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## Reactive oxygen species derived from NADPH oxidase regulate autophagy under nitrogen and carbon deficiency in wheat root tips

H. JING\*, Z. WANG, L. ZHANG, G. ZHOU, Z. GU, Y. SHAN, Y. ZHOU, Z. YANG, and Z. KONG

College of Biological Engineering, Henan University of Technology, Zhengzhou 450001, P.R. China

### Abstract

Reactive oxygen species (ROS) originating from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) play vital roles in regulating autophagy. However, the relationship between autophagy and NOX in common wheat (*Triticum aestivum* L.) is still unknown. In order to clarify the mechanism of autophagy in wheat, ROS content, NOX activity, and autophagy levels in root tip cells under condition of N or C deficiency were measured. The results showed that the N and C deficiency increased the production of superoxide anions ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) via the NOX activation, leading to an increase in the number of autophagic bodies, in comparison to the control group. This showed that autophagy constitutively existed in the root tip cells of wheat seedlings. Interestingly, imidazole, a NOX inhibitor, inhibited the  $H_2O_2$  and  $O_2^{\cdot-}$  content and reduced the accumulation of autophagic bodies under N deficiency. Hydrogen peroxide and  $O_2^{\cdot-}$  derived from NOX possibly regulated autophagy in wheat root tip cells. In order to further verify whether ROS regulated autophagy, exogenous ROS or a reductant was applied to the roots of wheat seedlings under N deficiency. The results showed that exogenous  $O_2^{\cdot-}$  and  $H_2O_2$  both induced the accumulation of autophagic bodies in the vacuoles of root tip cells. On the contrary, glutathione and ascorbic acid significantly decreased the accumulation of autophagic bodies. Therefore, this study confirmed that  $H_2O_2$  and  $O_2^{\cdot-}$  derived from NOX could regulate autophagy in root tip cells of wheat seedlings under stress.

*Additional key words:* imidazole,  $H_2O_2$ , superoxide anion, *Triticum aestivum*.

### Introduction

Wheat is one of the most important crops in the world. To date, there is little information available in the literature about the mechanism and physiological functions of autophagy in crop species. Macroautophagy (referred to in this paper as autophagy) is a conserved biological process by which unwanted proteins or organelles are engulfed to form the double-membrane structure of autophagosomes in the cytoplasm. The autophagosomes then fuses with the vacuole at the outer membrane and the inclusion inside the autophagosome is disassembled inside the vacuole (Yang and Bassham 2015, Klionsky *et al.* 2016, Wang *et al.* 2018). Autophagy is generally maintained at a basal level for homeostasis and can be upregulated under stress to aid plant survival (Wang *et al.* 2018).

For example, constitutive autophagy has been proven in *Arabidopsis thaliana* root tip cells (Inoue *et al.* 2006). Previous studies have confirmed that deficiency stress, such as N or C deficiency, can induce autophagy (Floyd *et al.* 2012, Liu and Bassham 2012, Yoshimoto 2012). In addition, autophagy also participates in maturation and programmed cell death in the endosperm (Li *et al.* 2015). Autophagy plays vital roles in mobilizing nutrients from the endosperm of seeds for early growth and development of seedlings (Chung *et al.* 2009). Therefore, seedlings are the best model of constitutive autophagy.

Reactive oxygen species, as byproducts of common metabolism, play dual roles in plant cells (Suzuki *et al.* 2011, Marino *et al.* 2012, Liu and He 2016, Wang *et al.* 2016). Superoxide anion and  $H_2O_2$  are two kinds of ROS in cells. As signal molecules, moderate ROS production

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*Abbreviations:* ASA - ascorbic acid; DHE - dihydroethidium; NADPH - nicotinamide adenine dinucleotide phosphate; GSH - glutathione; MS - Murashige and Skoog; NBT - nitroblue tetrazolium; NOX - nicotinamide adenine dinucleotide phosphate oxidase; ROS - reactive oxygen species; TEM - transmission electron microscopy.

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\* Corresponding author, e-mail: hjjing@haut.edu.cn

regulates plant development, growth, and response to biotic and abiotic stresses (Suzuki *et al.* 2011, Marino *et al.* 2012, Wang *et al.* 2016, Liu and He 2016). Excess ROS destroys the normal structure of cells by oxidation of lipids, proteins, and nucleic acids.

In mammals,  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  are able to regulate autophagy. For example, nutrient deficiency leads to ROS accumulation, especially  $\text{H}_2\text{O}_2$  aggregation in the mitochondria, which induces autophagy (Scherz-Shouval *et al.* 2007, Poillet-Perez *et al.* 2015). Similar results have been found in plants. Xiong *et al.* (2007a,b) showed that methyl viologen, as the producer of ROS, activated autophagy in *A. thaliana*. Some research has shown that exogenous  $\text{H}_2\text{O}_2$  results in aggregation of  $\text{O}_2^-$  but not  $\text{H}_2\text{O}_2$  and showed that  $\text{O}_2^-$  induces autophagy (Chen *et al.* 2009, Poillet-Perez *et al.* 2015). However, the exact ROS molecule that induces autophagy is still unknown. On the other hand, autophagy affects the elimination of ROS. Hydrogen peroxide accumulated in the *A. thaliana* mutants *atg5* and *atg2*, which lack the autophagy related genes (*ATG5* or *ATG2*; Yoshimoto *et al.* 2010). In addition, the rice (*Oryza sativa* L.) mutant *atg10* was more sensitive to methyl viologen and accumulated more oxidized protein than the wild-type (Shin *et al.* 2009). Therefore, the complex relationship between ROS and autophagy is still the subject of ongoing research.

