MdATG18a overexpression improves tolerance to nitrogen deficiency and regulates anthocyanin accumulation through increased autophagy in transgenic apple

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Running title: MdATG18a enhances tolerance to N-deficiency

Summary

Autophagy plays a critical role in recycling and remobilization of nitrogen. Here, overexpression of *MdATG18a* enhanced tolerance to N-depletion in both *Arabidopsis* and apple. Overexpression of *MdATG18a* in apple accumulated more anthocyanin and improved nitrate uptake by up-regulating anthocyanin-related genes and some nitrate transporters, respectively. These results were possibly because of increased autophagy activity in transgenic lines under N deficiency.

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Abstract

Nitrogen (N) availability is an essential factor for plant growth. Recycling and remobilization of N have strong impacts on crop yield and quality under N deficiency. Autophagy is a critical nutrient-recycling process that facilitates remobilization under starvation. We previously showed that an important AuTophaGy (ATG) protein from apple, MdATG18a, has a positive role in drought tolerance. In this study, we explored its biological role in response to low-N. Overexpression of *MdATG18a* in both *Arabidopsis* and apple improved tolerance to N-depletion and caused a greater accumulation of anthocyanin. The increased anthocyanin concentration in transgenic apple was possibly due to up-regulating flavonoid biosynthetic and regulatory genes (*MdCHI*, *MdCHS*, *MdANS*, *MdPAL*, *MdUFGT* and *MdMYB1*), and higher soluble sugars concentration. *MdATG18a* overexpression enhanced starch degradation with up-regulating amylase gene (*MdAM1*), and up-regulated sugar metabolism related genes (*MdSS1*, *MdHXKs*, *MdFK1* and *MdNINVs*). Furthermore, MdATG18a functioned in nitrate uptake and assimilation by up-regulating nitrate reductase *MdNIA2* and three high-affinity nitrate transporters *MdNRT2.1/2.4/2.5*. *MdATG18a* overexpression also elevated other important *MdATG* genes expression and autophagosomes formation under N-depletion, which play key contributions to above changes. Together these results demonstrate that overexpression of *MdATG18a* enhances tolerance to N-deficiencies and plays positive roles in anthocyanin biosynthesis through greater autophagic activity.

Key words: autophagy, anthocyanin, apple, *MdATG18a*, N-deficiency

Introduction

Nutrients support growth and development, making them indispensable to the plant life cycle. Nitrogen (N) is one such essential element because it is a key component of important cellular constituents, including nucleic acids, proteins, chlorophyll, and phytohormones (Fukushima & Kusano, 2014; Martin *et al*., 2016). Plants acquire this nutrient through the soil, where nitrate and ammonium are the universal forms (Li *et al.*, 2017). However, under natural agricultural conditions, plants often encounter low-N availability because of soil physical properties, leaching, and microbial activity (Kiba & Krapp, 2016). This deficiency leads to reduced rates of cell division, cell expansion, photosynthesis, leaf production, and tillering (Chapin *et al.*, 1988), thus presenting an enormous environmental challenge to crop yields. In acclimating to this changing environment, plants must adjust their metabolism by employing several adaptive strategies, e.g., a reduction in photosynthesis, increases in root growth and lateral branching, chlorophyll degradation, N-remobilization from older to young tissues, and the accumulation of anthocyanins (Diaz *et al.*, 2006).

Anthocyanins are a major class of plant pigments necessary for multiple eco-physiological functions in response to developmental and environmental signals (Grotewold, 2006). Anthocyanins are biosynthesized via the phenylpropanoid pathway, in which phenylalanine ammonia-lyase (PAL) catalyzes the de-amination of phenylalanine to produce precursors. Enzymes involved in the subsequent flavonoid pathway include chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-dependent flavonoid 3-O-glycosyltransferase (UFGT) (Shirley *et al.*, 1995). These structural genes are modulated

by a conserved MYB-bHLH-WD40/WDR (MBW) regulatory protein complex that has been studied in many species (Allan *et al.*, 2008; Lin-Wang *et al.*, 2011; Xie *et al.*, 2012). In apple (*Malus domestica*), MdMYB1 regulates anthocyanin biosynthesis and fruit coloration (Takos *et al.*, 2006). MdbHLH3 binds to the promoters of the anthocyanin-biosynthetic genes *MdDFR* and *MdUFGT* and the regulatory gene *MdMYB1* to activate their expression (Xie *et al.*, 2012).

When plants are grown under an N-deficit, anthocyanins accumulate in different tissues (Lillo *et al.*, 2008; Masclaux-Daubresse *et al.*, 2014; Nemie-Feyissa *et al.*, 2014; Shi & Xie, 2010). During low N-induced leaf senescence, such accumulations can minimize stress-related oxidative damage and facilitate nutrient remobilization from older leaves to younger active tissues (Shoki *et al.*, 2014). This deficiency of N increases anthocyanin biosynthesis by regulating the transcript levels of structural genes or positive and negative transcription factors (TFs) in the MBW regulatory complex (Soubeyrand *et al.*, 2014). For example, *PAL* is induced in N-deficient plants of *Nicotiana tabacum* (Fritz *et al.*, 2006). Structural genes *PAL*, *CHS*, and *F3H* are expressed in response to low-N in tomato (*Lycopersicon esculentum*) (Larbat *et al.*, 2012; Lovdal *et al.*, 2010). In addition, sugars induce anthocyanin biosynthesis in various plant species (Zhang *et al.*, 2015). For example, *MdHXK1* phosphorylates and stabilizes MdbHLH3 to promote anthocyanin production in apple (Hu *et al.*, 2016). The interactive effect of carbon (C) and N seems to have more influence on the flavonoid biosynthetic pathway. Under high C/N growing conditions, MYB10 in *Malus* sp. positively regulates anthocyanin structural genes (Wan *et al.*, 2015). In the hypocotyls of *Raphanus sativus*, an increase in the level of soluble sugars, especially

sucrose, contributes to an accumulation of anthocyanin that results from an N-deficit (Su *et al.*, 2016).

