

HYPOXIA-INDUCED PROGRAMMED CELL DEATH IN ROOT-TIP MERISTEMATIC CELLS OF *TRITICUM AESTIVUM* L.

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In this study, wheat (*Triticum aestivum* L.) roots were treated with hypoxic water. The staining of cell preparations with DAPI revealed morphological changes of the cells such as nuclear condensation, deformation and fragmentation. Under TEM, cellular membrane shrinkage and breakage, chromatin condensation and apoptotic-like bodies were displayed. The number of mitochondria increased dramatically; their cristae were damaged; the interior became a cavitation and only some flocculent materials were distributed. Indirect immunofluorescence staining indicated that cytochrome C diffused from mitochondria to nucleoplasm and cytoplasm. TUNEL positive nuclei indicated double strand breaks of DNA. DAB staining was used for the identification of hydrogen peroxide and examination showed that the longer the treating time, the darker the staining of the meristematic zones of the roots which suggested the increased accumulation of these Reactive Oxygen Species (ROS). The elevation of hydrogen peroxide production was paralleled with the increase of SOD and POD activities. A negative correlation between the exposure time under hypoxia and the contents of soluble proteins was found. No obvious effect of hypoxia on MDA was established. The obtained results demonstrate that hypoxia causes programmed cell death in the root-tip meristematic cells of *Triticum aestivum* L. which is most probably attributed to the accumulation of large amounts of ROS.

Key words: Hypoxic water, reactive of oxygen species, programmed cell death, root-tip meristematic cell, *Triticum aestivum* L.

INTRODUCTION

Oxygen plays a vital role in the growth, development and survival of all living organisms. Nevertheless, hypoxia can be formed by lots of internal or external factors (Rabalais et al., 2010). Plants respond and adapt to stress conditions at the molecular and cellular levels as well as at the physiological and biochemical levels (Sanghera et al., 2011). One of the most significant morphological changes is the formation of aerenchyma in plant roots grown under flood or hypoxic conditions in order to provide an internal aeration system for the transfer of oxygen between roots and the aerial environment (Armstrong, 1979; Drew et al., 2000). Aerenchyma formation requires the death of the cells in the root cortex (Drew et al., 2000; Gunawardena et al., 2001) and other mechanisms, such as the increase of alcohol dehydrogenase (ADH) (Silva-Cardenas et al., 2003), non-symbiotic haemoglobin and nitric oxide gene expression linked to alternative type of respiration besides mitochondrial electron transport

(Sairamer et al., 2009), changes in cytosolic Ca^{2+} concentration, phosphoinositides (Drew, 1997; Drew et al., 2000) and ethylene (Colmer, 2003; Evans, 2003), which are also involved in the process.

Programmed cell death (PCD), or apoptosis, is an active, inherently controlled and intrinsic process (Kerr et al., 1972). There are several morphological and biochemical hallmarks of PCD both in plants and animals including cytoplasmic shrinkage, chromatin condensation, the formation of apoptotic bodies, cytochrome C leakage out of mitochondria, the fragmentation of DNA, altered nuclear morphology and activated proteases, etc. (Danon, 2000; Krishnamurthy et al., 2000). PCD also plays an important role during cell death in response to various abiotic stresses.

Aerenchyma formation has long been identified as a process of PCD in the root cortex (Brailsford et al., 1993; Drew, 1997). It is gradually clear that signaling events, such as phosphoinositides, Ca^{2+} , ethylene (Drew et al., 2000) and MAP kinases (Joshi

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and Kumar, 2012), etc., can regulate PCD during the development of aerenchyma.

Reactive oxygen species (ROS) are produced as byproducts during the mitochondrial electron transport of aerobic respiration or by oxidoreductase enzymes and metal catalyzed oxidation (Dinakar et al., 2010). It contains singlet oxygen (1O_2), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\cdot) (Dinakar et al., 2010). Since ROS can damage biological molecules including DNA, RNA, protein and lipid by inducing peroxidation (Shah et al., 2001), they are important for the induction of apoptosis or PCD (Simon et al., 2000).

There have been some reports involving PCD induced by hypoxia in plants in the past few decades. In the mid-1990s, Drew and his colleagues demonstrated that the transduction of an ethylene signal leading to an increase in intracellular Ca^{2+} is necessary for cell death and the resulting aerenchyma development in roots of maize subjected to hypoxia (He et al., 1996; Drew et al., 2000). When maize roots were grown hydroponically in 3% oxygen, $1 \mu l l^{-1}$ ethylene, Gunawardena et al. (2001) identified that most of the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) positive nuclei were distributed throughout the cortex as well as a few in the epidermis and stele. Since other typical phenomenon such as plasma membrane invagination, the formation of vesicles, cellular condensation, condensation of chromatin, the presence of intact organelles surrounded by membrane resembling apoptotic bodies and DNA ladder could also be observed, they concluded that the formation of aerenchyma initiated by hypoxia or ethylene appears to be a form of PCD that shows characteristics in part resembling both apoptosis and cytoplasmic cell death in animal cells. Later on, Virolainen et al. (2002) indicated that the treatment of isolated wheat root mitochondria with high concentrations of Ca^{2+} and anoxia condition could cause changes in the mitochondrial structure. Electrophysiological assay and western blot analysis demonstrated that oxygen deprivation and Ca^{2+} ions together could cause cytochrome C release from mitochondria, suggesting that PCD was initiated. However, their results found that anoxia alone failed to induce the release of cytochrome C.

It is obvious that all of the reports only indicated the formation of PCD and some signaling events involved in it under the hypoxia/anoxia environment. Their results are controversial and lack convincing evidence to evaluate the physiological mechanisms of why hypoxia/anoxia can induce PCD in plants.

