PI3K is involved in nucleolar structure and function on root-tip meristematic cells of *Triticum aestivum* L.

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**Abstract**

In this study, wheat (*Triticum aestivum* L.) seeds were used to detect the effect of wortmannin, a specific inhibitor of PI3K, on the nucleolar structure and function. When the germinated seeds were treated with wortmannin, it was shown that the root growth was suppressed and the mitotic index was decreased. The inhibition effects were positively correlated with the concentrations of the drug. The observations of light and transmission electron microscopy revealed that the nucleolar morphology became irregular and their fine structure disappeared. Some granules with a size range of 0.05–0.30 μm diffused from the nucleoli and gradually moved to the nucleoplasm between and around the chromatin. Indirect immunofluorescence staining indicated that B23 shuttled from the nucleoli to the nucleoplasm, or even, to the cytoplasm. RT-PCR technique demonstrated that the expression of C23 was severely down-regulated. Our results suggest, for the first time, that wortmannin treatment can not only damage nucleolar structure, but also inhibit its function, implying that PI3K is involved in nucleolar structure and function.

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**Introduction**

The nucleolus is the nuclear domain where ribosomal RNAs are synthesized, processed, and assembled with ribosomal proteins (Hernandez-Verdun et al., 2010). By transmission electron microscopy (TEM), the nucleoli mainly exhibit a tripartite organization with fibrillar centers (FC), dense fibrillar components (DFC) and granular components (GC) (Raska et al., 2006). Ribosomal DNA (rDNA) transcription occurs at the border of FC and DFC (Cmarko et al., 2008). In addition to DNA and RNA, the nucleoli also contain hundreds of proteins (Andersen et al., 2005).

Nucleolin (C23) and nucleophosmin (B23) are the two most abundant components of the nucleolus (Olson, 2011). Both of them are multifunctional proteins which playing important roles, not only in Pol I transcription and ribosome biogenesis, but also in many other biological processes such as cell cycle regulation and cell proliferation (Olson, 2011). Another phenomenon is that both of the proteins can shuttle between the nucleolus, nucleoplasm and cytoplasm during the cell cycle (Olson, 2011). This kind of movement is closely correlated with the cell and nucleolar activity as Yung et al. (1990) and Dundr et al. (1995) demonstrated, respectively, that when RNA synthesis was inhibited, the B23 would be relocated in the cells. Thus, the stability of nucleolar structure and function is very important in maintaining the normal functions of the cell.

PI3K is a crucial factor in intracellular signal transduction pathway. It can control the phosphorylation level of AKT kinase to regulate the expressions of a serials of factors downstream, such as BAD, FOXOs, p21, p27, GSK3, etc. and at last, directly or indirectly affect the cell growth, proliferation, survival, metabolism and autophagy (Vanhaesebroeck et al., 2012).

In plants, PI3K also has multiple functions including root hair growth, root nodule formation, pollen and vascular development (Xue et al., 2009). Bunney et al. (2000) reported that PI3K distributed within the plant nucleus and nucleolus, especially associated with active nuclear and nucleolar transcription sites. Unfortunately, there was no further information on the functions of PI3K in the nucleolus and what would happen to nucleolar structure and function if PI3K was blocked.

In this study, when the germinated seeds of wheat were treated with wortmannin, a specific inhibitor of PI3K, it was shown that the root growth was suppressed along with a significant decrease in the mitotic index. Cellular and molecular researches further verified that both nucleolar functions and structure were abnormal. Our results demonstrated that PI3K is involved in nucleolar structure and function of *Triticum aestivum* L.
Materials and methods

Plant material and reagents

Wheat (T. aestivum L., CB037-A) seeds were from Capital Normal University, China. Wortmannin was obtained from Selleckchem (Shanghai, China). Glutaraldehyde and osmium tetroxide were obtained from Sigma (Shanghai, China). Cellulase R-10, Pectolase Y-23, Triton X–100, Goat serum and BSA were from Solarbio (Beijing, China). B23 antibody (H-106) was from Santa Cruz (Shanghai, China). Alexa Fluor® 550 Goat Anti-Rabbit IgG (H+L) and Trizol were from Invitrogen (Beijing, China). DAPI was from Roche (Hong Kong, China). Antifade solution was from Applygen (Beijing, China). RevertAid First Strand cDNA Synthesis Kit was from Fermentas (Beijing, China).

