

Freezing wheat root tips causes hyperphosphorylation of histone H3 at serine10 in the cell during mitosis

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ABSTRACT. Plants are able to produce various responses to environmental changes. In this report, we demonstrate that freezing of wheat root tips results in hyperphosphorylation of Histone H3 at serine10. In normal conditions, the serine10 phosphorylation of histone H3 occurs at the condensed chromosomes at prophase and vanishes at telophase. The phosphorylated H3 is present mainly in the pericentromeric regions at metaphase and anaphase. However, in the frozen cells, the phosphorylation of histone H3 at serine10 is distributed throughout the chromosome arms at all of the phases during mitosis, even at interphase and cytokinesis. The results support the notion that hyperphosphorylation of Histone H3 at serine10 is related to a stringent response.

Keywords: Freezing; Hyperphosphorylation; Immunofluorescence microscopy; Phosphorylation of histone H3 at serine10; Stringent response.

INTRODUCTION

During the cell cycle, histones are subjected to a variety of post-translation modifications, including acetylation, methylation, phosphorylation, ubiquitylation, and ribosylation. These different modifications can generate synergistic or antagonistic interaction affinities for chromatin-associated proteins, which in turn dictate dynamic transcriptionally silent chromatin states. Therefore, the histone modifications and combinations represent a fundamental regulatory mechanism that has an impact on most chromatin-templated processes, and many cellular processes (Jenuwein and Allis, 2001). The cell cycle-dependent phosphorylation of histone H3 at serine10 (Ser-10 pH3) has been certificated to be conserved in eukaryotes (Hendzel et al., 1997; Li et al., 2005; Wei et al., 1998). This post-translational modification has been linked to transcription activation (Thomson et al., 1999) during the interphase and to chromosome condensation during mitosis (Van Hooser et al., 1998; Wei et al., 1998).

A similar post-translational modification of H3 has also been demonstrated for higher plants (Houben et al., 1999; Kaszas and Cande, 2000; Yang et al., 2002). Using a site-phosphorylation specific antibody, it was shown that in wheat root tips Ser-10 pH3 started at early prophase and

vanished at telophase. The modification was concentrated mainly in the pericentromeric regions at metaphase and anaphase during cell division (Yang et al., 2002). The function of the Ser-10 pH3 in wheat root cells is related to the chromosome condensation during mitosis.

It has been shown that plants encode Aurora-like kinases, analogous to the yeast aurora/Ipl1 founding member and the Aurora-related kinases of other organisms. In plant cells, the Aurora-like kinase is responsible for phosphorylation of the histone H3 at serine10 (Demidov et al., 2005). Some evidence has been published supporting the idea that the mitotic phosphorylation and dephosphorylation of H3 are governed by Ipl1/aurora kinase and Glc7/pp1 phosphatase in budding yeast and nematodes (Hsu et al., 2000). In these models, both enzymes are required for H3 phosphorylation and chromosome segregation. They are responsible for the balance of H3 phosphorylation during mitosis in *Sacharomyces cerevisiae* and *Caenorhabditis elegans*.

The hyperphosphorylation of the alfalfa cellular proteins at low temperature has been shown to be caused by differential sensitivity to cold between the protein kinase and phosphatase by using a cell-free system of the plant (Monroy et al., 1997). In the present study, immunofluorescence microscopy and western blot were used to analyze the level of the Ser-10 pH3 in the freezing treatment of wheat root cells. We found that the high level Ser-10 pH3 was present in the whole course of cell cycle in the freezing treatment cells. This kind of post modification of histone H3 involves a stringent reaction to the freezing stress.

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MATERIALS AND METHODS

Plant material

The wheat (*Triticum aestivum* cv. Chinese Spring) root tips were taken from seeds germinating on wet filter paper in Petri dishes at 25°C. The germinating seeds were maintained at -20°C for 1h for collecting the cold-treated wheat root tips.

