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The Response to Oxidative Stress Induced by Magnesium Deficiency in Kidney Bean Plants

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To understand the plant response to oxidative stresses, we studied the influence of magnesium (Mg^{++}) deficiency on the formation of hydrogen peroxide (H_2O_2), malondialdehyde (MDA), and protease activity in kidney bean plants. The expression pattern of proteins under Mg^{++} deficiency also was examined via two-dimensional electrophoresis. The formation of H_2O_2 and MDA increased in the primary leaves of plants grown in a nutrient solution deficient in Mg^{++}. Protease activity in Mg^{++}-deficient plants was also higher than in those grown with sufficient Mg^{++}. The expression pattern of the proteins showed that 25 new proteins were generated and 64 proteins disappeared under Mg^{++}-deficient conditions. Therefore, a deficiency in Mg^{++} may cause oxidative stress and a change in protein expression. Some of these proteins may be related to the oxidative stress induced by Mg^{++} deficiency.

Keywords: H_2O_2, kidney bean, magnesium deficiency, oxidative stress, protein alteration, proteolytic activity

In plants, active oxygen species (AOS), such as the superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and singlet oxygen ($^1O_2$), can be generated during normal photosynthesis and/or respiration, thereby causing oxidative stress (Smirnoff, 1993). Under extreme environmental conditions, the plant response is associated with free radicals or oxidative damage. Stress conditions can be caused by herbicides (Orr and Hess, 1982; Finch and Kunert, 1985; Babbs et al., 1989), air pollutants (Mehlhorn et al., 1987), toxic metals (De vos et al., 1992; Cho and Park, 1999; Gou and Lee, 1999), extreme temperature (Kendall and Mckersie, 1989), or salt stress (Smirnoff, 1993). The AOS react with lipids on the cell membrane to form lipid peroxides such as ethane (Matsumoto et al., 1994) or malondialdehyde (MDA; Kojima et al., 1991). The specific oxidation and modification of protein enzymes by AOS contribute to irreversible inhibition of enzyme activity. These invalidated enzymes then may be degraded by proteolytic enzymes (Levine et al., 1981; Rivett, 1985).

Plants have various systems for protecting against damage from AOS (Smirnoff, 1993). Defense mechanisms include 1) low molecular antioxidants, such as Vitamin C, Vitamin E, carotenoids, and glutathione; and 2) antioxidative enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR), monodehydro ascorbate reductase, and catalase.

Magnesium (Mg^{++}) is a main component in chlorophyll. It is also a cofactor for the chloroplast ribosome (a 70s ribosome), which is responsible for the biosynthesis of large subunits of ribulose 1,5-bisphosphate (RuBP) carboxylase (Rubisco; Goodwin and Mercer, 1983) and enzymes that participate in CO_2 fixation. In Mg^{++}-deficient chloroplasts, CO_2 fixation can be impaired by the inhibition of Rubisco biosynthesis. If the energy generated by Photosystem I and II is not used for CO_2 fixation, harmful AOS may be formed.

In a previous study, we showed that the activities of antioxidative enzymes, such as SOD, AP, and GR, increased rapidly in Mg^{++}-deficient cucumber (Lee et al., 1998). We also observed rapid chlorosis and a reduction in protein content. However, in Mg^{++}-deficient kidney bean plants, the reduction in chlorophyll and protein contents was less obvious. The activities of SOD and AP in the deficient plants increased greatly, but GR activity decreased (Lee et al., 1998). These results suggest that the response to Mg^{++} deficiency varies among plant species.

This present study was designed to understand the plant response to Mg^{++} deficiency. Here, we investigated the formation of H_2O_2, lipid peroxidation, and

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the varying activities of proteolytic enzymes in deficient kidney bean plants. In addition, we used two-dimensional electrophoresis to study the alteration in protein profiles because of Mg^{++} deficiency.