In apoplasts, ROS mainly originate from NADPH oxidases (NOXs), which are homologs of respiratory burst oxidase homolog proteins. The NADPH oxidases oxidize oxygen molecules to  $\text{O}_2^-$  in the apoplast by use of the electron donor of NADPH in the cytoplasm (Sagi and Fluhr 2006, Liu and He 2016). In addition, other studies have shown that ROS derived from NOX play important roles in tip growth (Evans *et al.* 2016). For example, root length in the *A. thaliana* mutant *rhd2* (root hair defective) shortened by 20 % and ROS content in the mutant also decreased (Foreman *et al.* 2003, Monshausen *et al.* 2007). Subsequent research showed that the *rhd2* mutant was caused by the mutation of *AtRbohC*, demonstrating that NOXs plays essential roles in root tip growth. Our research also demonstrated that ROS derived from NOX regulates wheat root growth (Jing *et al.* 2012). Notably, NOX participates in autophagy induced by nutrient deficiency and salt stress but not by osmotic stress in *A. thaliana* (Liu *et al.* 2009). Until recently, there has been a gap in our knowledge of ROS derived from NOX and how they regulate autophagy in common wheat.

In this study, in order to probe the relationship of ROS and autophagy, we explored the effect of deficiency stress on ROS content, NOX activity and autophagy in root tip cells of common wheat seedlings. The results may help us to understand the mechanisms of root growth under deficiency stress.

## Materials and methods

**Plants, growth conditions, and treatments:** Wheat (*Triticum aestivum* L. cv. Zhengmai 9023) seeds, obtained from the Henan Academy of Agriculture, China, were

surface-sterilized in 0.5 % (m/v)  $\text{NaClO}_3$  for 15 min and washed extensively with deionized water. Seeds were incubated in 9-cm Petri dishes (35 seeds per dish) containing two layers of moistened filter paper. Wheat seedlings were grown on distilled water under a 15-h photoperiod, an irradiance of  $?? \mu\text{mol m}^{-2} \text{s}^{-1}$ , and a temperature of 25 °C. For N deficiency or C deficiency, 3-d-old seedlings grown on distilled water were transferred to solid Murashige-Skoog (MS) nutrient medium lacking sucrose or N for an additional 6 d. Plants grown on sucrose deficiency plates were incubated in the dark. These seedlings were used for measurements of NOX activity and the lengths of shoots, roots, and coleoptiles. In order to further verify the function of NOX during deficiency stress, imidazole was used to treat 3-d-old seedling for 6 d.

In order to verify whether autophagy in the root cells of wheat seedling was regulated by ROS, reductants [ascorbic acid (ASA) and glutathione (GSH)] and exogenous ROS ( $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$ ) were used to treat 3-d-old seedlings under N deficiency.

In order to detect autophagy or ROS *in situ*, 3-d-old seedlings grown on distilled water were subjected to C deficiency or N deficiency for 2, 4, 6, and 24 h. In addition, 3-d-old seedlings were also transferred to MS liquid medium with or without 0.2 mM imidazole, 100  $\mu\text{M}$  ASA or GSH, 1 mM  $\text{H}_2\text{O}_2$ , or 0.01 mM  $\text{O}_2^-$  for the indicated times. The  $\text{O}_2^-$  production system (the methionine-riboflavin system) included: 26 mM *L*-methionine, 4  $\mu\text{M}$  riboflavin, and 20  $\mu\text{M}$  ethylenediaminetetraacetic acid- $\text{Na}_2$ . This system produced  $\text{O}_2^-$  when solutions of methionine and riboflavin were irradiated (El-Zahaby *et al.* 2004). To avoid possible damage caused by continuous action of  $\text{O}_2^-$ , an alternative system (1 h light and 1 h darkness) were used for  $\text{O}_2^-$  production and germinated seeds were grown under an irradiance of 120  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 25 °C. In order to detect whether NOX changed autophagy under deficiency stress, 3-d-old seedlings were treated with MS liquid medium without N but with 100  $\mu\text{M}$  aloxistatin (*Sigma-Aldrich*, St Louis, USA) and 0.2 mM imidazole.

***In-situ* detection and quantification of  $\text{O}_2^-$  and  $\text{H}_2\text{O}_2$ :** Hydrogen peroxide was monitored with 2,7-dichlorodihydrofluorescein (DCFH) diacetate (*Sigma-Aldrich*) as described elsewhere (Laureano-Marín *et al.* 2016). The formed DCFH-DA does not fluoresce but can freely pass through the cell membrane. The dye is hydrolyzed by esterase to DCFH, which is captured by an actively respiring cell. The DCFH was oxidized by ROS to a fluorescent compound DCF in cytoplasm. To detect  $\text{O}_2^-$ , dihydroethidium (DHE) (*Sigma-Aldrich*) was used as a probe. The fluorescence of DCF and DHE was detected by laser scanning confocal microscopy (*FV3000*, *Olympus*, Japan).