Specimen preparation

Root tips were fixed for 30 min in freshly prepared 4% (W/V) paraformaldehyde (PFA, Sigma) solution containing phosphate-buffered saline (PBS, pH 7), washed for 45 min in PBS and digested at 37°C for 30 min in a mixture of 2.5% (W/V) pectolyase Y-23 (Japan Yakult.) and 2.5% (W/V) cellulase (Japan Cal-Bio) dissolved in PBS. Root tips were washed 15 min in PBS and squashed between a glass slide and a coverslip in 45% (V/V) acetic acid. After being frozen in liquid nitrogen, the coverslips were removed, and the slides were transferred immediately into PBS.

Immunofluorescence staining

Slides were permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature (RT). To avoid non-specific antibody binding, Slides were blocked for 30 min in 4% (w/v) bovine serum albumin (BSA) in PBS at RT prior to two washes in PBS for 5 min each and incubated with the primary antibodies in a humid chamber. Polyclonal affinity purified rabbit antibody against histone H3 phosphorylated at serine10 (Upstate Biotechnology, catalog no. 06-570) and mouse monoclonal antibody against microtubules (Zymed Laboratories Inc., catalog no. 32-2500) were diluted 1:400 in PBS with 3% BSA. After 12 h incubation at 4°C and washing for 15 min in PBS, the slides were incubated in FITC-conjugated anti-rabbit IgG (Upstate Biotechnology, catalog no. 02-15-06) and TRITC-conjugated anti-mouse IgG (Upstate Biotechnology, catalog no. 1090-03) diluted 1:200 in PBS, 3% BSA for 1 h at 37°C. After final washes in PBS, the preparations were mounted in DAPI as counterstain.

Confocal laser microscopy

Confocal scanning microscopy was performed using a TCS-NT Leica microscope (Lasertechnik, Heidelberg, Germany); an argon-krypton ion laser was adjusted at an excitation wavelength of 345 nm, 488 nm, and 568 nm. Fluorescent images were captured in sequential mode. Serial optical sections were taken. Selected paired sections were then processed to produce single composite, color-merged overlay images.

Plant histone extraction and Western blot analysis

We collected the same weight of normal/freezing wheat root tips for analysis. Histone-enriched protein extracts were prepared essentially as described (Yu et al., 2004).

Briefly, the wheat tips were collected and ground in liquid N₂ into fine powder. After homogenization of the powder in buffer A (0.4 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 0.1 mM PMSF, 5 mM β-mercaptoethanol) (approximately 5 ml buffer per gram of powder), the resulting slurry was filtered through 200 and 100 μm nylon meshes. The filtrate was centrifuged at 1,500 g for 20 min. The pellet was washed in buffer B (0.25 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1% Triton X-100, 0.1 mM PMSF, 5 mM β-mercaptoethanol), then homogenized in 1 ml buffer C (1.7M sucrose, 10 mM Tris-HCl pH 8.0, 2 mM MgCl₂, 0.15% Triton X-100, 0.1 mM PMSF, 5mM β-mercaptoethanol). The chromatin was treated twice for 45 min with 0.4 M H₂SO₄, and the proteins were precipitated with 5 volumes of ethanol for 48 h at -20°C. After centrifugation at 12,000 g for 10 min, the pellet was washed with 80% ethanol, dried, and resuspended in 0.01N HCl. The samples were resolved on 15% SDS-PAGE and transferred onto PVDF membrane (Amersham Biosciences). The rabbit anti-phospho-Histone H3 (Ser-10) (Upstate Biotechnology, catalog no. 06-570), diluted 1:1000, was used to detect the target protein. Membranes were exposed to a horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (Upstate Biotechnology, catalog no. 474-1506, 1:5000 dilution). A DAB detection system was used for protein detection and visualized in a UVP Bio-imaging system.