In this paper, wheat roots were treated with self-made hypoxic water. It was demonstrated that hypoxia could induce PCD in root-tip meristematic cells of wheat after analysis by a combination of light, electron microscopy, indirect immunofluores-

cence staining and TUNEL assay. DAB (3,3'-diamino-benzidine) staining and examination of several biochemical indexes of the physiological state of the cells (malondialdehyde – MDA, superoxide dismutase – SOD and peroxidase dismutase – POD) showed that ROS were accumulated dramatically. So this lead us to make the conclusion that the reason why hypoxia can induce PCD in plants is because of the sharp increase of ROS during hypoxic treatment.

MATERIALS AND METHODS

SEED GERMINATION AND THE TREATMENT OF ROOTS WITH HYPOXIC WATER

Wheat seeds (*Triticum aestivum* L., CB037-A) were pre-imbibed for 30 min and then placed in 10 cm Petri dishes on moistened filter papers for germination at 25°C in darkness. When the length of the roots was approximately 0.5~1.0 cm, the whole germinated seeds were totally immersed in self-made hypoxic water in darkness at 25°C for 2, 4 and 6 h. The control specimens were placed in distilled water with continuous aeration for the same times (Sato and Yamada, 1996; Pang and Zhang, 2013).

Hypoxic water was made by boiling distilled water for 5 min, then cooling down immediately to 25°C with cold tap water. The concentrations of the oxygen (O_2) in the water were measured with an 810A dissolved oxygen meter (Thermo Orion, USA).

DAPI STAINING

The root-tips of wheat were excised and fixed in freshly prepared 4% paraformaldehyde (Solarbio, Beijing, China) in PBS (phosphate buffer solution, pH 7.4) for 4 h. After being washed thoroughly with distilled water, they were hydrolyzed with 95% ethanol/concentrated hydrochloric acid (1:1v/v) at room temperature for 6 min. After a thorough wash with distilled water, the tissue was macerated in a drop of methanol/glacial acetic acid (1:1) and squashed. Air-dried specimens were stained with $1 \mu g/ml$ of DAPI (4',6'-diamidino-2-phenylindole) (Roche, Hong Kong, China) for 5 min then examined under a Leica DMRE fluorescence microscope (Leica Microsystems Ltd., Beijing, China).

OBSERVATION OF CONVENTIONAL ULTRATHIN SECTIONS

The wheat root-tips were carefully excised and fixed immediately in 3% glutaraldehyde (Sigma, Shanghai, China) in 0.2 M PBS (pH 7.4) for 12 h at room temperature. After rinsing with the same buffer, the specimens were postfixed in 2% osmium

tetroxide (Sigma, Shanghai, China) in distilled water for 2 h. After a thorough wash with distilled water, the specimens were dehydrated in an ethanol-acetone series and embedded in Epon 812 epoxy resin (Polysciences, Inc., PA, USA). Sections were cut on a Leica UC6 ultratome (Leica Microsystems Ltd., Beijing, China) at a thickness of 60–70 nm. After being stained with uranyl acetate and lead citrate, the sections were observed under an H-7500 transmission electron microscope (TEM) (Hitachi Instruments (Shanghai) Co., Ltd., Shanghai, China).

INDIRECT IMMUNOFLUORESCENCE STAINING

The wheat root-tips were fixed for 15 h with freshly prepared 4% paraformaldehyde in PBS, washed in PBS and digested with 2% cellulose R-10/2% pectolase Y-23 (1:1v/v) (Solarbio, Beijing, China) at 37°C for 2 h. The root tip meristems were spread onto the slides.

Samples were blocked with 10% goat serum (Solarbio, Beijing, China) and 1% BSA (Solarbio, Beijing, China) in PBS for 1 hour, incubated with an antibody for Cytochrome C (Cell Signaling, Shanghai, China), followed by an anti-rabbit secondary antibody conjugated to Alexa fluor 555 (Invitrogen, Beijing, China). Nuclei were counterstained with 1 µg/ml of DAPI for 5 min. Samples were examined under a Leica DMRE fluorescence microscope.

TUNEL ASSAY

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions. In brief, segments of wheat roots were fixed in FAA (Formalin-acetic acid-alcohol), dehydrated through a graded ethanol series, infused in a graded xylene series and then embedded in paraffin wax. Longitudinal root sections with the thickness of 10 µm were deparaffinized, rehydrated and stained with One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology, Nantong, China) to visualize 3'OH-labelled ends in the nuclei. Samples were examined under a Leica DMRE fluorescence microscope.

DAB STAINING

The cellular localization of H₂O₂ production was visualized according to Thordal-Christensen (1997)'s procedure. Wheat roots were stained with 0.1% DAB (3,3'-diamino-benzidine, pH 6.5) (Solarbio, Beijing, China) for 3h at 37°C in darkness. The stained samples were kept in 70% ethanol at 4°C prior to imaging. The images were taken with a Nikon SMZ1500 stereo-microscope.

EXAMINATION OF MDA CONTENTS

Malondialdehyde (MDA) content was examined as described previously (Heath and Packer, 1968). Briefly, 0.1 g of fresh roots were homogenized in 10 ml of 10% TCA (Trichloroacetic acid). After centrifuging at 4000 rpm for 10 min, 2 ml of the supernatant were mixed with 2 ml of 0.6% TBA (Thiobarbituric acid). The mixtures were heated in boiled water for 15 min and quickly cooled down on ice. After centrifuging at 4000 rpm for 10 min, the absorbance of the supernatant was recorded at 532 nm, 600 nm and 450 nm. The malondialdehyde (MDA) content was expressed as in µmol g⁻¹ of fresh weight.