When less soil-N is available, nutrient recycling and remobilization processes that are crucial for the sacrifice of healthy organs to feed and save the rest of the plant will have a strong influence on crop yield and grain quality (Masclaux-Daubresse *et al.*, 2017). Autophagy is one of the most important pathways that promote cell longevity and nutrient recycling through the degradation of unwanted or damaged organelles, proteins, and cytoplasmic constituents. The autophagy machinery, a conserved universal mechanism in eukaryotes, involves double membrane-bound vesicles, i.e., autophagosomes, which form autophagic bodies to engulf and deliver cytoplasmic components to the vacuole for degradation (Bassham, 2009; Noda & Inagaki, 2015). This machinery controls N-remobilization under limited-N conditions (Guiboileau *et al.*, 2012; Wada *et al.*, 2015). *Arabidopsis atg* mutants are hypersensitive to that deficiency, exhibiting reduced seed production and less efficient N-remobilization at the whole-plant level (Doelling *et al.*, 2002; Hanaoka *et al.*, 2002; Thompson *et al.*, 2005). An autophagy-disrupted rice mutant, *Osatg7-1*, shows diminished biomass production and less nitrogen-use efficiency because N-remobilization is suppressed during leaf senescence (Wada *et al.*, 2015). The maize *atg12* seedlings is severely arrested and, as the plants mature, they manifest early leaf senescence, stunted ear development, and impaired N-remobilization under N-starvation (Li *et al.*, 2015a). However, heterologous overexpression (OE) of some AuTophaGy (ATG) genes in *Arabidopsis* can confer improved tolerance to N starvation (Li *et al.*, 2015b; Wang *et al.*, 2016a; Wang *et al.*, 2016b; Wang *et al.*, 2017; Xia *et al.*, 2012). These study results

demonstrate the importance of autophagy in N-remobilization during periods of nutrient starvation or leaf senescence.

Recently, a few studies highlight a role has been described for autophagy in the formation of anthocyanic vacuolar inclusions (AVIs) during anthocyanin accumulation (Chanoca *et al.*, 2015). *Arabidopsis atg* mutants have fewer AVIs and decreased accumulations of anthocyanins (Pourcel *et al.*, 2010). A novel microautophagy mechanism has been hypothesized to mediate the transport of anthocyanin into the vacuoles (Chanoca *et al.*, 2015). Under low-N conditions, the *Arabidopsis atg5* mutant accumulates less anthocyanin in the rosettes, and transcriptome data has indicated that genes involved in flavonoid biosynthesis and regulatory pathways are down-regulated in that mutant (Masclaux-Daubresse *et al.*, 2014). However, few investigations have been made about the relationships between anthocyanin accumulation and autophagy under N-starvation. An *ATG* gene from apple, *MdATG18a*, has been cloned and shown to respond transcriptionally to N-depletions (Wang *et al.*, 2014). Its stably transgenic OE apple plants display improved drought tolerance and greater autophagosome formation (Sun *et al.*, 2017). In this study, we employed *MdATG18a*-OE apple plants to explore the function of this gene under N-starvation conditions. We found it particularly interesting that, when N was limited, more anthocyanin was accumulated in the OE plants than in the WT, possibly because the former had enhanced autophagy activity. Therefore, more efficient recycling of N and C by autophagy in those transgenics led to better growth under those adverse conditions. Furthermore, the increased levels of soluble sugars promoted anthocyanin biosynthesis in the OE lines.

Materials and Methods

Plant materials and growing conditions

Seeds from *Arabidopsis thaliana* 'Col-0' and two homozygous T_3 transgenic lines over-expressing *MdATG18a* were sterilized and plated in MS media. Growing conditions included 22°C, 100 μmol photons m^{-2} s^{-1}, 70% relative humidity, and a long-day (LD; 16-h) photoperiod. For N-depletion treatments, 5-day-old uniformly sized seedlings were transferred to MS control or N-depleted agar plates. The latter were prepared by replacing KNO_3 and $Ca(NO_3)_2$ with KCl and $CaCl_2$, respectively. After 10 d of growth, plants from all treatments were photographed under a light microscope (DM2000; Leica, Germany), and fresh weights and root lengths were recorded.

Tissue-cultured plants of *Malus domestica* cv. 'Roya Gala' were initially grown on an MS agar medium containing 0.3 mg L^{-1} 6-BA and 0.2 mg L^{-1} IAA. They were cultivated for 20 d under conditions of 23°C, 60 μmol m^{-2} s^{-1}, and a 14-h photoperiod. Afterward, the main shoots of transgenic and WT plantlets were cut into 1.5-cm segments that included the first four leaves, and were rooted on an MS agar medium containing 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} IAA. After 7 d, they were transferred to an MS medium that lacked growth hormones for testing differences in performance when exposed to either MS control or N-depleted conditions (-N). Plants were photographed and growth parameters were recorded after 20 d. After 10 d of treatment, various tissue types were sampled and frozen in liquid nitrogen for RNA isolation. On Day 15, leaves were collected for autophagosome observations.

Construction of plant-overexpressing vector for *MdATG18a*

The coding region of *MdATG18a* was introduced into the pCambia2300 vector by *Xba*I and *Kpn*I. Primers with restriction sites are listed in Table S1. This vector was driven by the

CaMV 35S promoter and carried the kanamycin (Kan) selectable marker in plants. The sequencing-confirmed plasmid was transformed to *Agrobacterium* EHA105 by electroporation.

RNA extraction, cloning, and real-time PCR

Total RNA was extracted according to a CTAB method (Chang *et al.*, 1993). The DNA was removed by treating with RNase-free DNase I (Thermo Scientific, Waltham, MA, USA). Apple genomic DNA was extracted by a modified CTAB method (Modgil *et al.*, 2005), and first-strand cDNA was synthesized by using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) with the same amount of mRNA (1 μg). Real-time PCR was performed with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Green Master Mix (Takara, Dalian, China). Transcripts of the *Malus* elongation factor 1 *alpha* gene (*EF-1α*; DQ341381) were used to standardize the cDNA samples for different genes. Specific gene primer sequences for all expression analyses are shown in Table S1. Each experiment was repeated three times biologically, based on three separate RNA extracts from three repeats.

