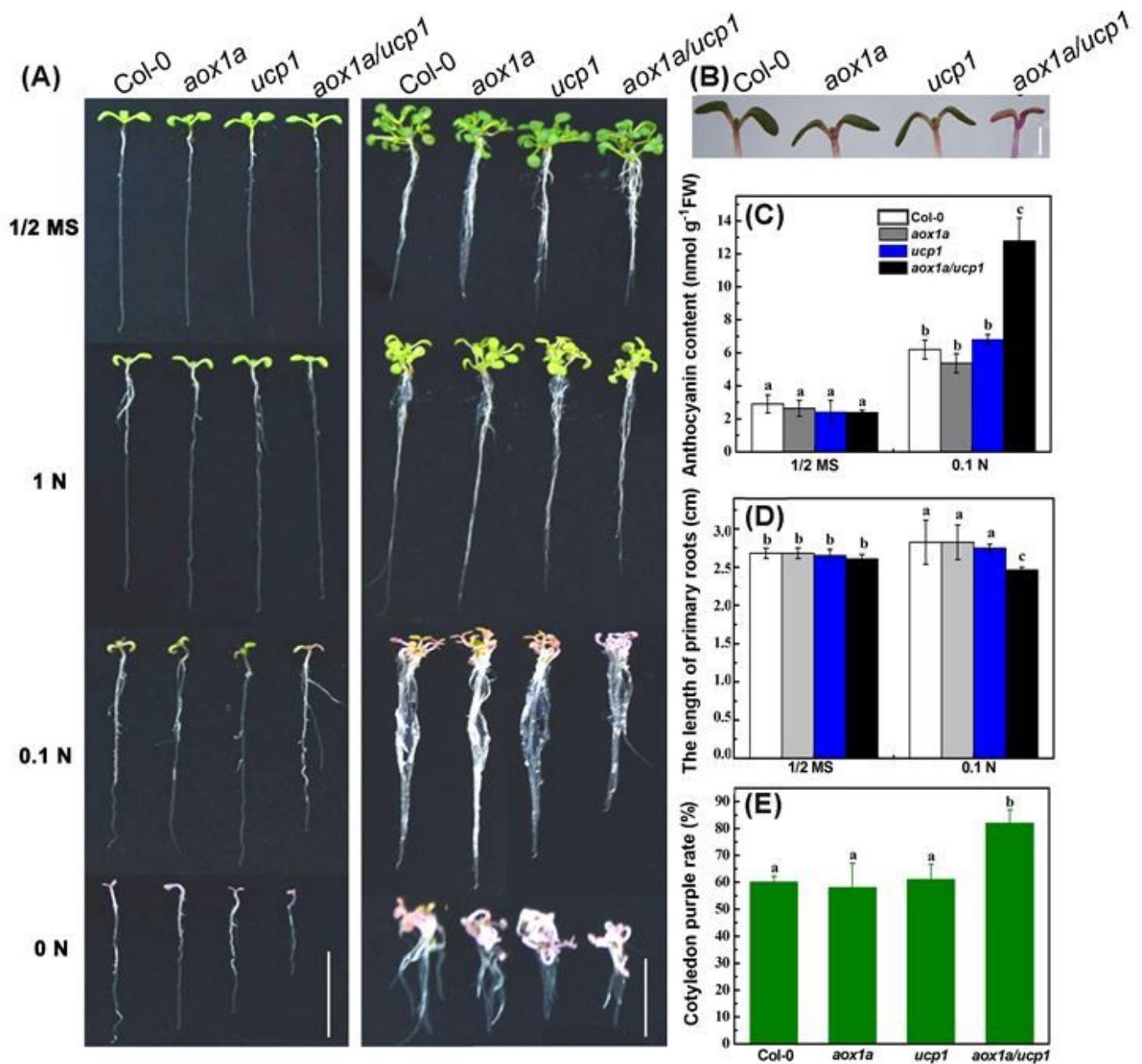


Figure 1

Effects of low N on Col-0, *aox1a*, *ucp1*, *aox1a/ucp1* seedlings. (a) Phenotypes of individual and clustered seedlings grown in different media for 8 d. Bar = 1 cm. (b) Magnified images of cotyledons under 0.1 N condition for 8 d. Bar = 50 μ m. (c) Anthocyanin content in cotyledons under 0.1 N condition for 8 d. (d) Primary root length under 0.1 N condition for 8 d. (e) Purple ratio of cotyledons under 0.1 N condition for 8 d. Data were expressed as mean \pm SD ($n \geq 3$, $P < 0.05$).