EXAMINATION OF SOD, POD AND SOLUBLE PROTEIN CONTENTS

The activities of SOD, POD and soluble protein content in roots treated with hypoxic water were performed with a superoxide dismutase detection kit, a peroxidase dismutase detection kit and a Coomassie brilliant blue detection kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), respectively, according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Data is presented as mean ± standard error of the mean (SEM). Between-group comparisons were analyzed using a Student's T-test or ANOVA and Tukey's multiple comparisons test and significance was assigned at p<0.05. Analyses were performed using SPSS software. Images were appropriately processed by Photoshop CS5 and Illustrator CS software.

RESULTS

NUCLEAR MORPHOLOGICAL CHANGES OF STRUCTURES

Hypoxic water was effectively prepared according to Sato and Yamada (1996). The O₂ concentration in the distilled water was 4.9033 mg/l. It was decreased to 0.6933 mg/l after boiling for 5 min then cooling immediately and sealed with Parafilm. Those standing for 1, 2, 4 and 6 h were 1.030, 1.167, 1.367 and 1.481 mg l⁻¹, respectively.

Cell specimens prepared from hypoxic water treated wheat roots were stained with DAPI and it was shown under a light microscope that the nucleus in control was round with an integrate structure and the nucleolus (Nu) was clearly visible (Fig. 1a). The nucleus in 2 h treatment became irregular, protruded (Fig. 1b, triangle arrows) and depressed (Fig. 1b, short arrows). Some non-nucleolar hollows

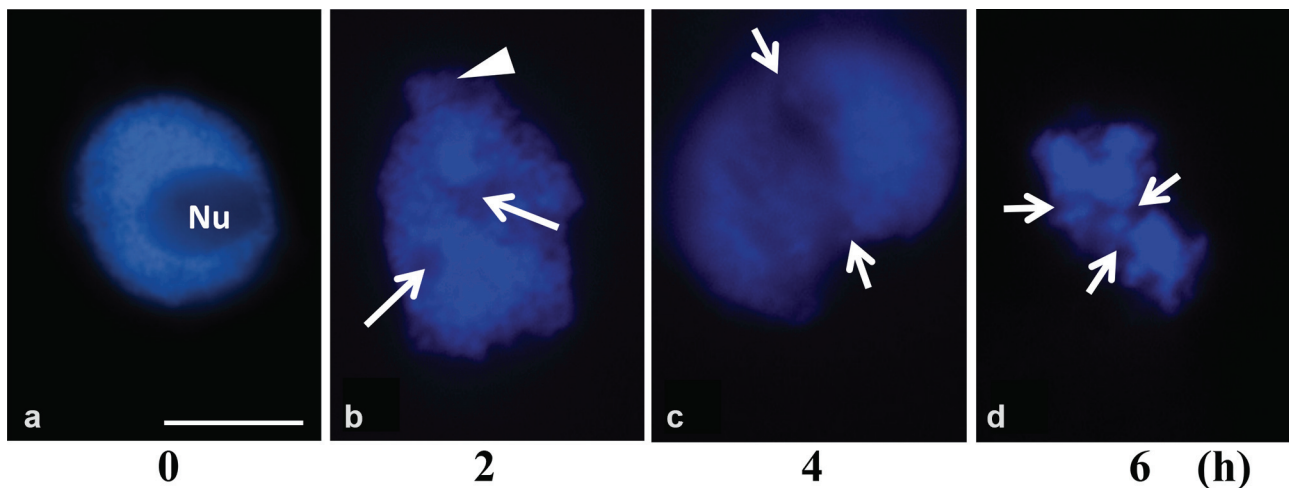


Fig. 1. The morphological and structural changes of the nuclei under a light microscope in root-tip meristematic cells of wheat treated with hypoxic water for 0, 2, 4 and 6 h. **(a)** The nucleus in the control, showing a round shape and a typical nucleolus, **(b)** Two h treated nucleus with irregular shapes. The triangle arrows, short arrows and long arrows indicate the protruded, depressed regions and non-nucleolar hollows, respectively, **(c)** Four h treated, swollen nucleus. The short arrows and long arrows indicate the stretched region and a large hollow between the two parts, respectively, **(d)** Six h treated nucleus which is split into several parts. The short arrows indicate the apoptotic-like bodies. Nu: nucleolus. Bar = 10 μ m.

appeared in the middle (Fig. 1b, long arrows). A 4 h treated nucleus swelled and stretched into two parts (Fig. 1c, short arrows) displaying a large hollow (Fig. 1c, long arrows) between the two parts. A 6 h treated nucleus was split into several parts forming apoptotic-like bodies (Fig. 1d, short arrows).

Under the TEM, the nucleus in control was elliptical with a smooth and integrated double-layered membrane (Fig. 2a, black arrows). The nucleolus (Nu) was large and its fine structures, including fibrillar centers (FC), dense fibrillar components (DFC) and granular components (GC) could easily be distinguished. The chromatin (CC) was distributed over the entire nucleus as condensed status (Fig. 2a). The nuclear structure in the treated samples changed dramatically. The nucleolus was small and localized at the edge of the nucleus. Its fine structures disappeared. The chromatin was highly condensed and concentrated into three parts (Fig. 2b, long black arrows). There were many depressed regions on the entire membrane (Fig. 2b, white arrows) so the whole nucleus was divided into three regions with condensed chromatin and one region with few chromatin. The middle of the nucleus also contained scattered, decondensed chromatin (Fig. 2b). What's more, the nucleolus vanished, the invaginations on the membrane were deep and at the end the whole nucleus was divided into four blocks (Fig. 2c, long arrows) with some membrane fragments around the edges (Fig. 2c, white arrows). The chromatin in the blocks was highly twisted together. These structures are similar to the apoptotic bodies reported in animals.