**Cell viability assay:** Root tip cells of wheat seedlings were observed using propidine iodide staining (Chen and Fluhr 2018). The roots were stained with 15  $\mu\text{M}$  propidine iodide (*Thermo Fisher Scientific*, Shanghai, China) in the dark for 30 min and then washed three times with a phosphate buffer. The fluorescence of propidine iodide was detected

by laser scanning confocal microscopy.

**Determination of NOX activity:** The NOX activity was assayed according to the protocols reported by Sarath *et al.* (2007). Roots from seedlings (1.0 g f.m.) were ground in a mortar and pestle with 3 cm<sup>3</sup> of cold (4 °C) sodium-phosphate buffer (pH 6.0, 10 mM), transferred to 10-cm<sup>3</sup> tubes, kept on ice, and sonicated with a microtip (*SCIENTZ-950E*, Ningbo Xinzhi Biotechnology, Ningbo, China) for 15 s. The extracts were centrifuged at 14 000 g for 15 min. The crude seedling homogenates were precipitated with acetone (9:1 acetone/homogenate) at -20 °C for 15 min. Precipitated proteins were recovered by centrifugation at 14 000 g and 4 °C for 10 min. Protein pellets were resuspended in a buffer [50 mM Tris(hydroxymethyl)-aminomethane-HCl, 0.1 mM MgCl<sub>2</sub>, 0.25 M sucrose, and 0.1 % (m/v) *Triton-X-100* (*Sigma-Aldrich*, pH 8.0)] and were used for measurement of NOX activity.

The NOX activity was determined according nitroblue tetrazolium (NBT) reduction. The reaction solution contained 0.5 cm<sup>3</sup> of a protein solution, 0.5 cm<sup>3</sup> of 730 μM NBT, and 1 cm<sup>3</sup> of 100 μM NADPH to initiate reaction. The products of NBT reduction were determined with a spectrophotometer at 530 nm. The coefficient of absorbance of 12.8 mM cm<sup>-1</sup> was used for calculation of the NOX activity. Protein content was detected with Coomassie brilliant blue (*Sigma-Aldrich*).

The in-gel assay for NOX activity was performed according to the procedures described by Sarath *et al.* (2007). The protein solution was electrophoretically separated on 10 % (m/v) native polyacrylamide gels. After electrophoresis, gels were incubated in the darkness in a reaction mixture containing 50 mM Tris(hydroxymethyl)-aminomethane-HCl (pH 7.4), 0.75 mM NBT, and 0.1 mM MgCl<sub>2</sub> for 10 min. Next, 0.2 mM NADPH was added and the appearance of blue formazan bands was monitored. The reaction was halted by immersion of the gels in distilled water.

**Transmission electron microscopy (TEM)** was performed using the large equipment management system in Huazhong Agricultural University, China. For TEM, samples were collected, fixed with 2 % (m/v) glutaraldehyde and 1 % (m/v) formaldehyde in a 100 mM sodium cacodylate-HCl buffer (pH 6.9) at room temperature for 1 h, and then at 4 °C overnight, and post-fixed with 1 % (m/v) osmium tetroxide for 2 h at room temperature. The samples were stained with 2 % aqueous uranyl acetate for 30 min, dehydrated in a graded ethanol series (30, 50, 70, 90 and 100 % ethanol), cleared with ultra-pure acetone, and infiltrated and embedded using Spurr's epoxy resin (*Hitachi*, Tokyo, Japan). Ultrathin sections (60 - 80 nm) were cut with a *Reichert UC6* ultramicrotome (*Leeds Precision Instruments*, Minneapolis, USA), followed by collection onto copper grids and counterstaining with 2 % uranyl acetate for 30 min. Images were captured via scanning microscopy and TEM (*H-7650*, *Hitachi*, Tokyo, Japan).

**Statistical analysis:** The number of autophagic bodies

in each cell was manually counted, and only individual dots were counted as autophagosomes. The fluorescence data were summarized by the software *PDQuest 8.0*. Each value was presented as a mean ± SE from a minimum three replicates. Statistical analysis was carried out by one-way *ANOVA* with the *t*-test being used to evaluate whether the means were significantly different taking  $P < 0.05$  and  $P < 0.01$  as significant or very significant, respectively.

## Results

In order to detect the effects of N or C deficiency on the growth of wheat seedlings, shoot length, root length, root number, and the viability of root tip cells were tested. The results showed that N deficiency markedly inhibited the length of shoots and roots, whereas the number of roots in of each seedling was not altered (Fig. 1A). In detail, shoot length and root length decreased to 78.2 and 65.4 % under N deficiency, respectively. In contrast, C deficiency did not change the shoot length, the root length, or the number of roots in wheat seedlings (Fig. 1A). However, N deficiency and C deficiency both increased the rate of death in root tip cells (Fig. 1B). Therefore, N deficiency and C deficiency increased cell death in roots and only N deficiency inhibited the early growth of wheat seedlings.