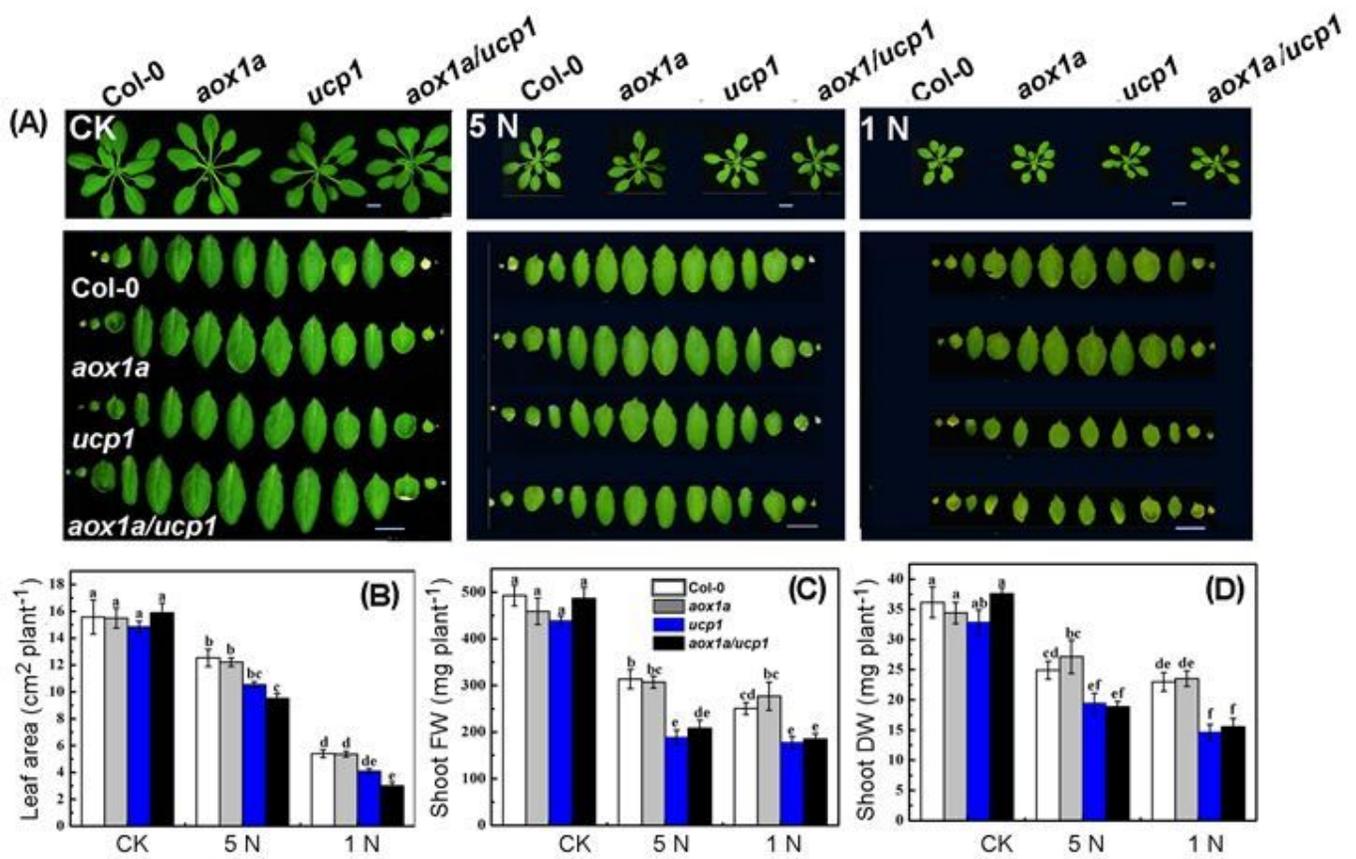


Figure 2

Effects of AOX1a and UCP1 mutation on leaf area and biomass of Arabidopsis under low N stress. (a) Phenotypes of plants and images of rosette leaves under the different N conditions. Bar = 1 cm. (b) Rosette leaf area. (c) Fresh weight of shoots. (d) Dry weight of shoots. 14-day-old Col-0, aox1a, ucp1, and aox1a/ucp1 seedlings were grown under low-(1 N), moderate-(5 N), or high-N (10 N, CK) conditions for 16 d. Data were expressed as mean \pm SD ($n \geq 3$, $P < 0.05$).