ULTRASTRUCTURAL CHANGES IN MITOCHONDRIA

Another striking phenomena was also observed under the TEM in the treated cells, that was, the mitochondria swelled, their outer membranes were not smooth and most of them were damaged (Fig. 3b, white arrows); their inner membranes were also severely damaged and the inner structures had vanished, only some flocculent materials were randomly distributed there (Fig. 3b, black arrows). In contrast, the mitochondria in the control cells had regular shapes. Their double membranes were clear with smooth outer membranes and cristae folded from the inner membrane (Fig. 3a, short arrows).

Our results also indicated that the number of mitochondria in treated cells were much higher than those in the control. This special phenomenon has not been reported before. It might be because cells have to proliferate rapidly in order to get more energy when they are under stress during hypoxic treatment.

SUBCELLULAR DISTRIBUTION OF CYTOCHROME C

To understand the subcellular distribution of cytochrome C in the cells treated with hypoxic water, the indirect immunofluorescence staining technique was performed and it indicated that the cytochrome C, originally localized in the mitochondria around the nucleus (Fig. 4, 1a and c, red signals), was released gradually to nucleoplasm and cytoplasm (Fig. 4, 2a and c, red signals) and finally, most of them were diffused but only a few signals could be detected around the nucleus (Fig. 4, 3a and c, red

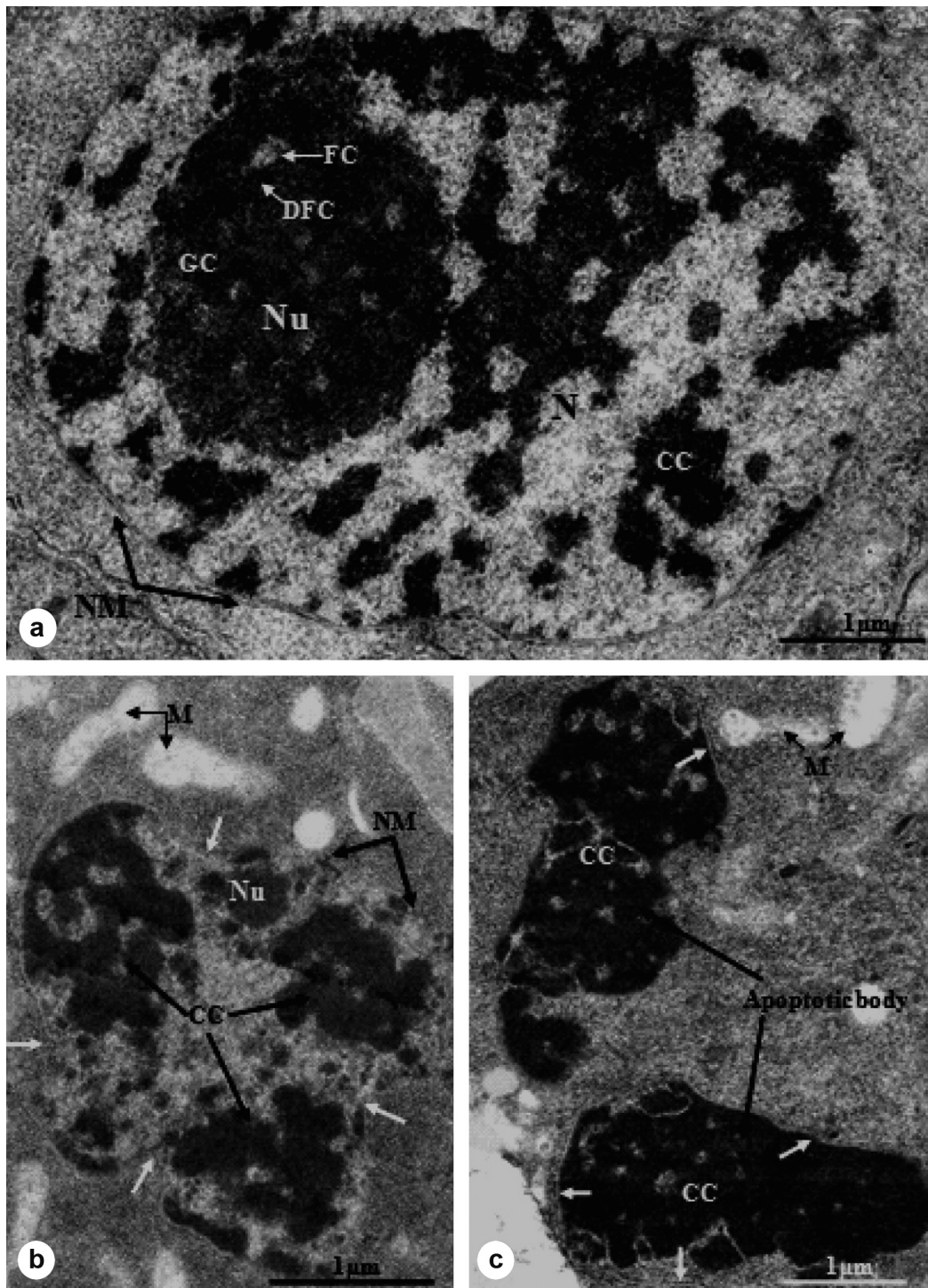


Fig. 2. The morphological and structural changes of the nuclei under a TEM in root-tip meristematic cells of wheat treated with hypoxic water for 6 h. (a) The control cell showing the nucleus is elliptical with normal cellular membrane, nucleolus and condensed chromatin. The black arrows indicate the double-layered membrane. (b) The treated cell showing that the nucleus has dramatically changed and is divided into four parts. The fine short arrows, short black arrows, long black arrows and white arrows indicate mitochondria, cellular membrane, condensed chromatin parts and depressed regions on the membrane, respectively. (c) The treated cell showing the whole nucleus is divided into four fragments. The fine short arrows, long arrows, and white arrows indicate mitochondria, apoptotic bodies and membrane fragments, respectively. CC – condensed chromatin; DFC – dense fibrillar component; FC – fibrillar center; GC – granular component; M – mitochondria; N – nucleus; NM – nuclear membrane; Nu – nucleolus. Bar = 1 μ m.