Seed germination and treatment

Wheat seeds (T. 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. Different concentrations of wortmannin (0, 10, 20 and 30 μM) were used to treat the germinated seeds (the radicles pierced the seed coat for 1 mm, hereafter) in darkness at 25 °C for 36 h. Distilled water was used for the control experiment.

Statistics on the mitotic index

The root-tips of wheat were excised and fixed in methanol/glacial acetic acid (3:1 v/v) at 4 °C for 24 h. After washing thoroughly with distilled water, the root tips were hydrolyzed with 95% ethanol/concentrated hydrochloric acid (1:1 v/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 v/v), then cut up with a razor blade and squashed in a drop of modified carbol fuchsin. The mitotic index was calculated as the ratio between the number of cells in mitosis and the total 2000 cells.

Silver staining for observation by light microscopy

The preparations of chromosomes were made by squashing the root tips in 45% glacial acetic acid and then air-dried. Specimen slides were stained with 50% AgNO3/2% gelatin (in 1% formal acid) (2:1 v/v) at 65 °C for 5 min and observed under a Leica D 2500 microscope.

Observation of conventional ultrathin sections

The wheat root-tips were carefully excised and fixed immediately in 3% glutaraldehyde in 0.2 M phosphate buffer (PBS, pH 7.4) for 12 h at room temperature. After rinsing in the phosphate buffer (PBS), the specimens were postfixed in 2% osmium tetroxide in the same buffer 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. After heat polymerization sections were cut on a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) at a thickness of 60–70 nm. After staining with uranyl acetate and lead citrate, the sections were observed under a Hitachi H–7500 transmission electron microscope (TEM).

Observation of en bloc silver-stained ultrathin sections

The wheat root-tips were carefully excised and fixed immediately in 3% glutaraldehyde (0.2 mol/L PBS, pH 7.2) at 4 °C for 30 min, washed 3 times with the PBS, postfixed in methanol/glacial acetic acid (3:1 v/v) at 25 °C for 30 min and dehydrated in ethanol–water gradient. Samples were stained with a mixture of 50% AgNO3/2% gelatin (2:1 v/v) at 65 °C for 30 min, reduced in 5% Na2S2O3 for 5 min, rehydrated with ethanol–acetone gradient and embedded in Epon 812 epoxy resin. Sections (60–70 nm in thickness) were cut, observed and photographed as described above.

Indirect immunofluorescence staining

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

Samples were blocked with 10% goat serum and 1% BSA in PBS for 1 hour, incubated with antibody for B23, followed by an anti-rabbit secondary antibody conjugated to Alexa Fluor® 550. Nuclei were counterstained with 1 μg/ml of DAPI for 5 min. Samples were examined under a Leica DMRE fluorescence microscope.

RNA extraction and RT-PCR analysis

0.2 g of wheat root tips was ground into a fine powder in liquid nitrogen and transferred to the centrifuge tubes as soon as possible. Total RNA was extracted with Trizol reagent according to the manufacturer’s protocol and reversely transcribed using RevertAid First Strand cDNA Synthesis Kit. Samples were stored at −80 °C until use. PCR with primers specific for wheat C23 (nucleolin) (forward primer 5′-GACTGATCGCTGCTGAA, reverse primer 5′-CTTTCGACCAATCCA) as well as the Actin 2 gene product (forward primer 5′-GCTGATCTGCTGATGCTGAG, reverse primer 5′-CAATTGAGATGGCGTGAAGGGG) (Siva et al., 2008) was performed using one tenth of the RT reaction. C23 and Actin 2 were amplified for 28 and 38 cycles, respectively.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Between-group comparisons were analyzed using a Student’s T-test or ANOVA and Tukey multiple comparisons test and significance was assigned at p < 0.05. Analyses were performed using SPSS software (SPSS, IBM, Chicago, IL, USA). Images were appropriately processed by Photoshop CS5 (Adobe Systems, San Jose, CA, USA) and Image-Pro Plus 6.0 software (Bethesda, MD, USA).