Generation and characterization of transgenic *Arabidopsis* and apple plants that over-express *MdATG18a*

The *MdATG18a/*pCambia2300 construct was transformed into *Arabidopsis thaliana* 'Col-0' via the floral dip method (Zhang *et al.*, 2006). After T_1 seeds were harvested and surface-sterilized in 50% bleach, they were screened on an MS medium supplemented with 50 mg L^{-1} Kan. The Kan-resistant plants were PCR-confirmed and further selected for single-copy insertion homozygous lines. Seeds of the T_3 generation were collected from two

independent T_2 lines and the WT.

Transgenic 'Royal Gala' apple plants were generated from leaf segments through *Agrobacterium*-mediated transformation, as previously described (Dai *et al.*, 2013; Sun *et al.*, 2017).

Determination of total anthocyanins

Anthocyanins were extracted by placing 0.1 g of tissue in 1 mL of 1% (v/v) HCl-methanol for 24 h at room temperature in the dark. After centrifugation for 5 min at 13,000 *g*, the upper aqueous phase was quantified at 530, 620, and 650 nm with a UV spectrophotometer (Shimadzu, UV-1750, Japan). Relative anthocyanin concentrations were determined with the following formula: optical density (OD) = (A530 – A620) – 0.1*(A650 – A620). One unit was expressed as a change of 0.1 OD (unit $*10^3$ g^{-1} FW) (Lee & Wicker, 2010; Xie *et al.*, 2012).

Soluble sugar contents measurement

Soluble sugars were extracted and derivatized as previously described (Wang *et al.*, 2010; Wei *et al.*, 2014). Briefly, leaf samples (0.1 g) were extracted in 1.4 mL of 75% methanol, with ribitol added as the internal standard. After the non-polar metabolites were fractionated into chloroform, 2-μL aliquots of the polar phase were transferred into 2.0-mL Eppendorf vials for measuring the metabolites (fructose, glucose, and sucrose). Samples were dried under vacuum without heating and then derivatized sequentially with methoxyamine hydrochloride and N-methyl-N-trimethylsilyl-trifluoroacetamide (Lisec *et al.*, 2006). Afterward, the metabolites were analyzed with a Shimadzu GCMS-2010SE (Shimadzu Corporation, Tokyo, Japan).

Iodine staining and determination of starch concentrations

Detached leaves were boiled in 95% ethanol for thorough removal of their pigments, and then washed twice with deionized water. Rehydrated leaves were stained in 5% Lugol's solution (5% [w/v] I_2 and 10% [w/v] KI) for 10 min and then de-stained in water until a clear background was obtained (Wang *et al.*, 2013b).

For starch extracts, 0.1 g of leaf tissue was ground and then transferred to a 5-mL volumetric flask. The residue was washed with 2 mL of diethyl ether and the filtered liquid was discarded. This step was repeated three to five times. Afterward, the residue was washed with 80% ethanol three to five times to remove pigments, soluble sugars, and other non-starch substances. Finally, the residue was washed with water and transferred to a 10-mL beaker. It was boiled in 5 mL of water for 15 min until the gelatinized starch became a clear solution. For starch measurements, 0.98 mL of the clear starch solution was mixed with 20 μL of 5% (w/v) I_2 and 2% (w/v) KI. This reaction was subjected to colorimetric determination at 660 nm. The standard curve was performed with soluble starch (Sigma).

Measurements of leaf-N

After 20 d of N-depletion treatment, apple leaves were oven-dried for 3 d at 70°C. The amount of total N from 0.1 g of dry mass was obtained by treating the leaves with Nessler's reagent after Kjeldahl digestion (Wang *et al.*, 2013a).

Autophagosome detection by transmission electron microscopy (TEM)

Our TEM analysis was performed as previously described (Sun *et al.*, 2017). On Day 15 of the N-depletion period, leaves were excised from the apple plants and immediately cut into small pieces, then fixed with 2.5% glutaraldehyde in 0.2 M PBS buffer (pH 7.4) before being

placed under darkness for 12 h at 4°C. After washes with PBS buffer, the samples were fixed for 2.5 h in 1% (v/v) osmium tetroxide at room temperature. They were then dehydrated in a graded ethanol series (30 to 100%; v/v) and embedded in Epon 812. Ultrathin sections (70 nm) were prepared on an ultramicrotome (Leica ULTRACUT, Wetzlar, Germany) and collected on Formvar-coated grids. The sections were examined using a JEOL-1230 transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 80 kV to observe autophagosomes and autophagic bodies.

Statistical analysis

Three independent replicates were used for each determination. Experimental data were presented as means ± standard deviation (SD). The statistical analysis was performed via one-way ANOVA, followed by Tukey's multiple range tests, using the SPSS18 statistical package (Chicago, Illinois, USA). Differences among results were considered statistically significant at $P < 0.05$.

Results

***MdATG18a* overexpression enhances tolerance of *Arabidopsis* to N-depletion**

We previously reported that *MdATG18a* expression could be induced by N-depletion (Wang *et al.*, 2014). For further analysis of its biological function under N-deficit conditions, *MdATG18a* was over-expressed in *Arabidopsis* under the control of the CaMV35S promoter. Two homozygous lines (OE-13 and OE-46) with high *MdATG18a* transcript levels (Fig. 1a and b) and WT seedlings were transferred to N-depleted media for 10 d. As shown in Figure 1c, the transgenics had more and larger true leaves than the WT. They also had significantly higher fresh weights and root lengths than the WT after 10 d of growth (Fig. 1d and e). Roots

from OE-13 and OE-46 were 15.8% and 33.5% longer, respectively, than those from the WT (Fig. 1e). Meanwhile, the transgenic lines accumulated more anthocyanins (59.6% for OE-13 and 53.4% for OE-46) than did the WT after N-starvation, as indicated by the red coloration in Figure 1c. These results showed that *MdATG18a* could improve tolerance to N-depletion and increase anthocyanin accumulations in *Arabidopsis*.