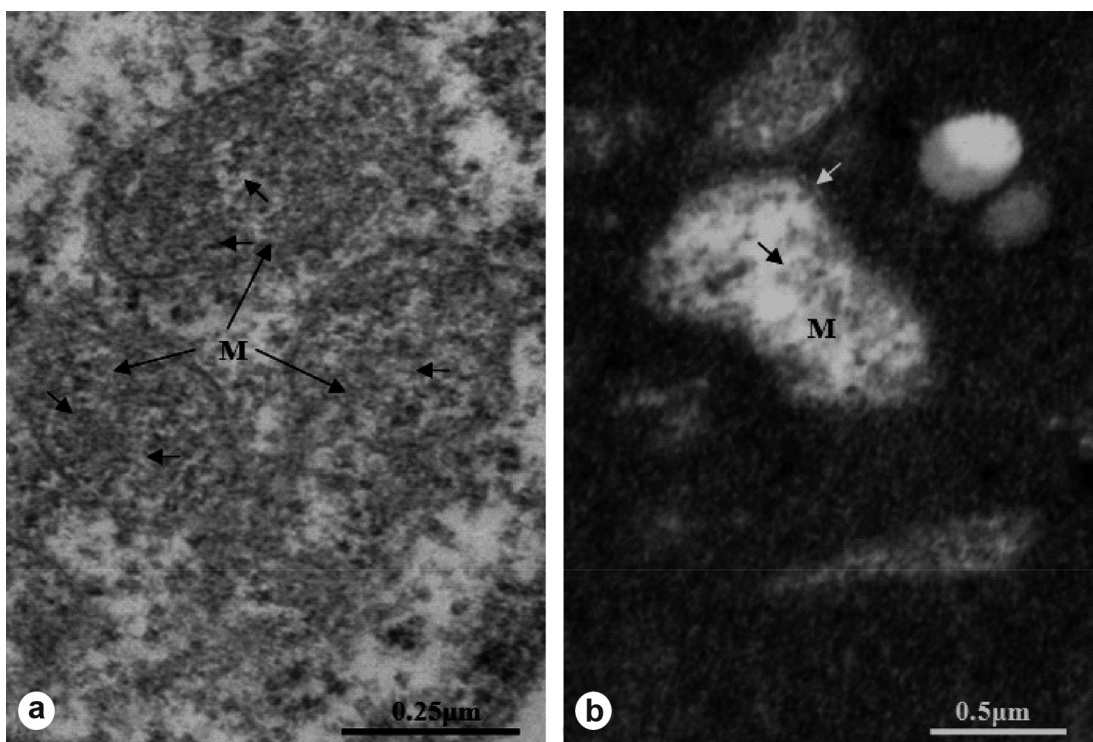


Fig. 3. The morphological and structural changes of the mitochondria under a TEM in root-tip meristematic cells of wheat treated with hypoxic water for 6 h. **(a)** The control cell showing three mitochondria. The long and short arrows indicate mitochondria and cristae, respectively. **(b)** The treated cell showing the mitochondria is damaged. The white and black arrows indicate the membrane fragments and flocculent materials, respectively. M – mitochondria. Bars in a and b represent 0.25 μm and 0.5 μm , respectively.

signals). To the best of our knowledge, it is the first time that this kind of phenomenon has been described by the indirect immunofluorescence staining technique. Nuclear condensation (Fig. 4, 2b), irregular membrane and nucleoli (Fig. 4, 2b and 3b) was clearly visible in the treated cells.

EXAMINATION OF TUNEL-POSITIVE NUCLEI

Since the TUNEL assay of DNA fragments is routinely used for the detection of apoptotic cells in various tissues, the One Step TUNEL Apoptosis Assay Kit was performed in our study. As shown in Fig. 5, there were no TUNEL-positive nuclei in the control cells (Fig. 5, 1b and c). In contrast, there were about 30% TUNEL-positive nuclei in the treated cells (Fig. 5, 2b and c, red signals). It is clear that PCD happened after the hypoxic treatment in root-tip meristematic cells of *T. aestivum* L.

DETERMINATION OF ENDOGENOUS H_2O_2

To further explore the reasons involved in PCD induced by hypoxic treatment, endogenous H_2O_2 in treated roots was detected by DAB staining. As shown in Figure 6, when the roots were treated for 2 h, the

meristematic zones were stained brown. The colours became deeper along with the treating time (comparing 2 with 4 and 6 h). However, no staining degree change was observed for the root caps and the elongation zones. It means that the endogenous H_2O_2 contents in the meristematic zones increase sharply after being stressed by hypoxia.

EFFECTS OF HYPOXIC TREATMENT ON ACTIVITIES OF ANTIOXIDANT ENZYMES

When compared to the control (17.702), the activities of SOD in roots treated with hypoxic water for 2, 4 and 6 h were 24.938, 35.492 and 45.896, being increased 40.88%, 100.50% and 159.27%, respectively (Fig. 7a). Similar results were obtained for the activities of POD which were increased 17.53%, 40.80% and 70.46% when treated for 2, 4 and 6 h, respectively (Fig. 7b). All of the treatments for both of the enzymes were significantly different ($P < 0.05$).