Results

The effects of wortmannin on root growth and mitosis in root tip meristem of T. aestivum L.

To test the role of wortmannin on wheat root growth, different concentrations of wortmannin (0, 10, 20 and 30 μM) were used to treat the germinated seeds in darkness at 25 °C for 36 h. It was shown in Fig. 1A that the root growth was progressively inhibited with wortmannin concentrations. The mean root length treated with 10, 20 and 30 μM of wortmannin decreased to 71.66%, 51.09% and 24.74% compared with the control, respectively (Fig. 1B). The mitotic index also decreased from 7.9% (control) to 5.0%, 3.8% and 1.9% (10, 20 and 30 μM of wortmannin treatment), respectively (Fig. 1C). The data suggested that the inhibition of root tip growth resulted from the inhibition of cell division.

Both 20 and 30 μM of wortmannin treatment displayed significant inhibitory effects (Fig. 1B and C) after statistical analysis.
The effect of wortmannin on nucleolar structure

The nucleolus in a control cell was round and the silver-stained granules were distributed evenly (Fig. 2A). In cells treated with 10 μM or 20 μM of wortmannin (Fig. 2B and C), the nucleoli began to swell and their morphologies were irregular. The silver-stained granules were distributed unevenly in the nucleoli. Some deeply stained blocks around the inner edges of the nucleoli (Fig. 2B and C, white arrows) and some unstained hollows in the middle area of the nucleoli (Fig. 2B and C, black arrows) were depicted. The nucleolus of 30 μM wortmannin treatment was much larger. The deeply stained blocks were disappeared instead of thinner fibers distributed as fibrous network throughout the nucleolus (Fig. 2D, triangular arrows). The number of hollows increased in the whole nucleolus (Fig. 2D, black arrows). According to the results, the concentration of 20 μM of wortmannin was chosen in the following experiments.

Under the TEM, the nucleolus in untreated cell was elliptical (Fig. 3A) and its fine structures, fibrillar centers (FC), dense fibrillar components (DFC), granular components (GC) and nucleolar...
vacuole (NV) could be easily distinguished. In cells treated with 20 \( \mu \)M of wortmannin, the nucleoli became irregular and their fine structures were disappeared (Fig. 3B and C). Another striking phenomena could be seen, that was, nucleolar materials diffused from the nucleoli (Fig. 3B and C, triangular arrows).

Since the contrast between the chromatins and nucleoli in conventional stained ultrathin sections was not strong for them to be distinguished easily, an “en bloc” silver-staining technique used in TEM studies was performed here. In this case, the nucleoli were stained darkly but chromatin lightly (Fig. 3D–F). It could be seen in Fig. 3D that the nucleoli in control cell was elliptical with typical FC, DFC, GC and NV. Most of the FCs, DFCs were in the center surrounded by GCs (Fig. 3 D). The nucleoli in 20 \( \mu \)M of wortmannin treatment were irregular (Fig. 3E and F). One of them showed almost the same phenomenon as seen in Fig. 2C, i.e. it was swollen and protruded (Fig. 3E); most of the FCs and DFCs moved to the edge so the area was stained darkly; there were some unstained hollows that appeared in the middle area (Fig. 3E). The nucleolar materials with diameters 0.05–0.30 \( \mu \)m began to diffuse from the nucleoli (Fig. 3E, triangular arrows) and gradually moved to the nucleoplasm between or around chromatin (Fig. 3F, triangular arrows).