MATERIALS AND METHODS

Plant Growth

Kidney bean plants (*Phaseolus vulgaris* L.) were grown in a controlled-environment chamber (light/dark regimes 14/10 h at 27/22°C, relative humidity 65-75%). A light intensity of 250 $\mu Em^{-2}s^{-1}$ was provided by fluorescent and incandescent lamps, as described previously (Lee et al., 1998). The primary leaves were harvested 5 to 11 days after the plants were transferred to a nutrient solution described by Lee et al. (1998). Harvested samples were stored at –80°C until they could be analyzed.

Determination of H_2O_2 Content and the Activity of Proteolytic Enzymes

A 0.5-g sample of leaf material, without the main midrib, was ground with liquid nitrogen and homogenized with 5 mL of 50 mM potassium phosphate buffer (pH 7.0). This homogenate was centrifuged at 15,000*g* for 15 min at 4°C. The supernatant was used for determinations of H_2O_2 content and proteolytic-enzyme activity. H_2O_2 content was determined by measuring the absorbance at 436 nm and comparing it with the H_2O_2 standard (Bernt and Bergmeyer, 1974). Proteolytic enzyme activity was assayed according to the method of Matsuzawa et al. (1988). One unit of protease activity was defined as the amount of enzyme that produced 1 µM of tyrosine for 1 h under assay conditions.

Measurement of Malondialdehyde (MDA)

Our assay for MDA followed the method of Ohkawa et al. (1979), with a slight modification. A 0.5-g sample of primary leaves was ground with liquid nitrogen, then homogenized with 5 mL of 50 mM potassium phosphate buffer (pH 7.0) and 0.1% (w/v) butylated hydroxytoluene. The homogenate was centrifuged at 12,000*g* for 20 min at 4°C, and the resulting supernatant was used for MDA determinations. Lipid peroxidation was determined as the absorbance at 532 nm, and the amount of MDA was calculated by its extinction coefficient (155 $mM^{-1}cm^{-1}$).

Two-Dimensional Electrophoresis

Protein samples were prepared according to the method of Wessel and Flügger (1984), with some modification. One gram of plant tissue was ground and homogenized with 5 mL of 100 mM potassium phosphate buffer (pH 7.0) that contained 5 mM EDTA, 1 mM ascorbic acid, and 20% (w/w) polyvinylpyrrolidone. The homogenate was centrifuged at 12,000*g* for 20 min, and the resulting supernatant was used for protein preparation. Protein content was determined by using the method of Bradford (1976).

Our IEF gel electrophoresis followed the methods of Kawakami and Watanabe (1988) and Laemmli (1970), with a slight modification. The gel contained 8.5 M urea, 4% acrylamide-bis, 2% triton X-100, 1.6% ampholine (pH 5-8), and 0.4% ampholine (pH 3.5-10). Protein was loaded at a concentration of 60 µg/10-20 µL on each gel. The electrophoresis was conducted at 400 V for 12 h, then at 800 V for 1 h.

The SDS-PAGE was slightly modified from the method of O'Farrell (1975). The separating gel contained 11.6% acrylamide-bis and 372 mM Tris-HCl buffer (pH 8.8), and the staking gel contained 4.1% acrylamide-bis and 127 mM Tris-HCl (pH 6.8). The SDS-PAGE was conducted at a fixed electric current of 2 mA per gel. After the electrophoresis, the gel was stained and destained with a silver staining kit (Bio-Rad®), and then photographed.

RESULTS AND DISCUSSION

We showed previously that Mg^{++} deficiency greatly increased the activities of SOD and AP, two antioxidative enzymes, in kidney bean plants (Lee et al., 1998). These enhanced activities suggested that the production of $\cdot O_2^-$ and/or H_2O_2 was increased by the deficiency, because SOD and AP are $\cdot O_2^-$- and H_2O_2-scavenging enzymes, respectively. In the present study, H_2O_2 increased when bean plants were grown in a nutrient solution deficient in Mg^{++} (Fig. 1). The H_2O_2 concentration in the deficient plants was about 40% higher after 5 and 11 days compared with plants with sufficient Mg^{++}. Therefore, kidney bean plants grown under Mg^{++}-deficient conditions formed more H_2O_2 than those supplied with adequate Mg^{++}.