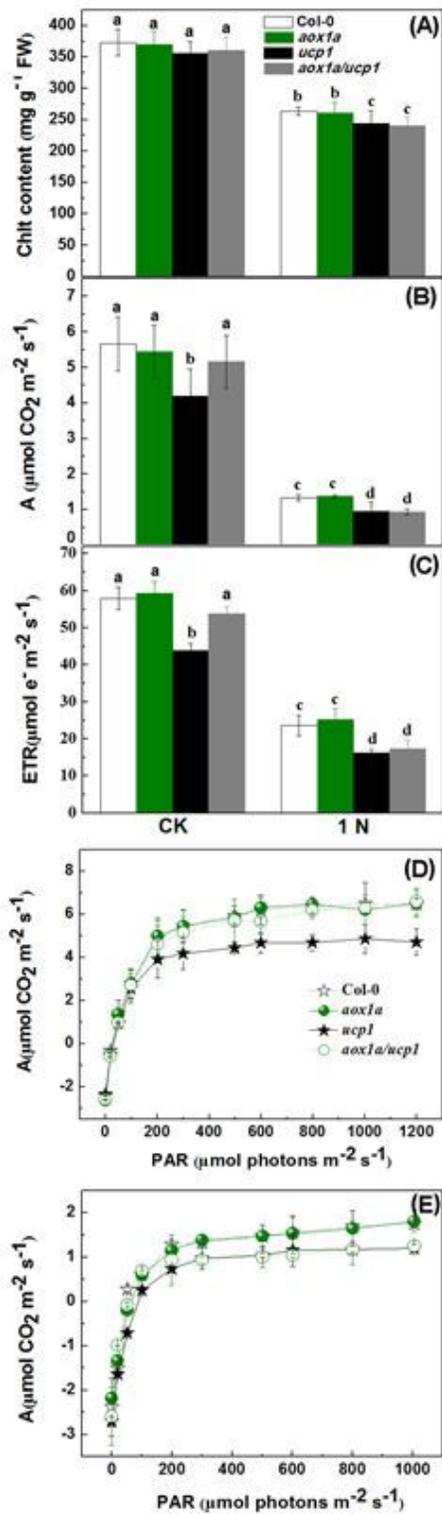


Figure 3

Effects of low N on chlorophyll content and photosynthetic parameters in Col-0, *aox1a*, *ucp1*, *aox1a/ucp1*. (a) Total chlorophyll (Chl) content. (b) Net photosynthetic rate (A). (c) Electron transport rate (ETR). (d, e) Light responsive curves under CK (d) and low N (e) conditions. The concentration of CO₂ is 400 ppm. 14-day-old seedlings were grown under low-(1 N), moderate-(5 N), or high-N (10 N, CK) conditions for 16 d. Data were expressed as mean ± SD (n≥3, P<0.05).