***MdATG18a* overexpression enhances tolerance of apple plants to N-depletion**

We used two previously obtained lines of *MdATG18a*-OE apple (Sun *et al.*, 2017) for further examination of the function of that gene when the N supply was limited. Under normal growing conditions, the phenotypes did not differ significantly between OE lines and the WT (Fig. 2a). However, after transfer to N-depleted media for 20 d, the OE lines exhibited more and larger leaves and longer roots than the WT. The lower leaves from the WT became yellow, while leaves from Lines OE-3 and OE-11 remained vigorous and green (Fig. 2a and b). Under deficit treatment, fresh weights were 47.6% to 49.9% higher from the OE plants than from the WT (Fig. 2b). Although root numbers did not differ significantly among genotypes, the OE lines had longer roots than the WT (Fig. 2b). Similar to the phenotype observed in transgenic *Arabidopsis*, the transgenic apple plants accumulated more anthocyanins in their roots when compared with the WT under the N-depleted conditions (Fig. 2c and d). These results suggested that apple *MdATG18a* plays a positive role in the plant response to N-depletion.

***MdATG18a* overexpression activates flavonoid biosynthetic and regulatory genes in apple and increases anthocyanin accumulation in response to N-depletion**

Transgenic plants over-expressing *MdATG18a* in *Arabidopsis* and apple showed increased

anthocyanin accumulations upon N-depletion (Figs. 1 and 2), presenting an interesting phenotype. The anthocyanin biosynthesis pathway is controlled by several structural genes and regulated by MBW TFs (Allan *et al.*, 2008; Hichri *et al.*, 2011; Koes *et al.*, 2005; Takos *et al.*, 2006; Xie *et al.*, 2012). To improve our understanding of how this accumulation is enhanced in transgenic apple, we examined the expression patterns of *MdMYB1* and anthocyanin structural genes *MdCHI*, *MdCHS*, *MdANS*, *MdPAL*, and *MdUFGT*. As shown in Figure 3, all of them were more strongly and significantly induced in OE plants than in the WT by N-starvation. These results indicated that *MdATG18a* activates anthocyanin biosynthesis by up-regulating the expression of anthocyanin-related genes.

MdATG18a overexpression promotes the accumulation of soluble sugars and degradation of starch in apple in response to N-depletion

Because autophagy contributes to the degradation of leaf starch (Wang *et al.*, 2013b), we used histochemical staining to examine the differences in starch concentrations between OE lines and WT plants that were treated with N-depletion. Under deficit conditions, starch was greatly accumulated in all genotypes (Fig. 4a), but those levels were significantly lower in the OE lines, where values were 69.9% and 73.1% of those measured in the WT (Fig. 4b). Meanwhile, levels of sucrose, fructose, and glucose were increased in response to N-depletion, with concentrations of all three soluble sugars raising more in OE lines than in the WT (Fig. 4d). For example, glucose levels were slightly higher in OE lines even under control conditions but were approximately 1.25-fold higher when compared with the WT plants under the deficit treatment (Fig. 4d). The genes encoding glucose sensor hexokinase (*MdHXK1*, *MdHXK2*, *MdHXK3*, *MdHXK5* and *MdHXK6*) were up-regulated more in the

transgenics than in the WT plants during the experimental period (Fig. 4c; Fig. S1). Fructokinase *MdFK1* also showed similar up-regulation in OE lines while other members *MdFK2/3/4* did not differ too much (Fig. S1). The expression level of amylase *MdAM1*, which played a key role in starch degradation, was higher in OE lines than in the WT under the N deficit treatment. Sucrose synthase (*MdSS1*) and neutral invertase (*MdNINV1/2*) also showed significantly more transcription in OE lines, while vacuolar acid invertase (*MdAINV1/2/3*) did not (Fig. S1). These results suggested that *MdATG18a* overexpression regulates several genes involved in sugar metabolism and might contribute to more soluble sugar accumulation and starch degradation in response to N depletion.

***MdATG18a* overexpression increases nitrate absorption and assimilation in apple**

Autophagy plays an important role in nitrogen remobilization (Guiboileau *et al.*, 2012; Li *et al.*, 2015a; Li *et al.*, 2015b; Wada *et al.*, 2015; Wang *et al.*, 2016a). To examine whether *MdATG18a* influences N-signaling in apple, we monitored nitrate concentrations and the transcript levels of several nitrate-related genes. Although the level of total nitrates was greatly reduced in all genotypes in response to N-depletion, the OE lines continued to have significantly more nitrate when compared with the WT under deficit conditions (Fig. 5a). We also investigated the expression of genes involved in nitrate uptake, including members of the NITRATE TRANSPORTER1/2 (NRT1/2) families. We found it interesting that expression of low-affinity nitrate transporters *NRT1.1* and *NRT1.7* (Lezhneva *et al.*, 2014) did no differ significantly between OE lines and the WT under either control or deficit conditions (Fig. 5b). In contrast, upon N-depletion treatment, high-affinity nitrate transporters *NRT2.1*, *NRT2.4* and *NRT2.5* (Lezhneva *et al.*, 2014) were significantly up-regulated in OE lines when

compared with the WT (Fig. 5b), while transcript levels of *NRT2.2*, *NRT2.6* and *NRT2.7* did not vary among genotypes (Fig. 5b). Nitrate reductase (NIA) assimilates nitrate after its uptake. Whereas *NIA1* transcripts did not differ among genotypes under deficit conditions, *NIA2* expression was up-regulated in OE lines when compared with the WT (Fig. 5c). These data demonstrated that overexpression of *MdATG18a* results in increased nitrate utilization by up-regulating some genes responsible for nitrate uptake and assimilation.

MdATG18a overexpression up-regulates other _MdATG_s in apple and increases autophagic activity in response to N-depletion

To determine whether overexpression of *MdATG18a* affects other autophagy-related genes, we used qRT-PCR to examine the transcripts of several other core *MdATG*s. As shown in Figure 6, under control conditions, expression of *MdATG3a*, *MdATG5*, *MdATG7a*, *MdATG7b*, *MdATG8c*, *MdATG8f*, and *MdATG10* did not differ among genotypes but *MdATG8i* and *MdATG9* were up-regulated in OE lines when compared with the WT. After the plants were transferred to N-depleted media for 10 d, most of the tested genes were induced by N-starvation, but their expression levels were significantly greater in the OE lines than in the WT (Fig. 6).

