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Distribution of Ubiquitin Protein in Meristematic Mesophyll Cells of Barley Leaves

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The distribution of ubiquitin protein in meristematic mesophyll cells of barley (*Hordeum vulgare* L.) leaves was investigated by using immunofluorescence microscopy. Simultaneous observation of nuclei was achieved by DAPI (4',6-diamidino-2-phenylindol-dihydrochloride) staining. A strong correlation between the chromatin organisation and the ubiquitin distribution could be observed. Interphase nuclei revealed an intense content of ubiquitin and accumulation of ubiquitin at the nuclear envelope, whereas condensed chromosomes of dividing cells excluded any ubiquitin appearance. During cell division, the aggregation of ubiquitin protein was detected in the area of the mitotic spindle in anaphase as well as the area of the cell plate in the late telophase.

Keywords: *Hordeum vulgare*, ubiquitin, DAPI, mitosis, cell cycle

INTRODUCTION

All eukaryotes contain a highly conservative intracellular system for nonlysosomal protein degradation, mediated by ATP dependent ubiquitin conjugation to target proteins first described by Goldstein (1974), Ciechanover *et al.* (1978) and Hershko *et al.* (1979). Ubiquitin is involved in far more processes than degradation of proteins such as cell differentiation, responses to stress and proceeding in cell cycle (Varshavsky, 1997).

Ubiquitin is a small heat stable polypeptide consisting of 76 amino acids with a molecular weight of 8.6 kD (Vierstra *et al.*, 1986). The sequence deviates in only 3 amino acids between higher plants and animals (Pollmann and Wettern, 1989). Therefore, ubiquitin is designated to have the highest homology in amino acid sequences and structure during evolution beside the histone H4. The ubiquitin-mediated degradation of proteins by the 26S proteasome, a large ATP dependent protease complex, was elucidated during the last decade and remains well understood and reviewed in many reports (Finley and Chau, 1991; Hochstrasser, 1996; Weissman, 1997). Most investigations have been carried out with animals and yeast, but all features of the ubiquitin-system have been found in plant cells as well (Vierstra, 1987; Vonkampen *et al.*, 1996). Even the exchange of purified ubiquitin-activating enzyme from animal to plant system did not alter its activity *in vitro* (Hatfield and Vierstra, 1989). The first protein in plants known to be degraded preceding ubiquitin conjugation was phytochrome (Shanklin *et al.*, 1987; Jabben *et al.*, 1989a; Jabben *et al.*, 1989b).

Investigations about the regulation of chromatin structure by ubiquitin were initiated by Goldknopf and Busch (1977) and Levinger and Varshavsky (1982). They first discussed the participation of ubiquitin in regulation of chromatin structure and the process of transcription. Ciechanover *et al.* (1984) and Finley *et al.* (1984) suggested an involvement of ubiquitin in the progression of cell cycle. Up to the present there are strong evidences that ubiquitin and ubiquitin-activating enzymes are involved in cell cycle and localised inside the nucleus in a cell cycle-dependent manner (Banerjee *et al.*, 1995; Osaka *et al.*, 1997). Ubiquitination of cyclins may play an important role in anaphase progression (Zachariae and Nasmyth, 1996; Hershko, 1997).

We now demonstrate an immunofluorescent approach to detect ubiquitin protein distribution in the nuclei of meristematic and dividing mesophyll cells of a cereal leaf. This approach was feasible because adequate quantities of meristematic cell populations can be obtained simply by harvesting

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the basal section of growing leaves.

MATERIALS AND METHODS

Leaf Material

Barley plants (*Hordeum vulgare* L.) were grown in vermiculite in a growth cabinet at 23°C with 15 h per day photoperiod as described recently (Hellmann *et al.*, 1995). The second foliage leaves were selected 8 days after sowing for analysis. The basal 2.5 mm of the still growing leaf, which had not yet developed a sheath, was dissected and used for the investigations described below.