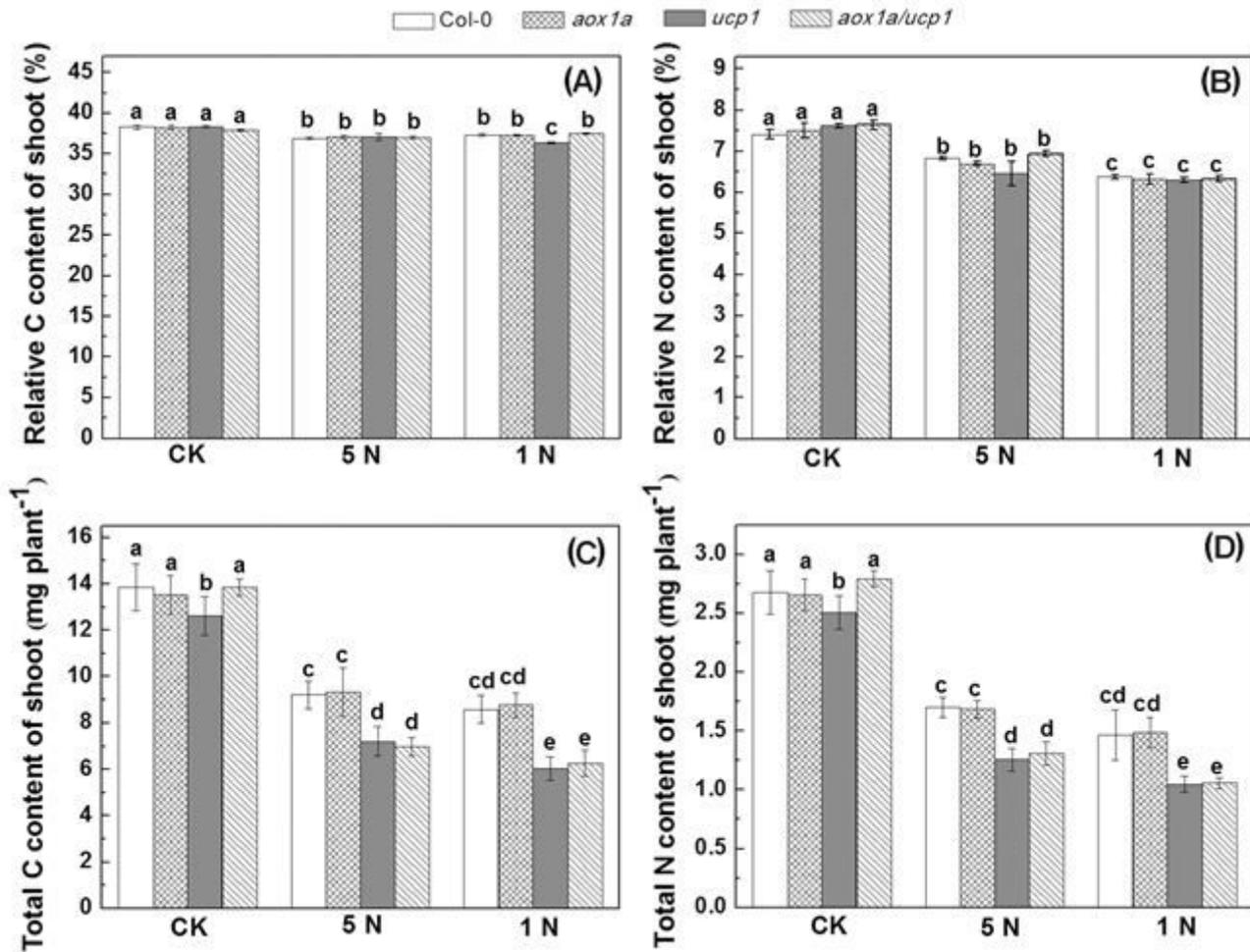


Figure 4

Effects of AOX1a and UCP1 mutation on C and N content upon low N condition. (a) Percentage of C content in shoots. (b) Percentage of N content in shoots. (c) Total C content in shoots. (d) Total N content in shoots. The 14-day-old seedlings were treated as in Fig. 2. Data were expressed as mean \pm SD ($n \geq 3$, $P < 0.05$).