We used TEM to observe autophagosome formation in response to N-depletion. Under MS-control conditions, the leaves contained very few autophagosome structures, regardless of genotype (Fig. 7a). However, 15 d of deficit treatment stimulated the production of more autophagosomes. Furthermore, up to four times as many autophagosomes and autophagic bodies had accumulated in the OE lines than in the WT (Fig. 7a, b). These data demonstrated that the occurrence of autophagy in apple is significantly enhanced by overexpression of

MdATG18a when plants are challenged by the N-depletion.

Discussion

When the soil is poor in nutrients, plants must be able to recycle and remobilize elements efficiently if they are to adapt to the starved environment. Autophagy is an increasingly significant mechanism in plant resistance to stress and starvation and in remobilization. Its activity is also regulated by nutrient and energy status (Masclaux-Daubresse *et al.*, 2017). *Arabidopsis* ATG18a is required for autophagosome formation during periods of nutrient deprivation, leaf senescence, and abiotic stresses (Liu *et al.*, 2009; Xiong *et al.*, 2005, 2007). We previously cloned the homologous *MdATG18a* from *M. domestica*, and found that this gene is responsive to various abiotic stress conditions, including leaf senescence, drought, heat, oxidative stress, N starvation or endoplasmic reticulum (ER) stress (Wang *et al.*, 2014). Its overexpression in tomato and apple improves their tolerance to drought (Sun *et al.*, 2017). In the study presented here, we continued to explore its biological function under N-starvation. As we had predicted, overexpression of *MdATG18a* in both *Arabidopsis* and apple enhanced their tolerance, with plants showing better growth performance and higher accumulations of anthocyanin. Upon N-starvation, the core machinery genes of autophagy were greatly up-regulated and more autophagosomes were accumulated in the transgenic apple lines than in WT plants. These findings indicated that the improved tolerance to N-depletion is associated with increased autophagic activity due to *MdATG18a* overexpression.

A strong accumulation of anthocyanins is an interesting phenotype in transgenic plants after N-depletion. N-deficit affects the secondary metabolic pathways, particularly those for

anthocyanin production (Diaz *et al.*, 2006). Those pigments usually serve as important antioxidant molecules for plant tolerance to various stresses, e.g., a diminished supply of N or excess light. Autophagy-deficient plants exhibit reduced levels of anthocyanin (Chanoca *et al.*, 2015; Masclaux-Daubresse *et al.*, 2014; Pourcel *et al.*, 2010). A role has been proposed for autophagy in specific trafficking of anthocyanin-related molecules from their synthetic organelles to the vacuoles for storage (Chanoca *et al.*, 2015; Pourcel *et al.*, 2010). In contrast, Masclaux-Daubresse (2014) has demonstrated that autophagy mutants are defective in anthocyanin production because genes involved in the anthocyanin pathway are down-regulated. Accordingly, we found here that *MdATG18a* transgenic apple plants accumulated more anthocyanins than did the WT when plants were N-starved. Our expression analysis showed that, under deficit conditions, five structural genes (*CHI*, *CHS*, *ANS*, *PAL*, and *UFGT*) involved in anthocyanin biosynthesis and one TF (*MdMYB1*) that modulates this biosynthesis were up-regulated in the transgenic lines when compared with the WT. These results suggested that *MdATG18a* plays a positive role in anthocyanin biosynthesis when N is limited.

The underlying reason or upstream regulator behind the relationship between autophagy and anthocyanin production remains to be determined. Masclaux-Daubresse (2014) has proposed a role for the C/N balance because anthocyanin biosynthesis requires carbon resources. For example, in *Arabidopsis atg* mutants that are carbon-depleted, plants have low levels of glucose, fructose, sucrose, and starch. We also found that, under the N-depletion treatment, the leaves of our transgenic plants accumulated more soluble sugars but less starch when compared with the control. Those increased levels of soluble sugars in the OE lines

might have contributed to their greater accumulation of anthocyanins. Simultaneously, expression level of *MdAM1* and *MdSS1* were significantly higher in OE lines than WT under N depletion. *MdHXK1/2/3/5/6*, *MdFK1* and *MdNINV1/2* were up-regulated more in OE lines than in WT plants under N depletion condition. These data might contribute to the enhanced starch degradation and soluble sugar accumulation in the OE lines under N depletion.

A restricted supply of N in the soil can induce sink limitations within the whole plant due to decreased growth (Paul & Foyer, 2001), which in turn leads to feedback downregulation of photosynthesis. This N-depletion results in the accumulation of carbohydrates (sugars and starch) in the leaves, and a high level of carbon is then allocated to the roots to assist in N-uptake under starvation conditions (Remans *et al.*, 2006; Scheible & Stitt, 2004). Accordingly, we noted that the N-deficit was associated with lower fresh weights and reduced leaf growth, along with increased root growth and a greater accumulation of carbohydrates. Furthermore, the *MdATG18a*-OE apple lines displayed more root growth and higher amounts of soluble sugars but less starch accumulation in the leaves. Autophagy contributes to starch degradation by sequestering into the vacuoles small starch granule-like structures for their subsequent breakdown to produce sugars (Wang *et al.*, 2013b). The decreased accumulation of starch in our transgenic plants might have resulted from accelerated starch degradation due to enhanced autophagic activity. This then led to greater sugar production that, in turn, promoted root growth and anthocyanin biosynthesis, thereby making those transgenics more tolerant to N-depletion conditions.

In addition to regulating carbon, *MdATG18a* positively controls nitrate uptake and assimilation by effecting the expression of genes for nitrate reductase and nitrate uptake. We

determined here that *MdATG18a* up-regulated the expression of *MdNIA2*, *MdNRT2.1*, *MdNRT2.4* and *MdNRT2.5* but did not influence *MdNIA1*, *MdNRT1.1*, *MdNRT1.7*, *MdNRT2.2*, *MdNRT2.6* and *MdNRT2.7*. Most members of the NRT1 family have a low affinity for nitrate (Léran *et al.*, 2014). In *Arabidopsis*, NRT2.1, NRT2.2, NRT2.4, and NRT2.5 account for most of the high-affinity nitrate influx activity under an N-limitation (Lezhneva *et al.*, 2014). Our results demonstrated that *MdATG18a* positively regulated three high-affinity nitrate transporters-*MdNRT2.1*, *MdNRT2.4* and *MdNRT2.5*. In addition, the increase in nitrate levels in the OE apple plants might have been associated with better growth performance during the period of N-starvation. Therefore, *MdATG18a* may play vital roles in coordinating the acquisition of C and N, thereby affecting anthocyanin biosynthesis and supporting plant survival under N-depletion conditions.