RESULTS

Dynamic distribution of Ser-10 pH3 in wheat cell during mitosis

To determine the localization of Ser-10 pH3, microtubules and DNA in the cell during mitosis, a three-color immunofluorescence label was used in this work. At preprophase (Figure 1A), the green signal of Ser-10 pH3 cannot be detected in the cell, but a red preprophase band composed of microtubule is present in middle of the cell surrounding by the inner-cytoplasmic membrane. At prophase; the green immunofluorescence signal of the Ser-10 pH3 appeared and spread along with the condensed chromosomes in the nuclei. This indicated that the phosphorylation of H3 at serine10 was global at this phase of mitosis in wheat cells (Figure 1B). At metaphase, the chromosomes line up at the equatorial plate of the cell and interact with the microtubules which arrange themselves in parallel perpendicular to the equator. The green labeling signal (Ser-10 pH3) was vivid mainly in the pericentromeric regions while it appeared weak on the chromosomal arms during the metaphase and anaphase (Figure 1C, D). The chromosomes began to decondense, and the green labeling signal was weak until it disappeared at telophase and cytokinesis (Figure 1E, F). This indicates that the distribution of the serine10 phosphorylation of histone H3 changes following the progression of mitosis.

In wheat root tip cells, microtubules show dynamic structural changes during cell cycle progression. The corti-