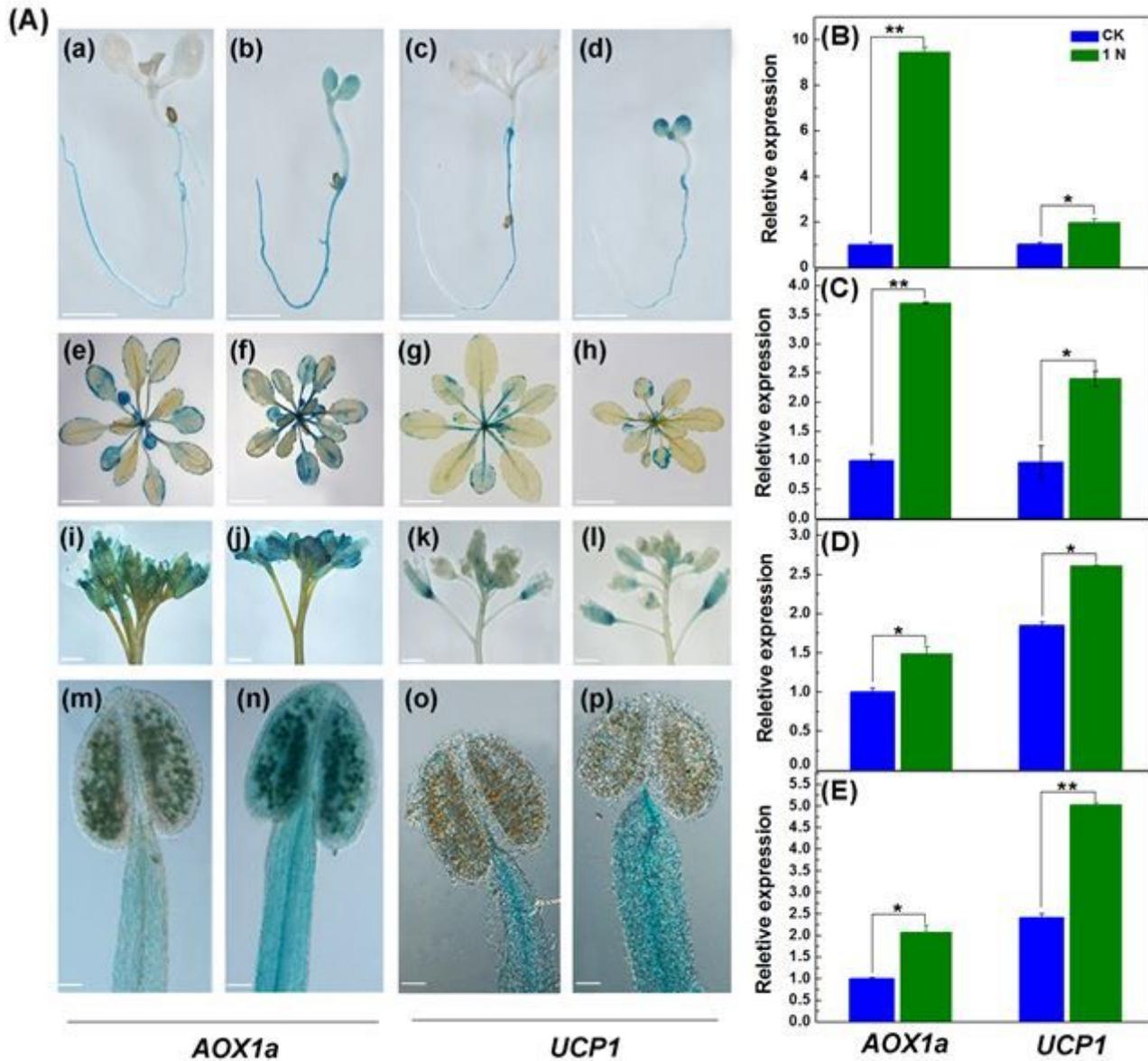


Figure 5

GUS-staining and qRT-PCR analysis of *AOX1a* and *UCP1* expression under low N condition. (a-d) 6-day-old seedlings under 1/2 MS (a, c) or 0.1 N (b, d) conditions, bar = 50 μ m. (e-h) Shoots under CK (e, g) and 1 N (f, h) conditions, bar = 1 cm. (i-l) Flowers under CK (i, k) and 1 N (j, l) conditions, bar = 20 μ m. (m-p) Stamens under CK (m, o) and 1 N (n, p) conditions, bar = 40 μ m. (q-t) qRT-PCR analysis of *AOX1a* and *UCP1* expressions in 6-day-old seedlings (q), leaves (r), flowers (s), and silques (t). In a-d and q, seeds were germinated and grown for 6 d under 1/2 MS or 0.1 N condition. In e-p and r-t, 14-day-old seedlings were transferred to CK (10 N) or low N (1 N) medium for 16 d (e-h, r) or 30 d (i-p, s, t). ACTIN 2 was used as the internal reference gene. Data were expressed as mean \pm SD (n = 3). *, P<0.05; ** P<0.01.