In summary, *Arabidopsis* and apple plants that over-express *MdATG18a* show improved tolerance to a deficit of N and they accumulate more anthocyanins. The apple transgenics also have increased autophagic activity, which might contribute to their better growth and higher starch degradation under depleted conditions. The accumulation of more soluble sugars and upregulation of anthocyanin-related genes by *MdATG18a* promotes anthocyanin biosynthesis in starved OE plants. This gene also positively regulates nitrate uptake and assimilation by up-regulating genes for high-affinity nitrate transporters NRT2.1/2.4/2.5 and NIA2. Based on these results, we suggest that enhanced autophagy due to overexpression of *MdATG18a* improves nitrogen acquisition and anthocyanin accumulation, which in turn support plant growth and survival in an N-deficient condition.

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Conflict of interest

The authors declare no conflicts of interest.

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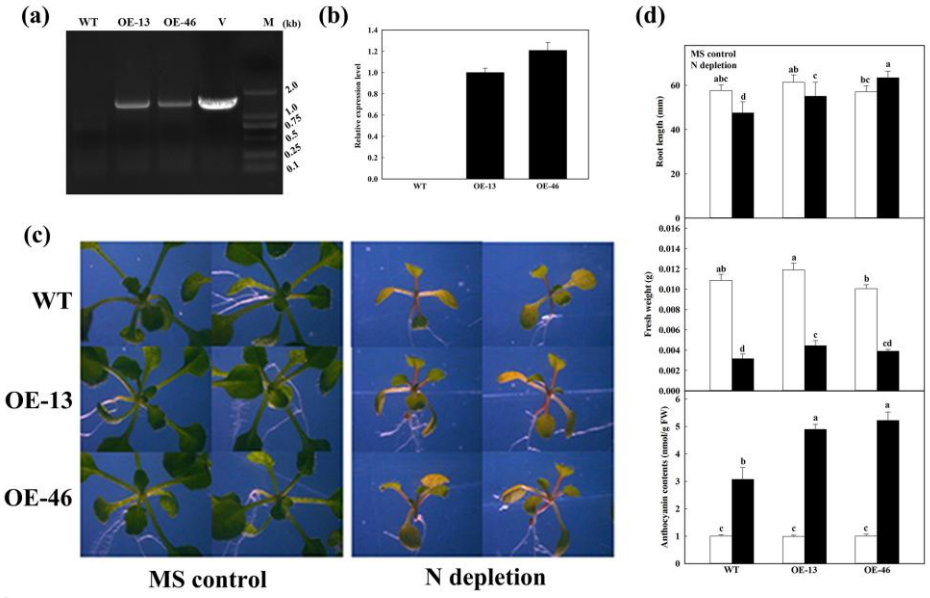

Fig. 1 *MdATG18a* overexpression enhances tolerance to N-depletion in *Arabidopsis*. (a) PCR confirmation of transgenic plants. Left panel: PCR with DNA; Lanes: M, molecular marker DL2000; V, positive vector containing pCambia2300-*MdATG18a* plasmid; WT, non-transformed wild-type; OE-13 and -46, MdATG18a-transgenic *Arabidopsis* lines. (b) Quantitative RT-PCR analysis of *MdATG18a* expression in leaves of WT and transgenic Lines OE-13 and OE-46. Five-day-old *Arabidopsis* seedlings were transferred to MS-control or N-depleted agar media. After 10 d under LD photoperiod, they were photographed (c) before recording fresh weights, root lengths, and anthocyanin concentrations (d). Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

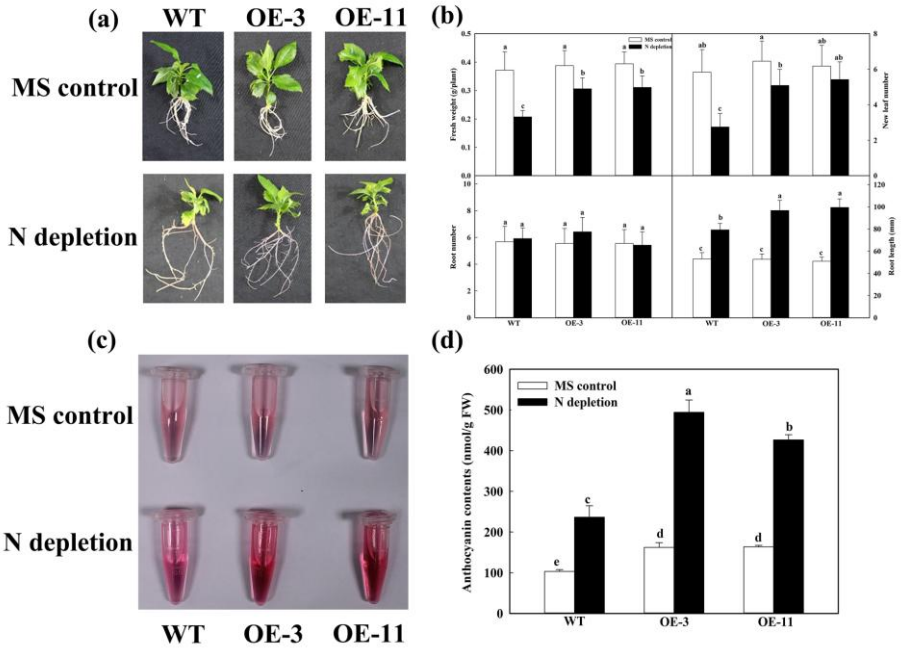

Fig. 2 *MdATG18a* overexpression enhances tolerance to N-depletion in transgenic apple. Segments were transferred to N-depleted MS media for 20 d. (a) Phenotypes of WT and OE lines in response to deficiency treatment. (b) Fresh weights, numbers of new leaves and roots, and root lengths were recorded at end of treatment period. (c-d) Extract colors and anthocyanin concentrations in roots. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