The dynamics of nucleolar protein B23 after wortmannin treatment

As B23 is one of the main components of the nucleolus and its shuttling can indirectly reflect the change of nucleolar function (Yung et al., 1990; Dundr et al., 1995), an indirect immunofluorescence staining technique to detect the dynamics of B23 was implicated after wortmannin treatment. It was shown that in the control cell, B23 signals were all located in the nucleolus (Fig. 4A3, white long arrows). In the treated cells, B23 signals were changed, few of them were still in the nucleoli (Fig. 4B3, white long arrows); some of them moved to the nucleoplasm (Fig. 4C3, triangular arrows) and most spread to the cytoplasm (Fig. 4B3 and C3, white short arrows).

The effect of wortmannin on C23 gene expression

Real time PCR was performed to evaluate the expression of C23, a nucleolus-specific gene, in control and wortmannin treated cells. It was obvious that the expression level of C23 in wortmannin treated
Discussion

There are several lines of evidence to prove that PI3K is involved in many biological processes in plants such as root hair growth, root nodule formation, pollen and vascular development (Xue et al., 2009). Recently, a study verified that PI3K plays a vital role in rice seed germination through regulating NADPH oxidase activity (Liu et al., 2012). The relationship between PI3K and plant nucleolus was first indicated by Bunney et al. (2000) who showed that the kinase was closely associated with active nucleolar transcription sites. Nevertheless, it is still unclear what would happen to nucleolar structure and function if PI3K was blocked.

In this study, when the germinated seeds of wheat were treated with wortmannin, a specific inhibitor of PI3K, it was shown that the root growth was suppressed along with a decreased mitotic index and the inhibition effect was positively correlated with the concentrations of the drug. Light and transmission electron microscopy observations indicated that nucleolar structure was damaged. In this case, indirect immunofluorescence staining and RT-PCR were performed to further understand nucleolar functional change after the treatment. It was obvious that B23 shuttled from the nucleoli to the nucleoplasm, or even, to the cytoplasm; the expression of C23 was severely down-regulated. B23 and C23 are the most abundant proteins in the nucleolus (Olson, 2011). In normally living cells, both of them should be localized in the nucleolus and expressed actively. If they shuttle from the nucleolus to other places or their expression is down-regulated, which means that the nucleolar function has been changed (Yang et al., 1990; Dundr et al., 1995). Our results demonstrated that PI3K is not only involved in nucleolar structure, but also in its function.
How does PI3K regulate nucleolar function?

PI3K was first identified in the late of 1980s. Over the past 20 years, its functions have been gradually elucidated. One of the important functions is that it can regulate cell growth and proliferation. The discovery of wortmannin as a specific inhibitor of PI3K made it possible to understand its functional mechanisms. Recently, Ohta et al. (2006) reported that wortmannin could induce Akt dephosphorylation and reduce Akt kinase activity. As a key effector of PI3K, Akt can control the expression of a series of factors downstream, such as BAD, FOXOs, p21, p27 and GSK3. Thus, cell growth, proliferation, survival, metabolism and autophagy are directly or indirectly affected by PI3K (Vanhaesebroeck et al., 2012).

The nucleolus is one of the most important organelles in the cell. Although it is primarily associated with ribosome biogenesis (Cmarko et al., 2008; Olson, 2011), several lines of evidence now show that it has additional functions, such as regulation of mitosis, cell-cycle progression and proliferation, many forms of stress response and biogenesis of multiple ribonucleoprotein particles (Boisvert et al., 2007). So it is reasonable that when cell growth and proliferation are depressed by wortmannin treatment, both nucleolar functions and structure will be abnormal.

On the other hand, since PI3K was closely associated with active nucleolar transcription sites (Bunney et al., 2000), when PI3K was blocked by wortmannin, the nucleolar transcription would be affected and certainly, its structure would be changed.

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References