Cell Isolation

Dissected leaf bases were cut into small pieces of approximately 0.5 × 0.5 mm and incubated in a fixing solution consisting of 3% formaldehyde in PME buffer (50 mM phosphate buffer, 5 mM EGTA, 1 mM $MgSO_4$, pH 7.3) for 90 min. Afterwards, the leaf pieces were washed with PME buffer to remove formaldehyde and incubated in a digestion solution consisting of 0.3% Pectolyase (Seishin Pharmaceutical, Tokyo, Japan), 0.3% bovine serum albumin in PME buffer (pH 5.8) for 2h. Thereafter, leaf tissues were rinsed three times carefully in PME buffer (pH 7.3) and the cells were then liberated by intense rinsing of the tissue through a wide mouth pipette for several times. The resulting cell suspension was filtered through a 100 µm nylon mesh to remove the undigested fragments. The separated mesophyll cells were sedimented by centrifugation in microcentrifuge tubes at 2000 × g for 5 min.

Immunological Staining of Cells

Sedimented cells were mixed with an equal volume of solubilized 2.5% ultra low gelling agarose in PME buffer at 35°C. Three µL of the cell-agarose mixture were transferred immediately onto glass slides and cooled to room temperature to immobilise the isolated cells. Immobilised cells were washed twice with PME buffer, lysed for 30 min with 1% Triton X-100 in PME buffer and washed repeatedly until all detergent was removed. Staining of ubiquitin was achieved by incubation with a polyclonal antibody against ubiquitin (Sigma-Aldrich, Seoul, Korea) in the dilution 1:50. The primary antibody was removed after one hour incubation by intense rinsing of the immobilised cells with PME buffer. Incubation of secondary biotinylated antibody (Amersham International plc, Seoul, Korea) and conjugation of streptavidin-fluorescein isothiocyanate (FITC) was carried out according to the manufacturers' protocol. For visualisation of stained cells a Zeiss Axioskop epifluorescence microscope with a filter combination for FITC exitation was used.

DAPI Staining of Nuclei

After completion of ubiquitin staining, immobilized cells were incubated with 0.1 µg/mL DAPI (4',6-diamidino-2-phenylindoldihydrochloride, Sigma-Aldrich, Seoul, Korea) in 5 mM Tris-HCl (pH 8.2) and 1 mM EDTA for 10 min and removed by extensive washing with PME buffer. Visualisation was carried out as described above using a filter combination for DAPI exitation.

RESULTS AND DISCUSSION

As ubiquitin is known to function in many different cellular processes such as ATP dependent protein degradation, responses to stress, organization of chromatin structure and progression in cell cycle, it was of great interest to investigate the ubiquitin distribution in meristematic plant cells. Leaves from monocotyledonous plants grow from their base and show a characteristic developmental gradient along their axis (Schnyder *et al.*, 1987; Hellmann *et al.*, 1995). Cells lose the ability for cell division and arrest in cell cycle with increasing distance to the leaf base and therefore provide a good system for cell cycle investigation. For the understanding of cessation in cell cycle activity and the feasible involvement of ubiquitin conjugation or ubiquitin itself, first the spatial distribution of ubiquitin protein and its conjugates during mitosis must be elucidated. With the polyclonal antibody used, it was not possible to distinguish between free nonconjugated ubiquitin and ubiquitin conjugates.

The presence of ubiquitin in monocotyledonous plants especially in grasses was demonstrated and has been reported (Vierstra *et al.*, 1985; Pinedo *et al.*, 1996) as well as in dicotyledonous plants (Seo *et al.*, 1996). The spatial distribution of ubiquitin in meristematic barley mesophyll cells depended on the developmental stage of the cells. Nondividing cells with nuclei fitting in nearly half of the cell yielded a weak but regular ubiquitin staining of nuclear areas avoiding the region of nucleoli, as demonstrated in DAPI-stained nuclei (Fig. 1a and 1b). Well-developed

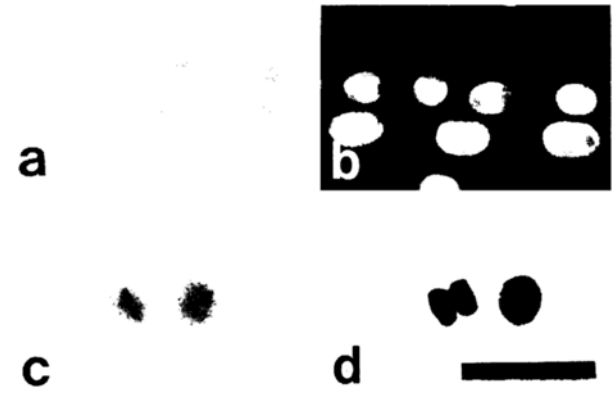

Fig. 1. Immunofluorographs of ubiquitin (a, c) and DAPI (b, d) staining in meristematic barley mesophyll cells. Nondividing young mesophyll cells with well-developed nucleoli, nuclei and their envelopes exhibit accumulation of ubiquitin (a, b) and accumulation of ubiquitin at higher rates in the nuclei and cell plate region short before completion of cell division (c, d; left cell); bar=25 μm.