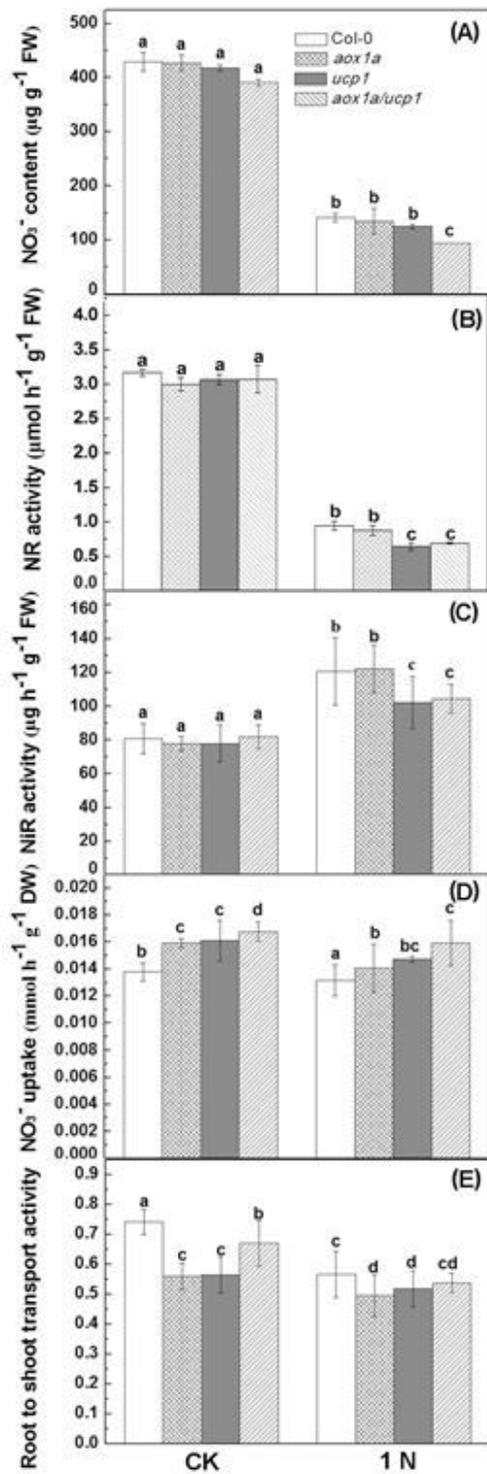


Figure 6

Effect of AOX1a and UCP1 mutation on N metabolism under low N condition. (a) NO₃⁻ content. (b) NR activity. (c) NiR activity. (d) NO₃⁻ uptake. (e) The root to shoot transport activity of NO₃⁻. In a, b and c, 14-day-old seedlings were cultured in low N (1 N) medium for 16 d. In d and e, CK: after the 24-day-old seedlings were starved under 0 N condition for 24 h, they were cultured in the medium containing 10 mM 15N-KNO₃ as the sole N source for 6 h. 1 N treatment: after the 14-day-old seedlings were treated under 1

N condition for 10 d and 0 N for 24 h, they were cultured in the medium containing $^{15}\text{N-KNO}_3$ (1 mM) as the sole N source for 6 h. Data were expressed as mean \pm SD ($n=3$, $P<0.05$).