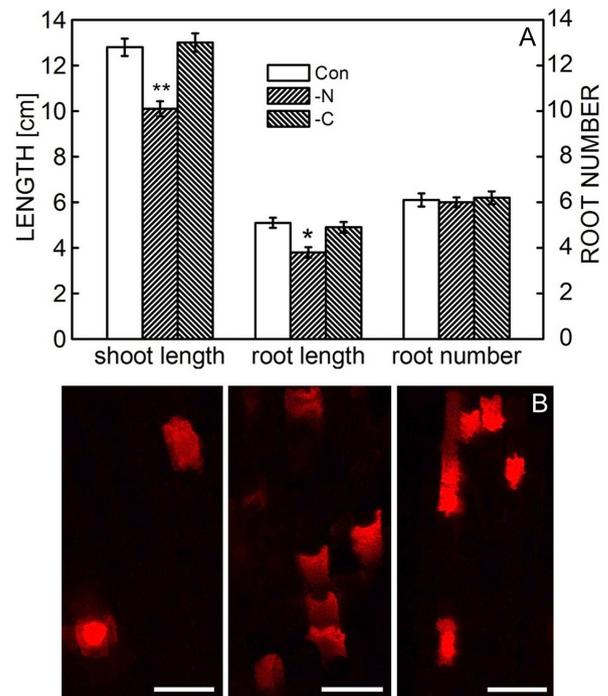


Fig. 1. Nitrogen deficiency (-N) and carbon deficiency (-C) inhibited early growth of wheat seedlings. A - The seedlings (3-d-old) were grown in a Murashige-Skoog medium without N or sucrose and under dark for 6 d, and the shoot length, root length, and number of roots were measured. Means ± SEs,  $n = 3$ , \* and \*\* indicate significant differences at  $P \leq 0.05$  and 0.01, respectively. B - Viability test in root tips. Dead cells were stained with propidium iodide. The scale bars: 20 μm.

It is well known that  $H_2O_2$  and  $O_2^{\cdot-}$  play vital roles in root growth (Han *et al.* 2015, Wang *et al.* 2016). In order to detect whether N or C deficiency induced ROS,  $H_2O_2$  and  $O_2^{\cdot-}$  produced in root tip cells were stained *in situ* with 2,7-dichlorodihydrofluorescein and DHE, respectively, for 2, 4, 6, and 24 h (Fig. 2). The fluorescence observation and quantification revealed that the production of  $O_2^{\cdot-}$  and  $H_2O_2$  in root tip cells of wheat seedlings was induced by N and C deficiencies in all analyzed time points.

It has been proved that the major ROS production in apoplast is connected with NOX. In order to verify if the  $O_2^{\cdot-}$  and  $H_2O_2$  in root tip cells of wheat seedling came from NOX, the effects of N and C deficiency on NOX activity were detected by treating 3-d-old seedlings with MS medium minus N or in the dark for 6 d. The in-gel assays and spectrophotometry showed that NOX activity was strongly activated by N and C deficiency and NOX activity increased to 183.3 and 176.7 % under N and C deficiency, respectively (Fig. 3B).

To verify the function of NOX under deficiency stress, imidazole which strongly decrease NOX activity was used (Fig. 4). The imidazole decreased NOX activity to 84.3 % under N deficiency and to 79.6 % under C deficiency (Fig. 4B). Correspondingly, imidazole decreased  $O_2^{\cdot-}$  content and  $H_2O_2$  content under N and C deficiency as well as in the control plants (Fig. 5). Therefore, the production of  $H_2O_2$  and  $O_2^{\cdot-}$  under deficiency stress was derived from NOX.

The accumulation of autophagic bodies in the vacuoles was detected and analyzed by TEM (Oh-ye *et al.* 2011). Ascorbic acid (ASA) or glutathione (GSH) both decreased  $H_2O_2$  and  $O_2^{\cdot-}$  production in the meristem and in the elongation zone of root tip, while exogenous  $O_2^{\cdot-}$  increased both  $H_2O_2$  and  $O_2^{\cdot-}$  production, and exogenous  $H_2O_2$  increased only  $H_2O_2$  production (unpublished results). Accordingly, when compared with the control, ASA and GSH markedly decreased the accumulation of autophagic bodies in the vacuoles of root cells of seedlings cultivated