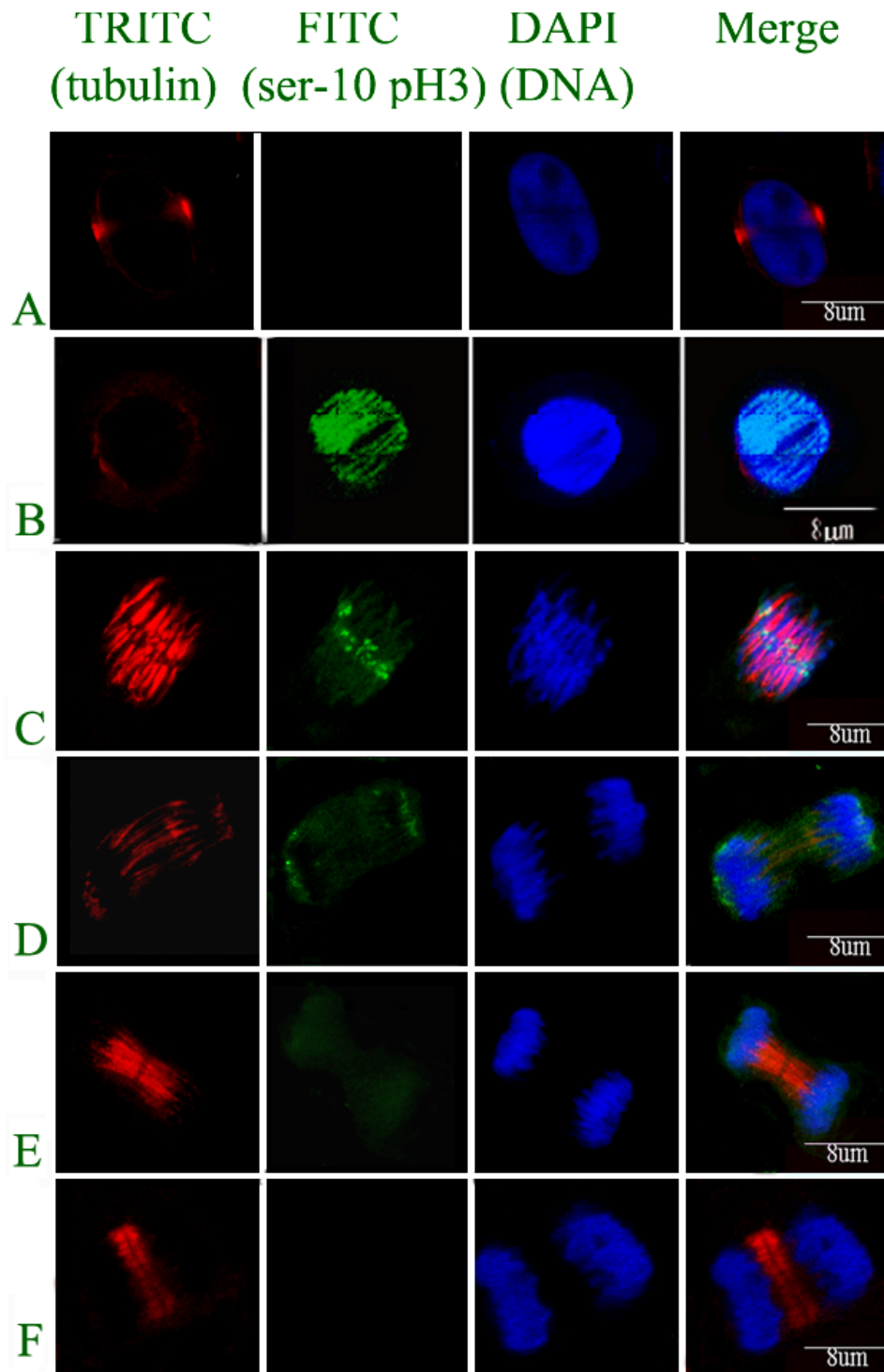


Figure 1. The dynamic distribution of the Ser-10 pH3 in wheat cell during mitosis. Ser-10 pH3 was labeled with FITC (green); α -tubulin and DNA were labeled with TRITC (red) and DAPI (blue), respectively. A: pre-prophase; B: prophase; C: metaphase; D: anaphase; E: telophase; F: cytokinesis.

cal microtubules, preprophase band, spindle microtubules, and phragmoplast, all of which are plant specific microtubule structures, can be observed from the late G2 phase through to telophase (Figure 1).

Abnormal distribution of Ser-10 phosphorylated histone H3 in freezing wheat cell

The wheat root tips were freezing at -20°C for 1 h before the cell specimen preparation and indirect immu-

nofluorescence staining. It is interesting that the dynamic distribution of Ser-10 pH3 was altered by cold treatment. The Ser-10 pH3 is distributed throughout the chromosome arms during mitosis, even in interphase and cytokinesis (Figure 2A, F). The regular dynamic distribution of Ser-10 pH3 cannot be found in the plant cell, but the organization of microtubules in freezing wheat cells is the same as in normal cells mostly at different phase (Figure 2A-F). Two hundred of the frozen mitotic cells were examined by con-