The H_2O_2 reaction to the bio-molecule is weaker than the reactions of other AOS. However, H_2O_2 can form the most toxic hydroxy radical (·OH) because of actions by transition metals such as Fe^{++} and Cu^+. Lipids that are rich in bio-membranes are degraded

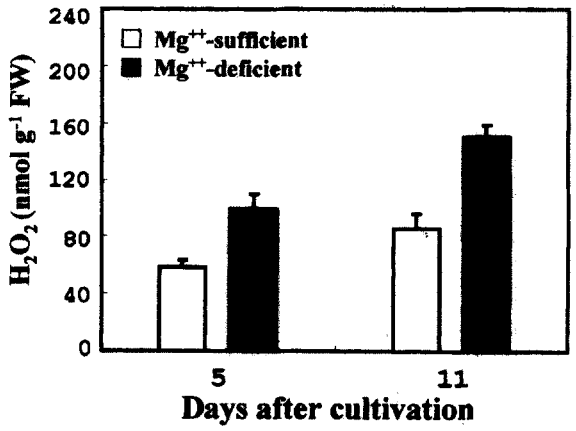

Figure 1. Formation of hydrogen peroxide (H_2O_2) in primary leaves of kidney bean plants grown in Mg^{++}-sufficient (1000 μM) and Mg^{++}-deficient (7 μM) nutrient solutions. Each bar represents the mean of four replicates plus SE.

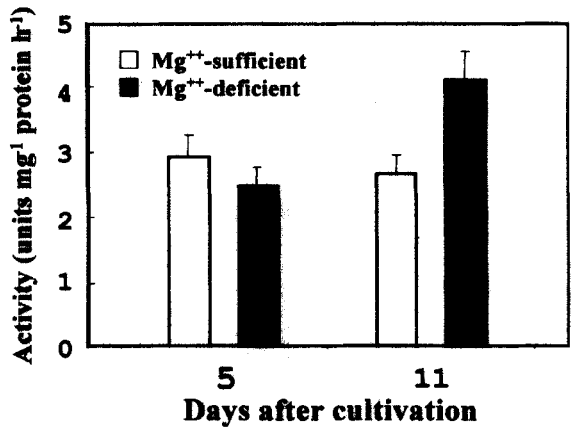

Figure 3. Proteolytic activity in the primary leaves of kidney bean plants grown in Mg^{++}-sufficient (1000 μM) and Mg^{++}-deficient (7 μM) nutrient solutions. Each bar represents the mean of four replicates plus SE.

into ethane or MDA by AOS peroxidation. The lipid peroxidation that was increased by Mg^{++} deficiency is shown in Figure 2. MDA formation, a product of lipid peroxidation, was nearly constant in the Mg^{++}-sufficient plant leaves, but increased over time in Mg^{++}-deficient plants. After 11 days, MDA content was approximately two times higher in the Mg^{++}-deficient leaves.

AOS can modify proteins to an oxidative form. The oxidized protein can then be decomposed by proteolytic enzymes (Levine et al., 1981; Rivett, 1985). Because the H_2O_2 concentration in Mg^{++}-deficient leaves increased (Fig. 1), we also measured the activity of alkaline protease to investigate the influence of increased H_2O_2 formation on protein degradation (Fig. 3). Protease activity increased over time only in