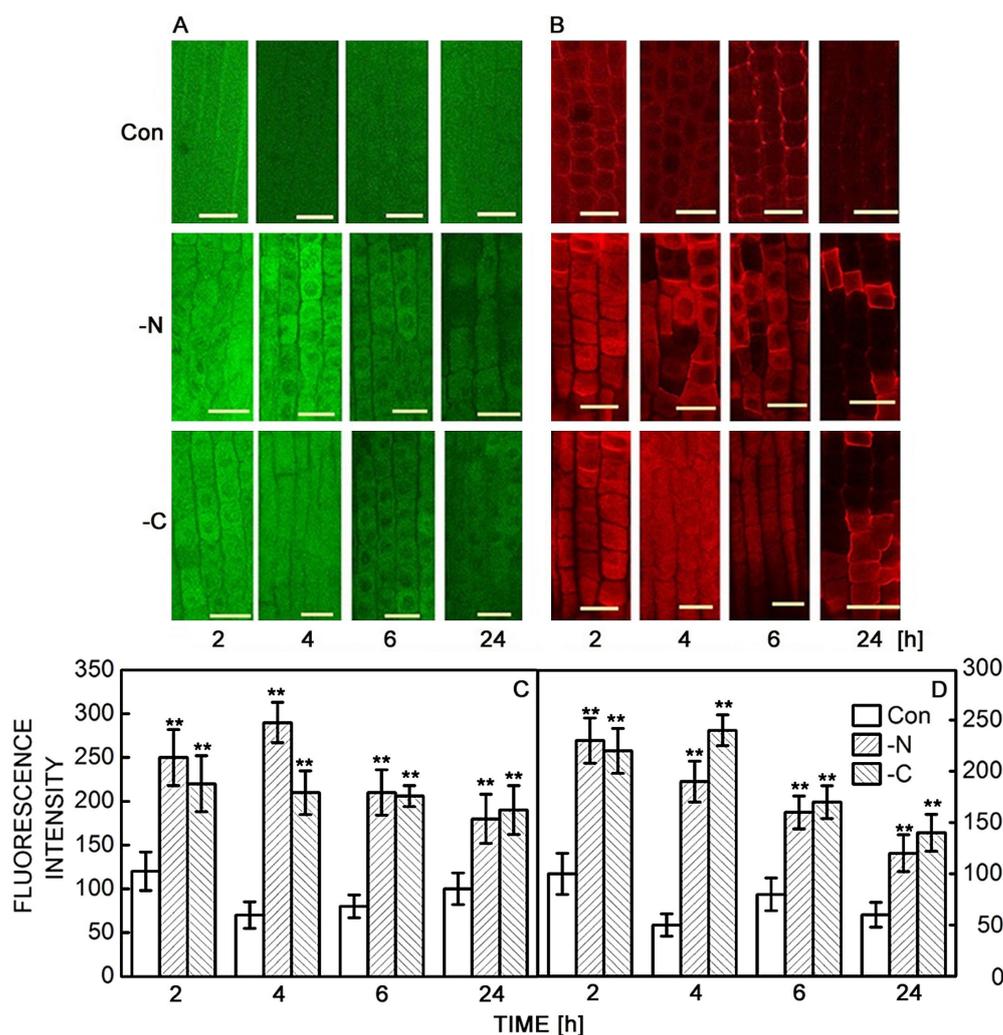


Fig. 2. Nitrogen deficiency (-N) and carbon deficiency (-C) induced the production of  $O_2^{\cdot-}$  and  $H_2O_2$  in root cells. To induce deficiency stress, 3-d-old wheat seedlings were transferred to a Murashige-Skoog medium without N or sucrose for 2, 4, 6, and 24 h. The roots of the seedlings were stained with 2,7-dichlorodihydrofluorescein (A) or dihydroethidium (B) to detect  $H_2O_2$  (A) and  $O_2^{\cdot-}$  (B) production in the meristematic zone. The scale bars: 20  $\mu$ m. Fluorescence intensities from A and B were summarized in C and D, respectively. Means  $\pm$  SEs,  $n = 8$ , \*\* indicates significant differences at  $P < 0.01$ .

under the N deficiency (Fig. 6). On the contrary, exogenous  $H_2O_2$  and  $O_2^-$  increased the accumulation of autophagic bodies (Fig. 6). Therefore,  $H_2O_2$  and  $O_2^-$  both had the ability to activate autophagy in wheat root tip cells under the N deficiency. Further on, the accumulation of autophagic bodies in the root cells of N-deficient seedlings was higher than in the control (Fig. 7). Most importantly, imidazole notably decreased the accumulation of autophagic bodies in the vacuoles of wheat seedlings (Fig. 7). These data suggest that autophagy constitutively exists in root cells of wheat seedlings, N deficiency induced autophagy, and NOX altered autophagy under N deficiency.

## Discussion

It is well known that N or C deficiency induces autophagy (Floyd *et al.* 2012, Liu and Bassham 2012, Yoshimoto 2012). Our results also showed that N and C deficiency increased the death of root tip cells, and only N deficiency inhibited root and shoot growth (Fig. 1). In addition, ROS derived from NOX plays vital roles in root growth (Foreman *et al.* 2003, Monshausen *et al.* 2007). In order to understand which mechanisms contribute to observed effect on the root growth, autophagy, ROS derived from NOX, and the relationships among them were studied.

To our knowledge, only few *ATGs* of wheat have been cloned because of the complexity of the wheat

genome. The *TdATG8* was the first cloned full-length gene from wild emmer wheat (*Triticum dicoccoides*) and its function was confirmed through yeast mutant functional complementation assay (Kuzuoglu-Ozturk *et al.* 2012). Further, two members of *ATG4* and nine members of *ATG8* have been cloned and their functions have been identified (Minibayeva *et al.* 2012, Pei *et al.* 2014, Ryabovol and Minibayeva 2014). Three members of the *ATG6* family (*ATG6a*, *ATG6b*, and *ATG6c*) are located on the homologous chromosomes 3DL, 3BL, and 3AL of allohexaploid wheat (Yue *et al.* 2015). The expression of *ATG6* was induced by powdery mildew or abiotic stress (Yue *et al.* 2015).