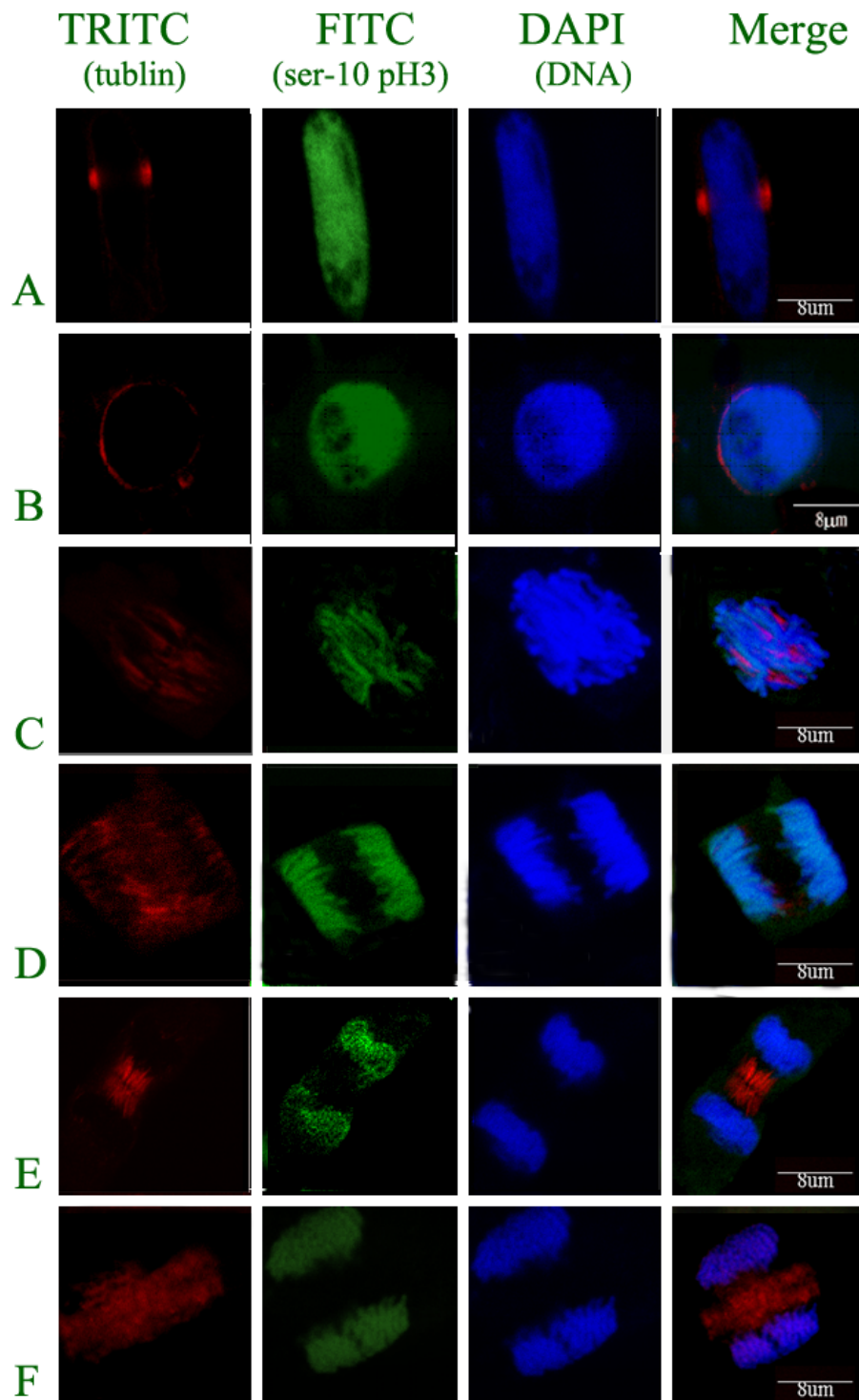


Figure 2. Abnormal distribution of Ser-10 p $H3$ in freezing wheat cell during mitosis. Ser-10 p $H3$ were labeled with FITC (green); α -tubulin and DNA were labeled with TRITC (red) and DAPI (blue), respectively. A: preprophase; B: prophase; C: metaphase; D: anaphase; E: telophase; F: cytokinesis.

focal microscopy; all of them showed irregularly strong immunofluorescence staining. Two important different points between frozen and untreated wheat tip cell are: 1) the strong phosphorylation signal appears at all phases from preprophase (Figure 2A, with a preprophase band in the cell) to telophase (Figure 2F, the phragmoplast formed in); 2) the Ser-10 p $H3$ appears all along the chromosome, not only the pericentromeric region. The results indicate that freezing treatment causes global phosphorylation of

histone H3 at Ser-10 in mitotic wheat cells and disturbs regular distribution of the modified histone. The cold treatment does not, however, destroy the constructions of microtubule that are mainly found in the cell.

The histones extracted from normal/cold treated cells were analyzed by SDS-PAGE and western blot

The histones of normal/cold treated cells were extracted

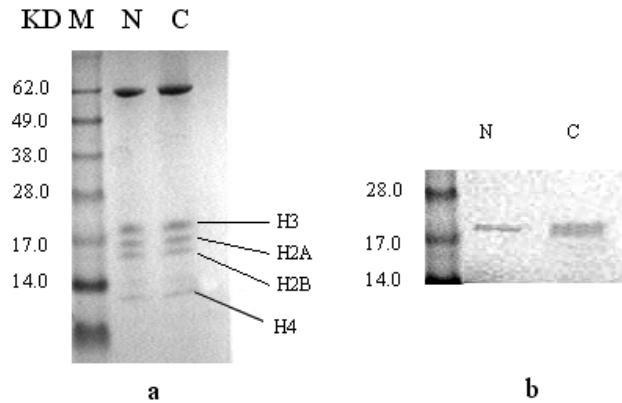


Figure 3. The SDS-PAGE (a) and Western blot (b) of the histone extracted from the normal/cold treated cells of the wheat root tips. M: Molecular weight marker; N: Histones of the normal cells; C: Histones of the cold-treated cells.

according to “Material and Methods.” The equal amounts of the two samples were resolved on 15% SDS-PAGE, and the core histones H2A, H2B, H3, and H4 bands lined up on the markers range of molecular weight 10.0 kDa–28.0 kDa (Figure 3a). The levels of the Ser-10 pH3 in cold-treated samples are much higher than those of the normal samples, as shown in analysis of the western blot (Figure 3b). These results are consistent with that of the immunofluorescence microscopy.