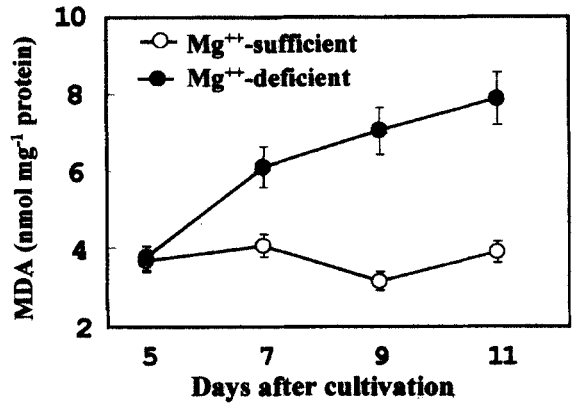

Figure 2. Malondialdehyde (MDA) formation in the primary leaves of kidney bean plants grown in Mg^{++}-sufficient (1000 μM) and Mg^{++}-deficient (7 μM) nutrient solutions. Each data point represents the mean of four replicates plus SE.

Mg^{++}-deficient leaves. After 11 days of growth in the deficient nutrient solution, the enzyme activity was approximately 35% higher. An elevated formation of AOS because of Mg^{++} deficiency may have increased the activity of proteolytic enzymes, thereby accelerating protein degradation.

In Mg^{++}-deficient plants, some of the excitation energy produced by the photosystem may have led to the formation of AOS; formation of H_2O_2 increased under the Mg^{++} deficiency (Fig. 1).In addition, many proteins were newly expressed in the deficient plants, but a large number of proteins also disappeared (Fig. 4). Up to 64 proteins were lost, with molecular weights ranging from 67.0 to 20.1 KDa. Approximately 55 KDa of the protein expected to be in the large subunit of Rubisco was not detected.

In contrast, 25 proteins were induced, most of them being less than 40 KDa in molecular weight. Therefore, we speculated that many of the proteins constitutively-expressed in deficient leaves may have been degraded into peptides with low molecular weights, because H_2O_2 formation and protease activity were higher under deficient conditions. Although the newly formed proteins may could be originated from the degradation of proteins with large molecular weights, some of the proteins would have been induced by the oxidative stress caused by the nutrient deficiency.

In conclusion, Mg^{++} deficiency increased the formation of H_2O_2 (Fig. 1). Because AOS can decompose unsaturated lipids, the increase in H_2O_2 formation apparently was accompanied by an increase in lipid peroxidation (Fig. 2). Oxidative stress