It has been proven that constitutive autophagy occurs in root tip cells of *A. thaliana* (Inoue *et al.* 2006) and *Triticum dicoccoides* (Kuzuoglu-Ozturk *et al.* 2012). The knockdown *taatg6* mutant of *T. dicoccoides* showed impaired constitutive autophagy in leaves under normal conditions (Yue *et al.* 2015). Our study also showed that constitutive autophagy existed in root tip cells of *T. aestivum* (Figs. 6, 7). During seed germination and early seedling growth, constitutive autophagy transferred nutrients from the endosperm to support the growth of seedlings. Therefore, any factor that changes autophagy, in principle, could alter the growth of seedlings. This study also showed that C or N deficiency induced autophagy in root tip cells of wheat seedlings (Fig. 7).

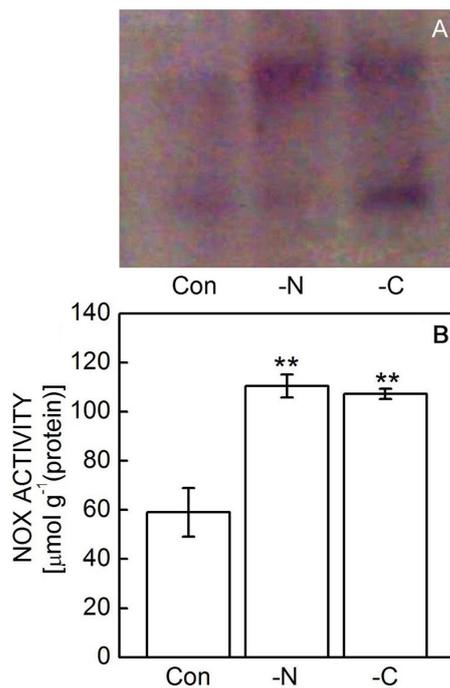


Fig. 3. Nitrogen deficiency (-N) and carbon deficiency (-C) increased the activity of nicotinamide adenine dinucleotide phosphate oxidase (NOX). For deficiency stress, 3-d-old wheat seedlings were transferred to a liquid Murashige-Skoog medium without N or sucrose for 6 d. The NOX activity in the roots of the seedlings was detected by electrophoresis (A) and spectrophotometry (B). Means  $\pm$  SEs,  $n = 3$ , \*\* indicates significant differences at  $P \leq 0.01$  according to the *t*-test.

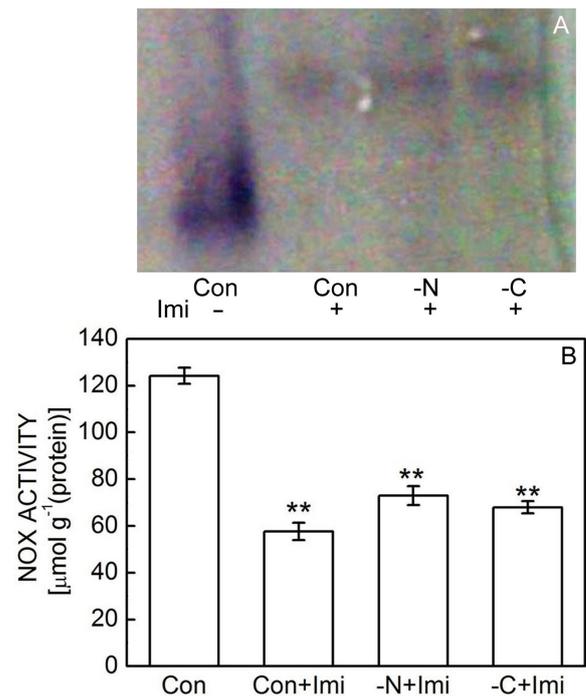


Fig. 4. Imidazole (Imi) inhibited the activity of nicotinamide adenine dinucleotide phosphate oxidase (NOX) in roots of wheat seedlings under N deficiency (-N) or carbon deficiency (-C). To induce deficiency stress, 3-d-old seedlings were transferred to a liquid Murashige-Skoog medium without N or sucrose for 6 d. The NOX activity in the seedling roots was detected by electrophoresis (A) and spectrophotometry (B). Means  $\pm$  SEs,  $n = 3$ , \*\* indicates significant differences at  $P \leq 0.01$  according to the *t*-test.

Many studies have shown that ROS derived from NOX regulates autophagy in *A. thaliana* (Liu *et al.* 2009, Liu and Bassham 2010, Morriss *et al.* 2017). The NOX inhibitors block the activation of autophagy under mineral deficiency and salt stress but not under drought (Liu *et al.* 2009) and they did not block constitutive autophagy (Liu and Bassham 2010). However, the null mutant *rns2-2* increased the amount of ROS, and the inhibitor of NOX diphenylene iodonium blocked the constitutive autophagy phenotype by inhibiting NOX (Morriss *et al.* 2017). Therefore, the view that ROS derived from NOX can regulate autophagy is not fully confirmed. Here, our results showed that nutrient deficiency activated the production of  $O_2^-$  and  $H_2O_2$  (Fig. 2) and they also promoted NOX activity in roots (Fig. 3). The NOX inhibitor not only blocked NOX activity (Fig. 4) and the production of  $H_2O_2$  and  $O_2^-$  under deficiency (Fig. 5), but also decreased the autophagy under N or C deficiency (Fig. 6). In this study, we concluded that  $O_2^-$  and  $H_2O_2$  originating from NOX possibly regulated the autophagy under nutrient deficiency in common wheat.