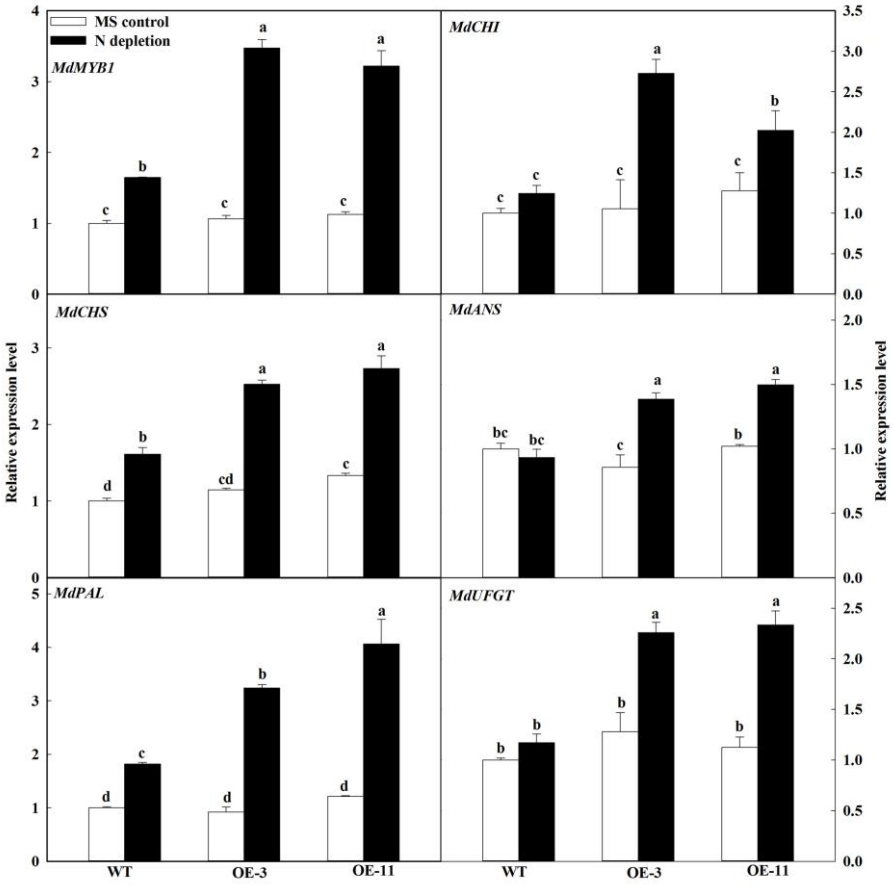

Fig. 3 Expression of anthocyanin-related genes under N-depletion. Total RNA was isolated from root samples collected at 10 d after transfer to deficient media, and expression levels were calculated relative to expression of *Malus EF-1α* mRNA. Expression in WT under MS control was set to '1'. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

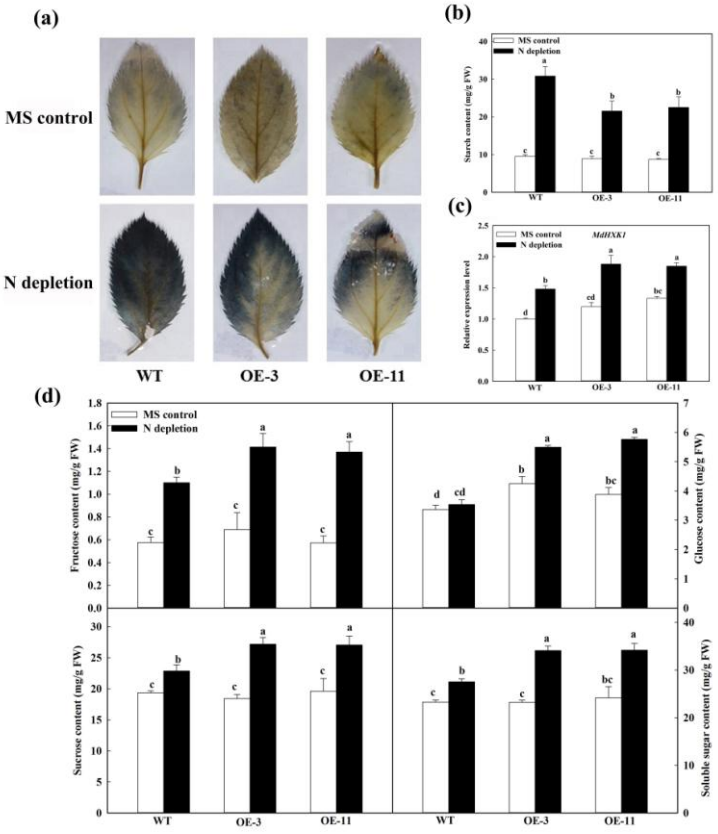

Fig. 4 Accumulation of soluble sugars and degradation of starch under N-depletion. (a) Staining of starch after placement on deficient media for 20 d. (b) Starch concentrations after 20 d on deficient media. (c) Expression of *MdHXK1* after 10 d on deficient media. (d) Levels of fructose, glucose, sucrose, and total soluble sugars measured 20 d after transfer to deficient media. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

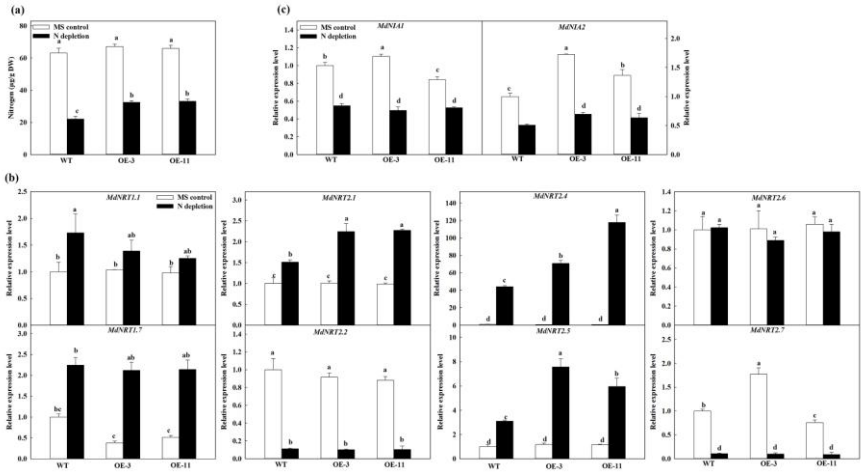

Fig. 5 Overexpression of *MdATG18a* enhances nitrate absorption and assimilation in apple. (a) Total leaf nitrate contents. Nitrate concentration was determined after 20 d of N-depletion. (b) Expression of *NRT1* and *NRT2* family genes. (c) Expression of *NIA1* and *NIA2* genes. Expression level was analyzed on Day 10 d of treatment. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