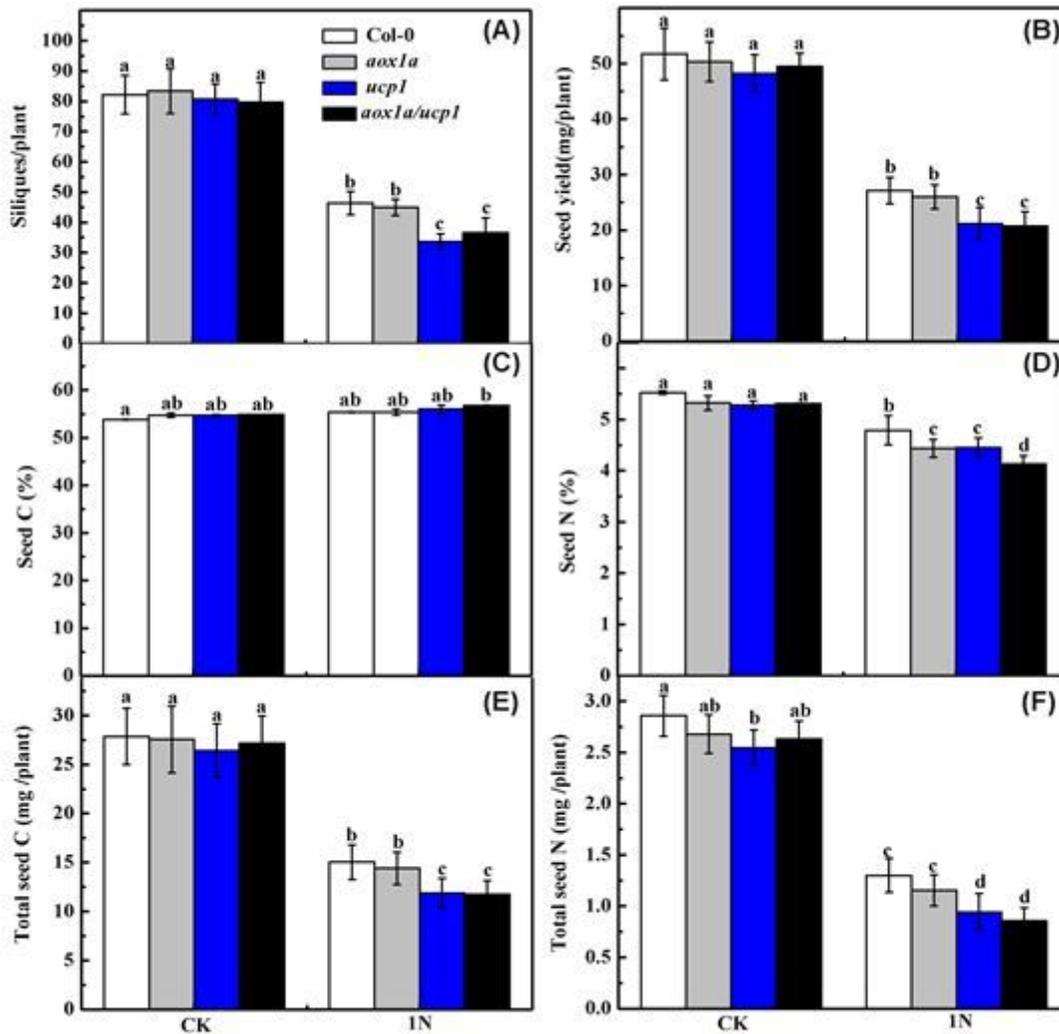


Figure 7

Effects of low N on seed yield and C/N content of Col-0, *aox1a*, *ucp1*, *aox1a/ucp1* plants. (a) Total silique number per plant. (b) Seed yield per plant. (c) The percentage of C content in seeds. (d) The percentage of N content in seeds. (e) Total seed C content per plant. (f) Total seed N content per plant. 14-day-old seedlings were transferred to low N (1 N) medium for treatment. Data were expressed as mean \pm SD ($n \geq 8$, $P<0.05$).