EFFECTS OF HYPOXIC TREATMENT ON MDA AND SOLUBLE PROTEIN CONTENTS

The MDA contents had no obvious difference when compared with the control though there was a slight increase in 2 and 4 h treatments (Fig. 7c).

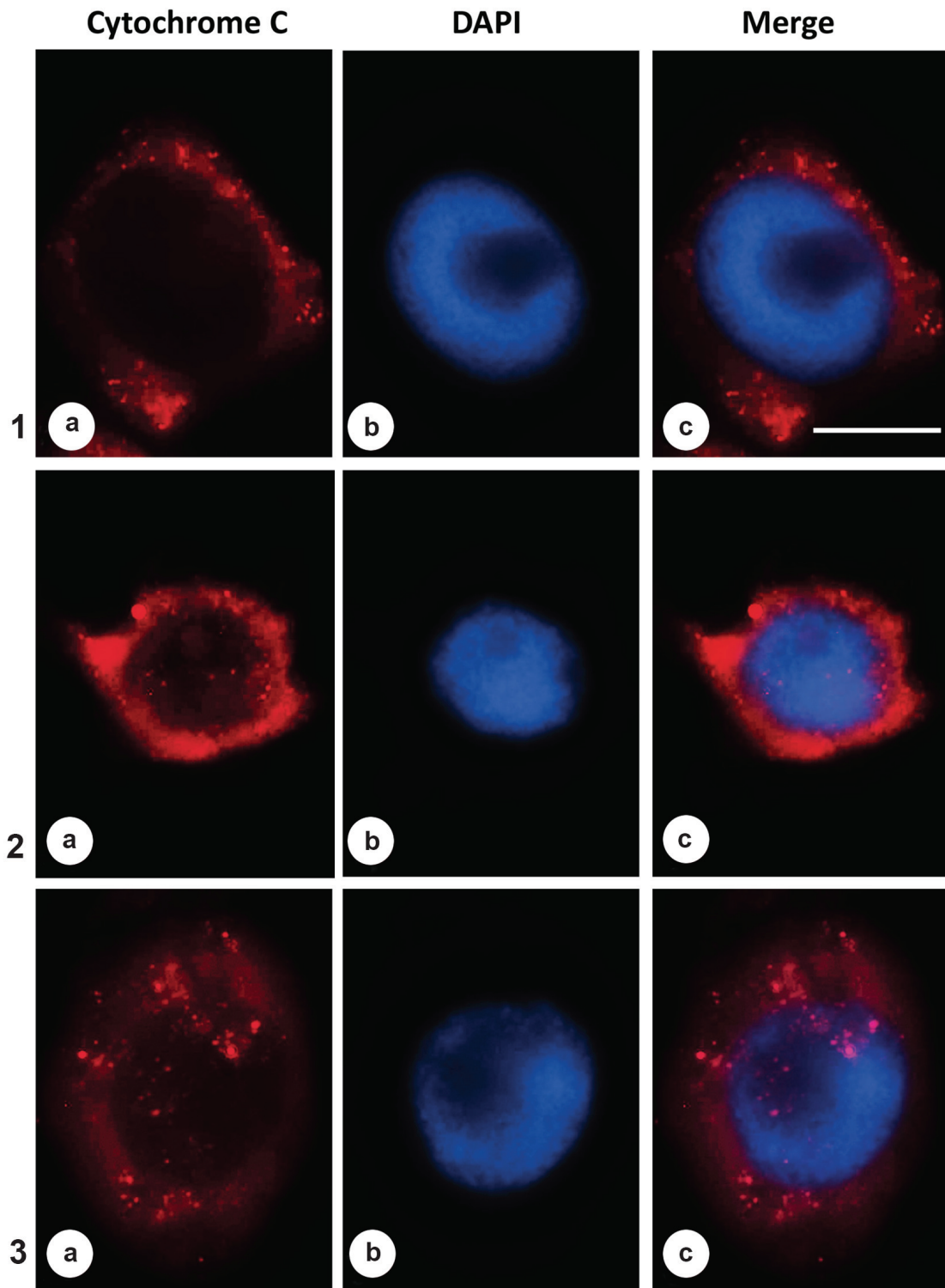


Fig. 4. Subcellular distribution of cytochrome C revealed by indirect immunofluorescence staining in root-tip meristematic cells of wheat treated with hypoxic water for 6 h. The red and blue signals indicate the distribution of cytochrome C and nuclei, respectively. In control cell (**1 - a and c**), the signals of cytochrome C are all located around the nucleus. In the treated cells, the signals of cytochrome C begin to release (**2 - a and c**) and at the end, most of them diffused out but only a few signals could be detected around the nucleus (**3 - a and c**). Bar = 10 μ m.