DISCUSSION

Ser-10 pH3 appeared in the eukaryotic cells as a mitosis marker during the cell cycle. It has been reported that these modified proteins are related to the chromosome condensation during mitosis (Van Hooser et al., 1998; Houben et al., 1999; Yang et al., 2002; Li et al., 2005). By using indirect immunofluorescence labeling and laser confocal microscopy, Ser-10 phosphorylation of histone H3 is proven to occur at prophase in the whole chromosomes of wheat cell root tips. Then, the phosphorylation signal is concentrated at the pericentromeric region in punctuate at metaphase and anaphase before disappearing at telophase. The dynamic distribution of Ser-10 phosphorylated histone H3 suggests that the modified protein at prophase and premetaphase may serve to promote chromatin condensation; and the Ser-10 pH3 is collected in pericentromeric region at metaphase, meaning that the Ser-10 pH3 may be involved in active kinetochore and direct the daughter chromosomes moving to the two poles of the dividing cell successfully. The distribution and function of Ser-10 pH3 at metaphase and anaphase is quite different in plants and mammals. The modified histone has been proved to be collected in the centre of the spindle at metaphase and to be involved in the midbody. The Ser-10 pH3 plays a role in cytokinesis in mammalian cells (Li et al., 2005; Song et al., 2007).

In the present study, we found that freezing causes hyperphosphorylation of histone H3 at Ser-10 in wheat

root tip cells during mitosis. The distribution of Ser-10 pH3 in the cell is neither the same as in normal cells (as described above) nor the same as in ice-water treated cells (Manzanero et al., 2002). The modified protein is present at all of the phases from preprophase to cytokinesis in the treated cells. The hyperphosphorylation of histone H3 in the frozen cell was demonstrated by SDS-PAGE and western blot of acid-extracted histone samples.

The hyperphosphorylation of H3 in wheat mitotic cell caused by freezing might be due to destruction of the balance between aurora-like kinases and phosphatase, if the phosphatase is also sensitive to cold stress.

As demonstrated above, the Ser-10 pH3 disappears in the later phases of the mitotic wheat cell. This means that the activity of aurora kinase has fallen to a low level. The Ser-10 pH3 is confined to the pericentromeric region at a late phase of the mitotic cell, so the irregular hyperphosphorylation of histone caused by freezing treatment resembles an initial regulating reaction of the cell. As the plant cell experiences freezing stress, it may conduct a stringent response against the severe conditions, and some kinases in the cell would activate histone phosphorylation and cause chromatin condensation. The cell reduces the metabolite level of the cell in order to survive the low temperature. How freezing induces the hyperphosphorylation of histone at Ser-10 should be a subject of intensive research.

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冷凍處理小麥根尖可以引起細胞中組蛋白 H3 ser-10 位點的超磷酸化現象

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植物對環境的改變會做出不同的反應。在本研究中，我們利用間接免疫螢光標記技術和共聚焦顯微鏡技術觀察到被冷凍處理的小麥根尖細胞在整個有絲分裂過程的不同相期中組蛋白 H3 的 ser-10 位點都處在超磷酸化狀態。在正常的小麥根尖細胞中組蛋白 H3 的 ser-10 位點的磷酸化現象出現在有絲分裂的前期，消失於分裂末期，在有絲分裂的中期和後期其主要定位在著絲粒區域。但是在冷凍的小麥根尖細胞中，在整個有絲分裂期中 ser-10 位點被磷酸化的組蛋白 H3 一直分佈在整條染色體上，甚至在胞質分裂期也沒有消失。實驗結果推斷，這種超磷酸化現象是植物在極端生長環境下的一種應急反應。

關鍵詞：免疫螢光顯微鏡；超磷酸化；ser-10 位點磷酸化的組蛋白 H3；冷凍；應急反應。