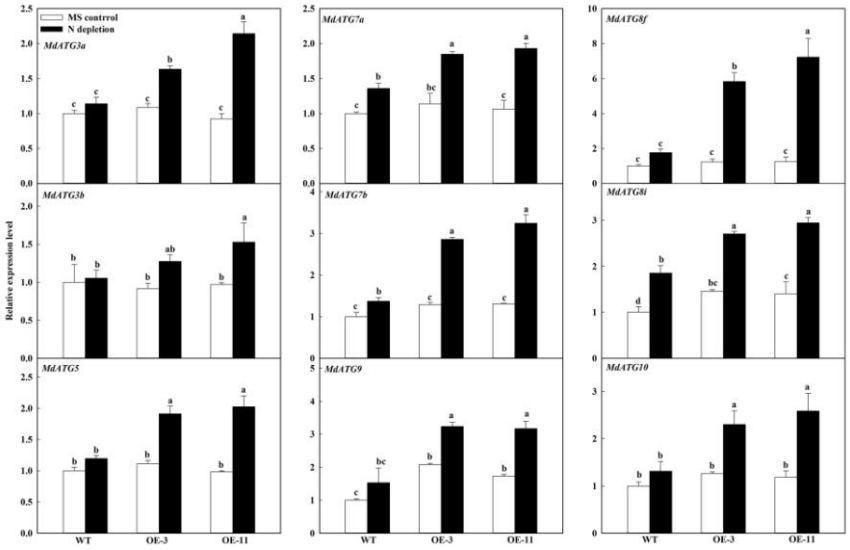

Fig. 6 Changes in transcription level of apple autophagy-related genes under N-depletion. Total RNA was isolated from leaf samples collected after 10 d of treatment, and expression levels were calculated relative to expression of *Malus EF-1α* mRNA. Expression in WT under MS control was set to '1'. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests (P <0.05).

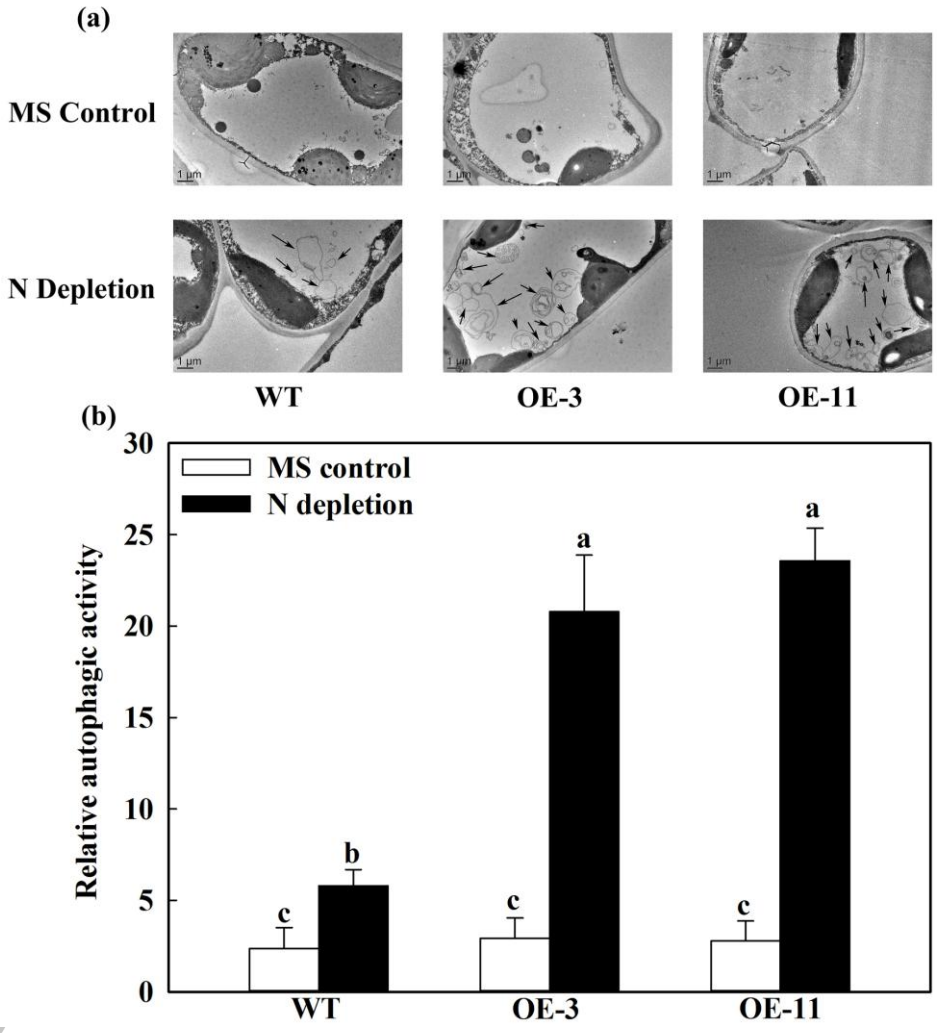

Fig. 7 Visualizing accumulation of autophagosomes under N-depletion by TEM. (a) Representative images of autophagic structures in mesophyll cells from WT and *MdATG18a*-OE plants on Day 15 of treatment. Autophagosomes and autophagic bodies structures are indicated by black arrows. Scale bars: 1 μm. (b) Relative autophagic activity normalized to activity of WT or *MdATG18a*-OE plants shown in (a). More than 10 cells were used to quantify structures. Data are means of 3 replicates with SD. Different letters indicate significant differences between treatments, according to one-way ANOVA Tukey's multiple range tests ($P < 0.05$).