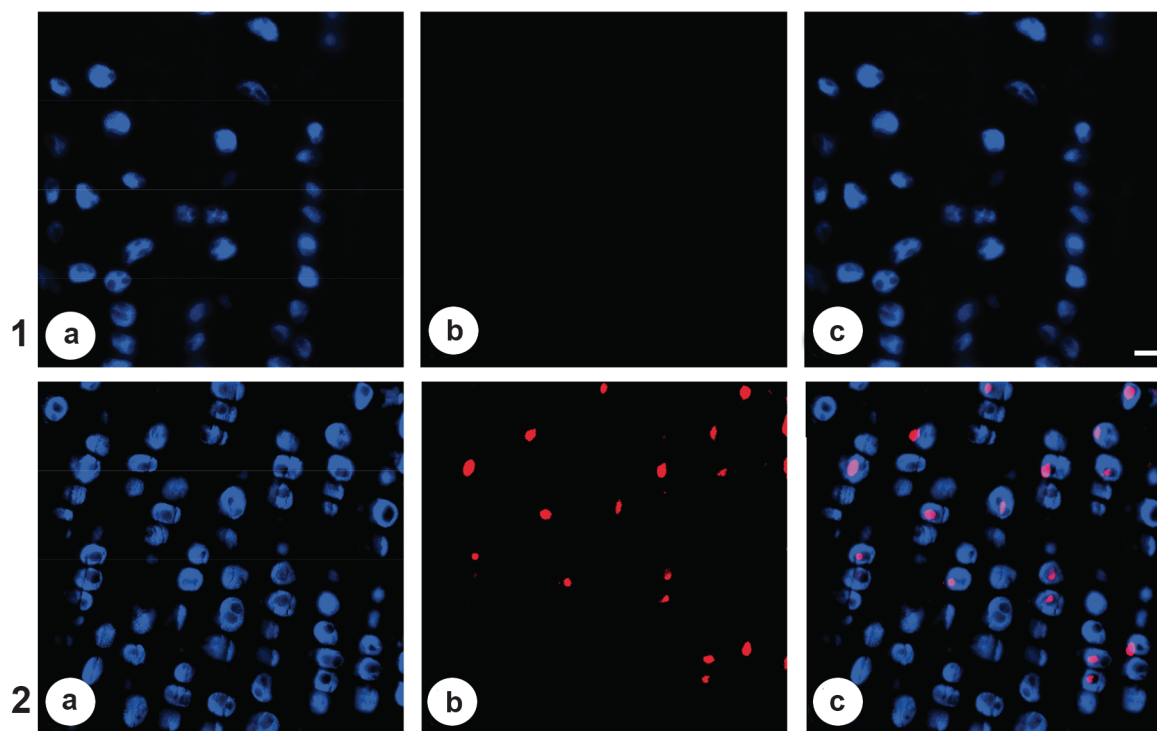


Fig. 5. TUNEL assay to root-tip meristematic cells of wheat treated with hypoxic water for 6 h. (1 – a to c) Control samples, no TUNEL-positive nuclei can be detected, (2 – a to c) Treated samples, about 30% TUNEL-positive nuclei can be seen. Red signals in 2-b indicate TUNEL-positive nuclei. Bar = 10 μ m.

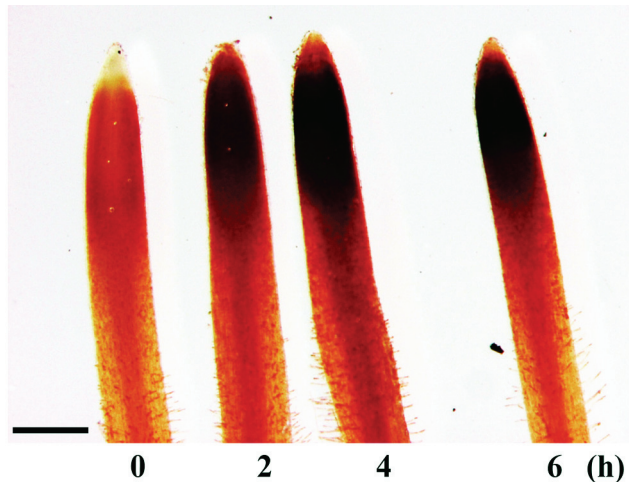


Fig. 6. DAB staining to detect the endogenous H_2O_2 in root-tip meristematic cells of wheat treated with hypoxic water for 0, 2, 4 and 6 h. No DAB-positive signals can be detected in the control roots (0). The staining degrees of roots become deeper along with the treating time (2, 4 and 6). The root caps and the elongation zones cannot be stained. Bar = 0.05 mm.

As could be seen in Figure 7d, the mean of the soluble protein contents in roots decreased along with the time of hypoxic treatment. The amount of 2,

4 and 6 h treatment decreased 0.77-, 0.74- and 0.51-fold, respectively. Statistical analysis indicated that the content in the 6 h treatment was significantly low ($P < 0.05$).

DISCUSSION

Programmed cell death (PCD) in plants is an active process leading to the selective elimination of unneeded or damaged cells during many developmental processes such as embryogenesis, tapetum degeneration, pollen selection due to self-incompatibility, organ senescence, and tracheary element differentiation, etc. (Serrano et al., 2012). So, it is essential for plant development, survival and maintaining tissue and organ homeostasis (Greenberg, 1996; Van Breusegem and Dat, 2006). In addition, many stressful environments such as drought, salt, heat shock, toxic chemicals and pathogens can also induce cell death in plants (Katsuhara and Kawasaki, 1996; Dannon and Gallois, 1998; Munné-Bosch and Alegre, 2004; Overmyer et al., 2005).