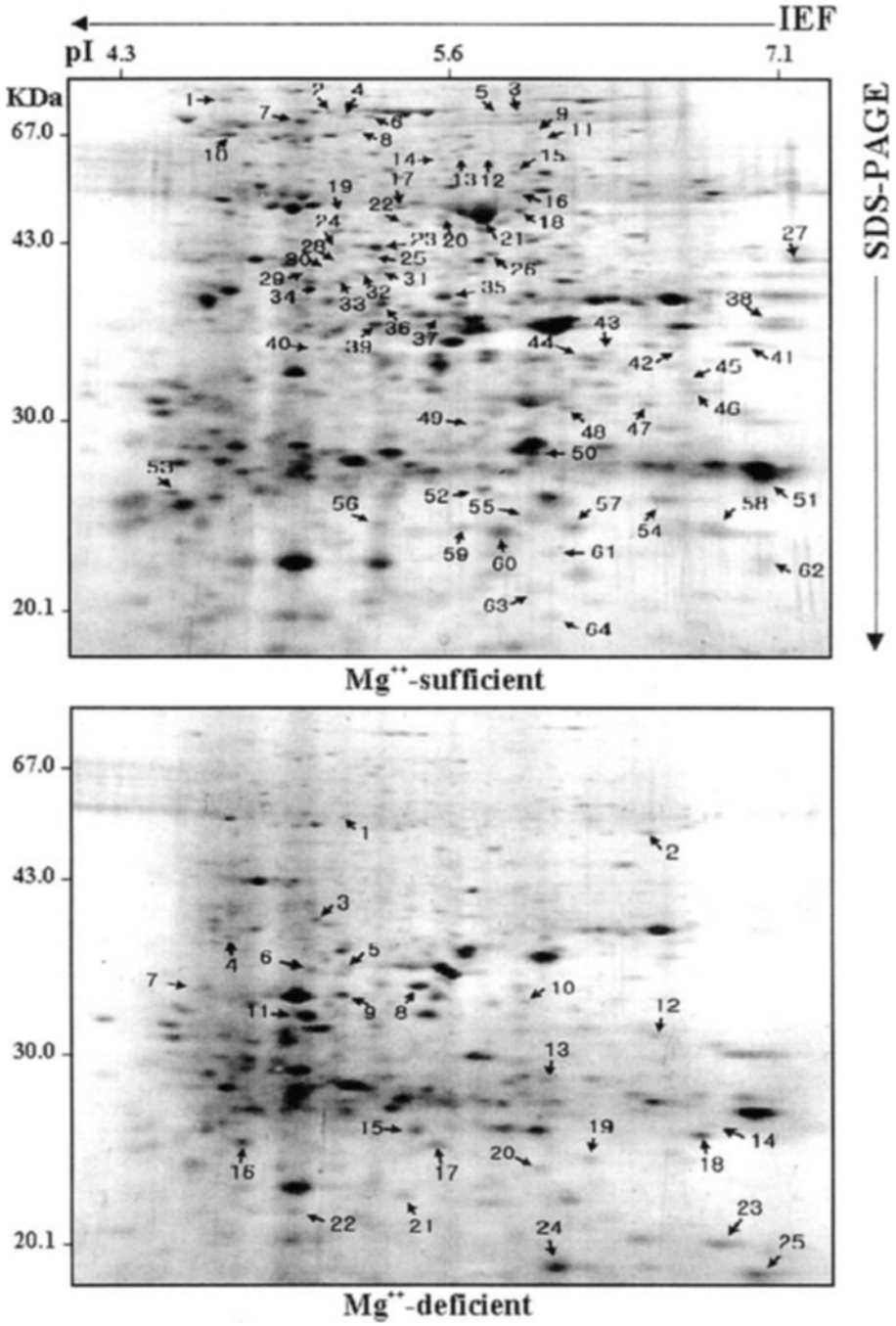

Figure 4. Protein expression patterns in the primary leaves of kidney bean plants grown for 11 days in nutrient solutions with different Mg^{++} supplies (1,000 μM, Mg^{++}-sufficient; 7 μM, Mg^{++}-deficient). The arrows in the Mg^{++}-sufficient pattern indicate the proteins disappeared in Mg^{++}-deficient plants; the arrows in the Mg^{++}-deficient pattern indicate the proteins induced by Mg^{++}-deficiency.

can modify proteins in cells, and these oxidatively modified proteins may be degraded by the proteolytic enzymes (Rivett, 1985). In our study, Mg^{++} deficiency promoted the activity of proteases (Fig. 3).

Two-dimensional electrophoresis showed that many proteins disappeared under deficient conditions (Fig. 4). At the same time, however, many proteins with low molecular weights were induced. Various environmental stresses can generate AOS. In order to acclimate to these oxidative stresses, organ-

isms can induce proteins (Demple and Halbook, 1983; Chan and Weiss, 1987; Mehlhorn et al., 1987; Cakmak and Marschner, 1992). This may be accomplished via de novo synthesis and/or by restoring target molecules such as proteins and nucleic acids, as well as antioxidative enzymes and low molecular antioxidants. In our study, some of the protein induction caused by Mg^{++} deficiency may have acted as an antioxidative mechanism. Our study will now focus on determining the amino acid sequences of proteins induced by Mg^{++} deficiency.