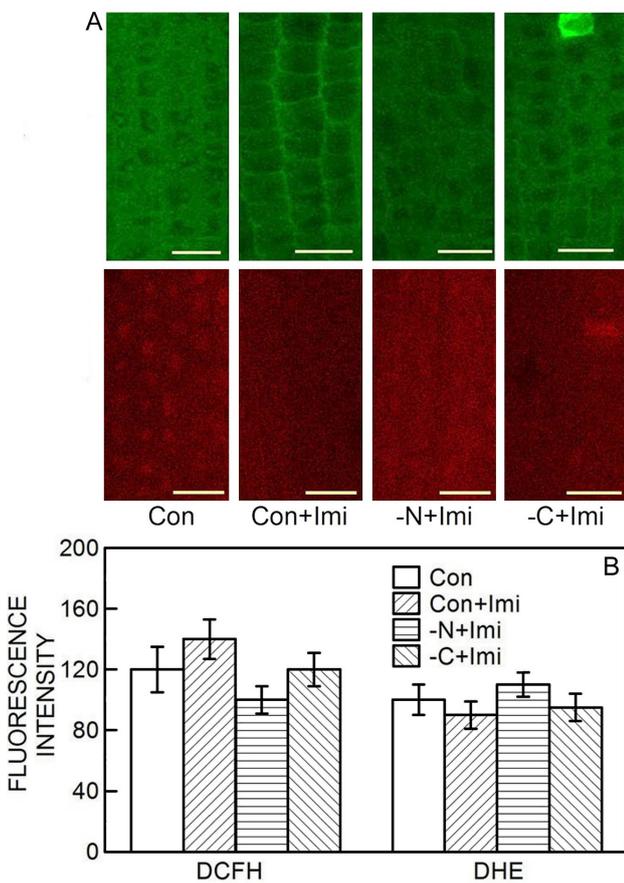


Fig. 5. Nitrogen deficiency (-N) or carbon deficiency (-C) induced an increase in  $O_2^-$  and  $H_2O_2$  content. To induce deficiency stress, 3-d-old wheat seedlings were transferred to a Murashige-Skoog medium with (Con) or without N or sucrose and with or without 20 mM imidazole (Imi) for 4 h. *A* - The roots of the seedlings were stained with 2,7-dichlorodihydrofluorescein or dihydroethidium to detect  $H_2O_2$  and  $O_2^-$  production, respectively, in the meristematic zone. The scale bar: 20  $\mu$ m. *B* - The fluorescence intensities of *A* were summarized in *B*. Means  $\pm$  SEs,  $n = 8$ .

Two conclusions can be drawn from our results. Firstly, the results confirmed that ROS produced by NOX regulated autophagy under nutrient deficiency. However, ROS produced from NOX had no influence on constitutive autophagy because a few autophagic bodies accumulated in root tip cells under imidazole treatment (Fig. 6). Secondly, the results further supported the view that autophagy is regulated by  $O_2^-$  and  $H_2O_2$  that probably originated from NOX. It is well known that ROS regulates autophagy (Ryter *et al.* 2012, Sciarretta *et al.* 2014, Rituraj *et al.* 2016, Zhu *et al.* 2016, Forte *et al.* 2017), although the exact ROS molecules controlling autophagy are still uncertain. For example, Scherz-Shouval *et al.* (2007) and Poillet-Perez *et al.* (2015) found that ROS induced autophagy under nutrient deficiency. Furthermore, an increase in  $O_2^-$  in