Hypoxia is also a common abiotic stress factor. It can affect the growth, development, metabolic regulation to multicellular organisms (Broker et al., 2005). Severe hypoxia can cause point mutations

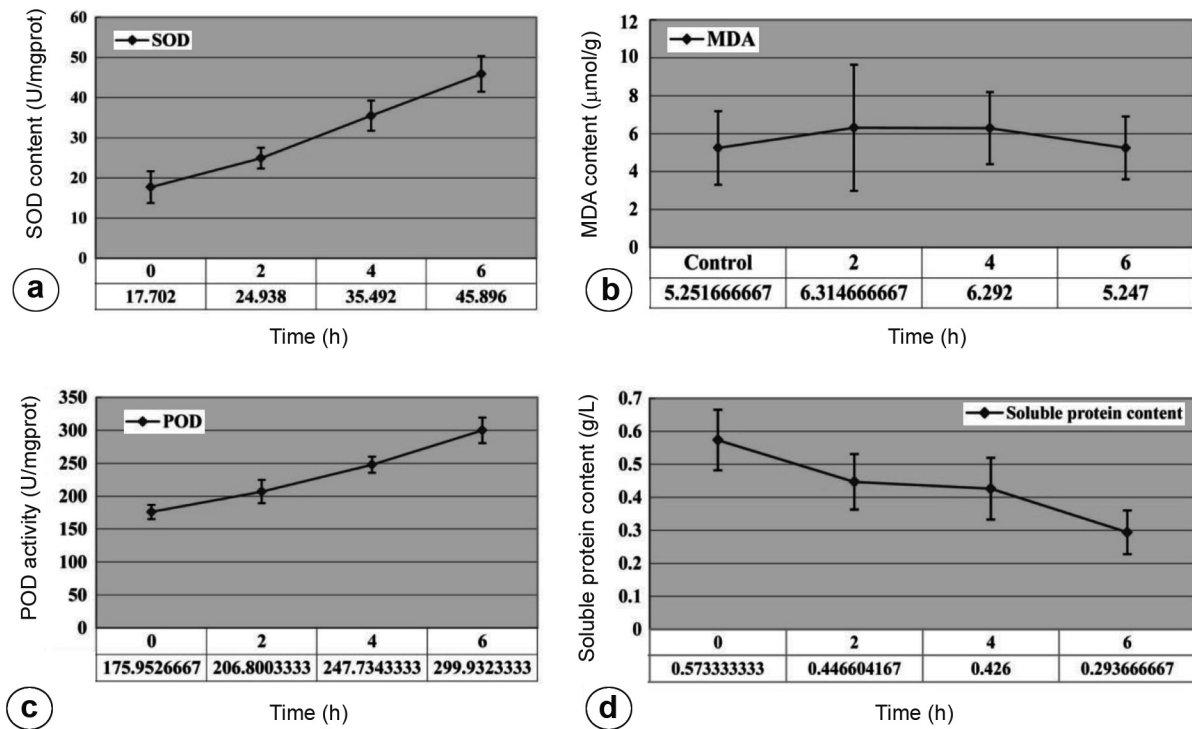


Fig. 7. The effects of hypoxia treatment on activities of antioxidant enzymes, MDA and soluble protein contents in root-tip meristematic cells of wheat treated with hypoxic water for 0, 2, 4 and 6 h. The activities of SOD (a) and POD (b) increase significantly ($P < 0.05$) when treated with hypoxic water for 2, 4 and 6 h. The MDA contents have no obvious differences when compared with the control (c). The soluble protein contents decrease when treated with hypoxic water for 2, 4 and those in 6 h treatment are significantly lower ($P < 0.05$) (d). Vertical bars are means \pm SD of three replicates.

(Perou et al., 2000) or genetic instability (Greiger and van der Wall, 2004).

So far, there have been multiple reports demonstrating that hypoxia can induce apoptosis or PCD in animals and the mechanisms are also deeply elucidated (Greiger and van der Wall, 2004). However, few such research studies have been carried out on plants. Though Drew et al. (2000), Gunawardena et al. (2001) and Virolainen et al. (2002) indicated that PCDs appeared in hypoxia-treated maize roots and isolated wheat mitochondria cultured in high concentrations of Ca^{2+} and anoxia, they made the conclusions mainly depending on the morphological and biochemical features and did not provide convincing evidence to evaluate the mechanisms.

In this study, when the wheat roots were treated with hypoxic water, typical morphological changes of PCD, such as cell shrinkage, membrane breakage, chromatin condensation, nuclear fragmentation and formation of apoptotic-like bodies, could be easily identified by a combination of light and electron microscope. Again, the release of cytochrome C from the mitochondria and TUNEL-positive nuclei were revealed by indirect immunofluorescence staining and TUNEL assay, respectively.

Our results could not identify the morphological features of autophagic-like PCD induced by hypoxia, such as the accumulation of autophagosome and small lytic vacuoles, the formation of actin cables, nuclear envelope disassembly, empty-walled cell corpse, etc. (van Doorn and Woltering, 2005; van Doorn et al., 2011). Hence, the evidence is convincing that hypoxia can really induce PCD in root-tip meristematic cells of *T. aestivum* L.

In order to explore the mechanisms of why hypoxia can induce PCD in wheat, hypoxic water treated roots were stained with DAB, a specific assay to detect endogenous H_2O_2 . It was shown that the meristematic zones were stained brown and the colours became deeper along with the treating time, suggesting ROS was induced after the treatment.

SOD and POD are effective antioxidant enzymes in the cells and can eliminate ROS such as H_2O_2 and O_2^- etc. to protect cells against destructive influences (Zhao and Xu, 2001; Neill et al., 2001). In the present investigation, both of the enzymes were significantly increased after hypoxic water treatment. This is consistent with what we have observed in DAB staining.

From these results we infer that, since ROS can damage biological molecules including DNA, RNA,

protein and lipid by inducing peroxidation (Shah et al., 2001), the mechanism of why hypoxia causes PCD is because large amounts of ROS are produced under hypoxic treatment.

CONCLUSIONS

The reported results indicate that ROS are most probably among the main factors underlying the occurrence of PCD under applied hypoxic condition.

AUTHORS' CONTRIBUTIONS

NP performed all of the experiments and drew Figures; FXZ was responsible for experimental design, advising and writing the manuscript. All the authors read and approved the final manuscript. The authors declare that they have no conflicts of interest.

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