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LITERATURE CITED

Babbs CF, Phans JM, Coolbaugh RC (1989) Lethal hydroxy radical production in paraquat-treated plants. Plant Physiol 90: 1267-1270

Bernt E, Bergmeyer HU (1974) Inorganic peroxides, *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis, Vol 4. Academic Press, New York, pp 2246-2248

Bradford MM (1976) A rapid and sensitive method for the quantitation of microorgan quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-255

Cakmak I, Marschner H (1992) Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase in bean leaves Plant Physiol 98: 1222-1227

Chan E, Weiss B (1987) Endonuclease IV of *Escherichia coli* is induced by paraquat. Proc Natl Acad Sci USA 84: 3189-3193

Cho UH, Park JO (1999) Changes in hydrogen peroxide content and activities of antioxidant enzymes in tomato seedlings exposed to mercury. J Plant Biol 41: 41-48

Demple B, Halbrook J (1983) Inducible repair of oxidative DNA damage in *Escherichia coli*. Nature 304: 466-468

De Vos CHR, Vonk MJ, Vooüs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Plant Physiol 98: 853-858

Finch BF, Kunert KJ (1985) Vitamins C and E: An antioxidative system against herbicide-induced lipid peroxidation in higher plants. J Agric Food Chem 33: 574-577

Goh C-H, Lee Y (1999) Aluminum Uptake and Aluminum-Induced Rapid Root Growth Inhibition of Rice Seedlings. J Plant Biol 42: 151-158

Goodwin TW, Mercer EI (1983) The Chloroplast Genome. Introduction to Plant Biochemistry, Ed 2, Pergamon Press, New York, pp 394-399

Kawakami N, Watanabe A (1988) Change in gene expression in radish cotyledons during dark-induced senescence. Plant Cell Physiol 29: 33-42

Kendall EJ, Mckersie BD (1989) Free radical and freezing injury to cell membranes of winter wheat. Physiol Plant 76: 86-94

Kojima S, Masumoto H, Ishizuka K (1991) Protoporphyrin IX accumulation in *Lemna pauciscostata* Hegelm. caused by diphenyl ether herbicides and their herbicidal activity. Weed Res Japan 36: 318-324

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 4007-4021

Lee JJ, Kim BC, Matsumoto H, Usui K (1998) Influence of magnesium deficiency on induction of antioxidation enzymes and increased tolerance to active oxygen generation herbicide in cucumber and kidney bean plants. J Weed Sci Tech 43: 349-358

Levine RL, Oliver CN, Fulks RM, Stadtman ER (1981) Turnover of bacterial glutamine synthetase: Oxidative inactivation precedes proteolysis. Proc Natl Acad Sci USA 78: 2120-2124

Matsumoto H, Lee JJ, Ishizuka K (1994) Variation in crop response to protoporphyrinogen oxidase inhibitors. Am Chem Soc Symp Ser 559: 120-132

Matsuzawa H, Tokugawa K, Hamaoki M, Mizoguchi M, Taguchi M, Terada M, Kwan ST, Ohta T (1988) Purification and characterization of aqualysin I (a thermophilic alkaline serine protease produced by *Thermus aquaticuse* YY-1). Eur J Biochem 171: 441-447

Mehlhorn H, Cottam DA, Lucas PW, Wellburn AR (1987) Induction of ascorbate peroxidase and glutathione reductase activities by interactions of mixtures of air pollutions. Free Radical Research Commun 3: 193-197

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of protein. J Biol Chem 250: 4007-4021

Ohkawa H, Dhishi N, Yagi K (1979) Assay for lipid peroxidase in animal tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358

Orr GL, Hess FD (1982) Mechanism of action of the diphenyl ether herbicide acifluorfen-methyl in excised cucumber (*Cucumis sativus* L.) cotyledons. Plant Physiol 69: 502-507

Rivett AJ (1985) Preferential degradation of the oxidatively modified form of glutamine synthetase by intracellular mammalian protease. J Biol Chem 260:300-305

Smirnoff N (1993) Transley review No. 52. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytol 125: 27-58

Wessel D, Flügge UI (1984) A method for quantitative recovery of protein in dilute solution in the presence of detergent and lipids. Anal Biochem 138: 141-143