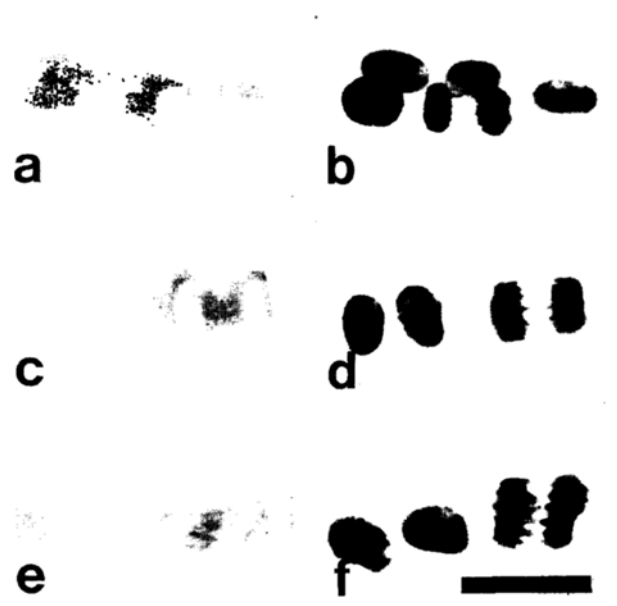

Fig. 2. Immunofluorographs of ubiquitin (a, c, e) and DAPI (b, d, f) staining in meristematic barley mesophyll cells. Dividing cells in the late anaphase (a-f; right cells) and adjacent nondividing cells with a more intense and granular ubiquitin staining of the nuclei (a, b; left cell), beginning of ubiquitin accumulation at the nuclear envelope (c, d; left cells) and a weak but regular ubiquitin staining of nuclei and nuclear envelope region (e, f; left cells); bar=25 μm.

nucleoli indicate high activity of transcription beginning short after completion of mitosis. Theses cells accumulate ubiquitin in the region of the nuclear envelope. Cytosolic ubiquitin could not be detected by the method used. Cells just after completion of cell division without any nucleoli revealed a more intense and granular ubiquitin staining inside the nuclei (Fig. 1c). The nuclear envelope could not be stained. It was reported that up to 15% of the histone H2A is covalently linked to ubiquitin (uH2A) and disappears before the beginning of metaphase during condensation of the chromosomes and arises after completion of cell division in G1 (Goldknopf and Busch, 1977; Levinger and Varshavsky, 1982). Theses findings are in accord with our results of intense staining of noncondensed chromatin. The highest degree of ubiquitin accumulation could be observed just after nuclear separation in late telophase in the area of the cell plate, introducing the new cell wall between the divided nuclei (Fig. 1c and 1d). Stephen *et al.* (1996) described a decrease in amount of ubiquitin conjugates by 50% during G2, shortly before condensation of chromosomes. Dividing cells with fully condensed chromosomes in anaphase represent a distinct exclusion of ubiquitin from chromosomes whereas ubiquitin condensed around them and accumulated between the separating chromosomes in the area of the mitotic spindle (Fig. 2a, 2c and 2e). The mitotic spindle as well as cell plate are structures mainly formed by the microtubular cytoskeleton, known as highly dynamic structures underlying high rates of construction and destruction (for review, see Seagull, 1989) but colocalization with ubiquitin was yet not observed.

At least it is well known that ubiquitination and ubiquitin-mediated degradation are involved or even essential in chromosome segregation and mitotic exit by degrading anaphase inhibitors and mitotic cyclins (Pagano, 1997).

Nevertheless, each stage of cell cycle exhibits a characteristic distribution of ubiquitin and ubiquitinated proteins inside or around the nucleus. Ubiquitin staining could be shown to give more detailed informations about proceeding in cell division and cell cycle compared to nuclear staining with DAPI. Further investigations must be done revealing the spatial distribution of ubiquitin in nondividing cells, arrested in either G1 and G2 or nondividing protoplast cultures which had been exposed to enormous stress during cell wall digestion.

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