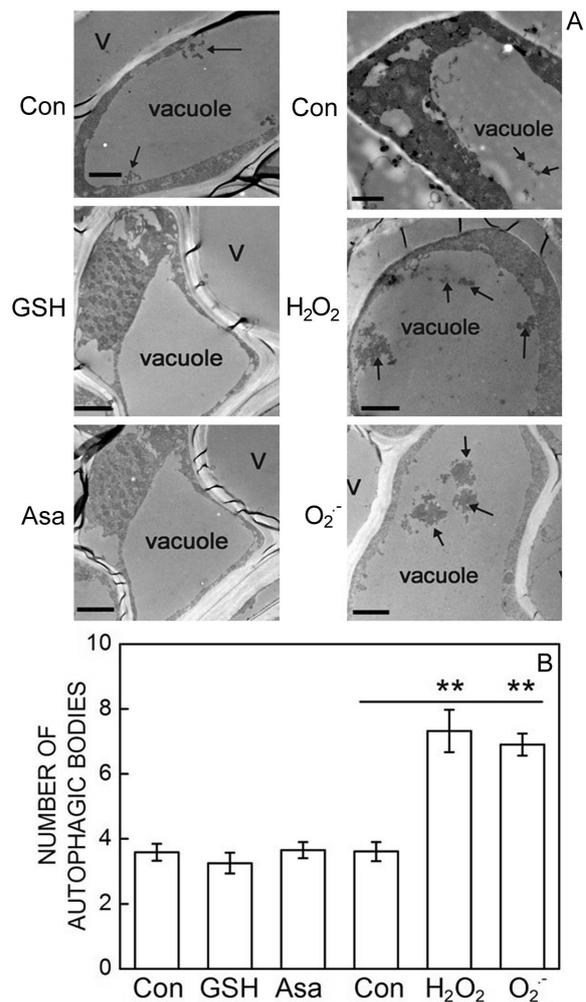


Fig. 6. Ascorbic acid (ASA) and glutathione (GSH) repress autophagy in the root tips of wheat seedlings. For control conditions (Con), 3-d-old seedlings were transferred to a liquid Murashige-Skoog medium with 100  $\mu$ M aloxistatin (E-64d) for 6 h. For the antioxidant treatment, 3-d-old seedlings were transferred to a liquid MS medium without N plus 0.1 mM ASA or GSH, 1 mM  $H_2O_2$  or 0.01 mM  $O_2^-$ , and 100  $\mu$ M E-64d for 6 h. *A* - Autophagic bodies (in vacuoles) were observed by transmission electron microscopy. The scale bar: 1  $\mu$ m. The number of autophagic bodies in each cell was summarized in *B*. Means  $\pm$  SEs,  $n = 6$ , \*\* indicates significant differences at  $P \leq 0.01$ .

response to exogenous  $H_2O_2$  also induced autophagy (Chen *et al.* 2009, Poillet-Perez *et al.* 2015).

In order to further explore whether  $H_2O_2$  and  $O_2^-$  induced autophagy in root tip cells, exogenous ROS, ASA, and GSH were applied to wheat seedlings under N deficiency in the study. Both ASA and GSH lowered the content of  $H_2O_2$  and  $O_2^-$  and decreased the autophagy under N deficiency in common wheat root tip cells (Fig. 6). Next, exogenous  $H_2O_2$  and  $O_2^-$  both induced autophagy in wheat root cells (Fig. 6).

In short, autophagy in *T. aestivum* root tips under N or C deficiency was regulated by  $H_2O_2$  and  $O_2^-$  produced by NOX.

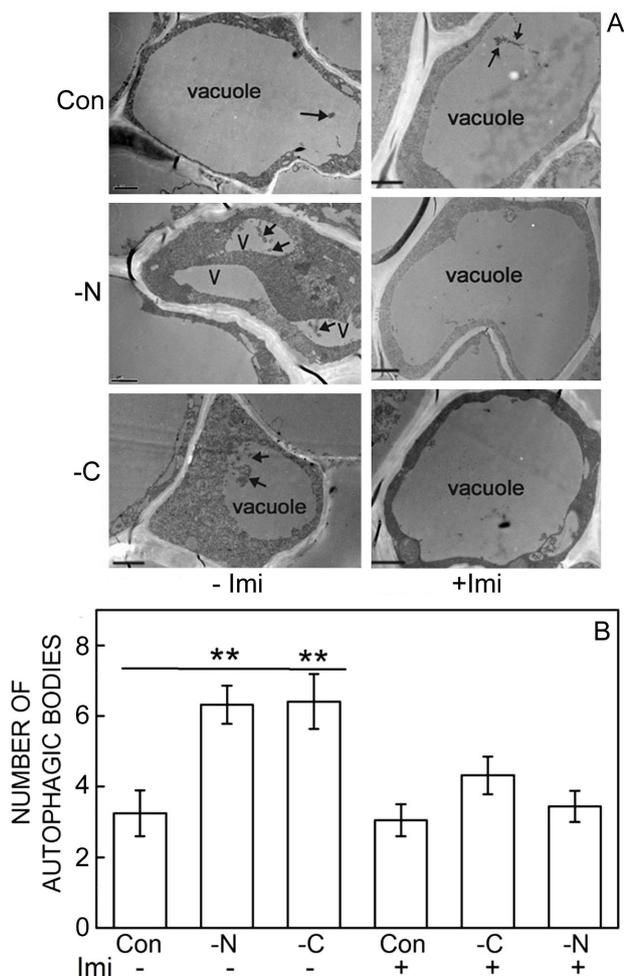


Fig. 7. Nicotinamide adenine dinucleotide phosphate oxidase regulated autophagy induced by nitrogen and carbon deficiencies. For control conditions (Con), 3-d-old wheat seedlings were transferred to a liquid Murashige-Skoog (MS) medium with 100  $\mu$ M aloxistatin for 6 h. To induce the deficiency stress, 3-d-old seedlings were transferred to a liquid MS medium minus N or sucrose with or without 0.2 mM imidazole (Imi) and with 100  $\mu$ M E-64d for 6 h. A - Autophagic bodies (in vacuoles) were observed by transmission electron microscopy. The bars: 1 $\mu$ m. The number of autophagic bodies in each cell was summarized in B. Means  $\pm$  SEs,  $n = 6$ , \*\* indicates significant differences at  $P \leq 0.01$ .

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