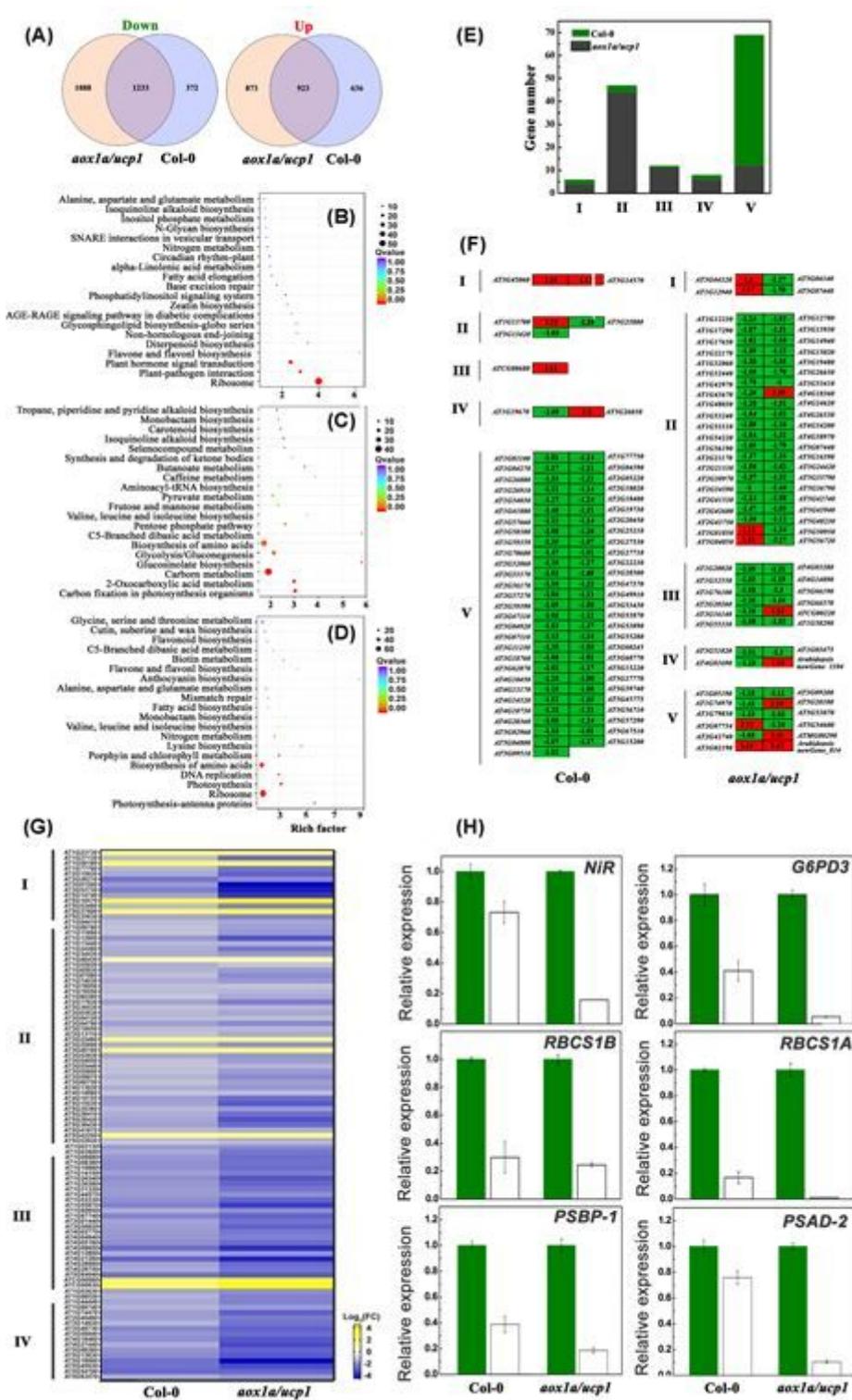


Figure 8

RNA-Seq analysis of the transcripts in *Col-0* and *aux1a/ucp1* shoots under low N condition. (a) Venn diagram of DEGs between *Col-0* and *aux1a/ucp1*. (b-d) KEGG enrichment of DEGs in *Col-0* (b), *aux1a/ucp1* (c), and both *Col-0* and *aux1a/ucp1* (d). (e) DEGs in C metabolism (I), N metabolism (II), photosynthesis (III), porphyrin (por) and chlorophyll (chl) metabolism (IV), and ribosome (V). (f) DEGs in e and their expressions. (g) Low-N

responsive patterns of DEGs in Col-0 and *aox1a/ucp1* involved in N metabolism (I), C metabolism (II), photosynthesis (III), and *por* and *chl* metabolism (IV). (h) Validation of DEGs in Col-0 and *aox1a/ucp1* by qRT-PCR. ACTIN 2 was used as the internal reference gene. Corresponding gene IDs are: NiR (AT2G15620), G6PD3 (AT1G24280), RBCS1A (At1G67090), RBCS1B (At5G38430), PSBP-1 (AT1G06680), PSAD-2 (AT1G03130). Data were expressed as mean \pm SE (n = 3, * P<0.05).

Supplementary Files

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

- [QiaoXYSupplementaldata20210417.doc](#)