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Research Article

Iron-mediated inhibition of H^+-ATPase in plasma membrane vesicles isolated from wheat roots

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Abstract. The mechanisms of iron-mediated inhibition of the H^+-ATPase activity of plasma membrane (PM) vesicles isolated from wheat roots were investigated. Both $FeSO_4$ and $FeCl_3$ significantly inhibited PM H^+-ATPase activity, and the inhibition could be reversed by the addition of the metal ion chelator EDTA-Na_2 or a specific Fe^{2+} chelator, indicating that the inhibitory effect was due to specific action of Fe^{2+} or Fe^{3+}. Measurement of the extent of lipid peroxidation showed that oxidative damage on the PM caused by Fe^{2+} or Fe^{3+} seemed to be correlated with the inhibition of PM H^+-ATPase activity. However, prevention of lipid peroxidation with butylated hydroxytoluene did not affect iron-mediated inhibition in the PM H^+-ATPase, suggesting that the inhibition of the PM H^+-ATPase was not a consequence of lipid peroxidation caused by iron. Investigation of the effects of various reactive oxygen species scavengers on the iron-mediated inhibition of H^+-ATPase activity indicated that hydroxyl radicals (•OH) and hydrogen peroxide (H_2O_2) might be involved in the Fe^{2+}-mediated decrease in PM H^+-ATPase activity. Moreover, iron caused a decrease in plasma protein thiol (P-SH), and Fe^{3+} brought a higher degree of oxidation in thiol groups than Fe^{2+} at the same concentration. Modification of the thiol redox state in the PM suggested that reducing thiol groups were essential to maintain PM H^+-ATPase activity. Incubation of the specific thiol modification reagent 5,5-dithio-bis(2-nitrobenzoic acid) with the rightside-out and inside-out PM revealed that thiol oxidation occurred at the apoplast side of the PM. Western blotting analysis revealed a decrease in H^+-ATPase content caused by iron. Taken together, these results suggested that thiol oxidation might account for the inhibition of PM H^+-ATPase caused by iron, and that •OH and H_2O_2 were also involved in Fe^{2+}-mediated inhibition.

Key words. H^+-ATPase; plasma membrane; iron; lipid peroxidation; P-SH oxidation; wheat.

The plasma membrane (PM) H^+-ATPase is a proton pump, which can pump protons from the cytoplasm to the apoplasm and generate an electrochemical potential difference across the membrane [1]. This electrochemical gradient provides the driving force for the transport of various minerals against a concentration gradient across the PM into the root cell from the soil [2]. In addition, this enzyme is involved in cell elongation, stomatal opening, and the response of plants to environmental factors, such as cold, drought, and salt [3–7]. The PM H^+-ATPase is encoded by a multigene family, and at least ten isoforms of the H^+-ATPase have been identified in plants [8]. The expression of the PM H^+-ATPase seems to be dependent on the plant species, developmental stage, and environmental stimuli [5–9].

Iron is the most abundant transition metal in the Earth's crust and is an essential element in the prosthetic groups of many constituents of photosynthetic electron transport

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carriers and enzyme factors [10]. Iron is a micronutrient that is very limiting to agricultural production worldwide [11]. Much research has been undertaken to identify mineral ion transporters and enzymes involved in nutrient assimilation [12–14]. Although iron is essential for plants, excess iron severely restricts cellular growth and results in a variety of morphological symptoms, among which the best characterized is leaf bronzing in rice [15–17]. Iron can act catalytically to generate radicals, which are potent oxidants of several organic molecules [17]. The root PM is the site of absorption of various minerals [8, 18]. The PM H^+-ATPase is an abundant PM protein accounting for approximately 5% of root PM protein [19]. Modulation of its activity is crucial for the survival of plants when they are under a variety of environment stresses [1]. A comprehensive study of the effect on PM H^+-ATPase activity caused by iron at the molecular and plant cell level would be of great help in understanding the processes controling plant growth and survival in a hostile environment. Therefore, we examined the effect of iron on the activity of PM H^+-ATPase and investigated the regulatory mechanism of H^+-ATPase activity in PM vesicles from wheat roots in response to iron.

Materials and methods

Plant material

Wheat [*Tritium aestivum* Longchun 20 (L-Ch20)] seeds (purchased from Gansu Agricultural Academy, China) were surface-sterilized with 0.1% $HgCl_2$ for 20 min, then soaked in water for 24 h and germinated in darkness for 24 h at 24 °C. The germinated seeds were planted in pots containing quartz sand, and irrigated with water. When wheat seedlings had been grown for 7–8 days at 24 °C with a light cycle of 14 h at a light intensity of 150 μmol m^{-2} s^{-1}, the roots were collected for the preparation of PM.

Purification of PM

The PM-enriched vesicles were prepared as described by Kasamo [20] with some modifications. All steps were carried out at 4 °C. Roots were cut into pieces and immediately homogenized in isolation medium (1:2 w/v) containing 250 mM sucrose, 25 mM Hepes-Tris, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), 1.5% polyvinylpyrrolidone (PVP), 1 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 g for 30 min. The supernatant was then centrifuged for 30 min at 80,000 g, crude membrane microsomes were collected and resuspended in a buffer containing 250 mM sucrose, 1 mM dithiothreitol (DTT), 1 mM EDTA, 2 mM Hepes-Tris, pH 7.2, then layered on top of a gradient consisting of 22/30/34/42% (w/w) sucrose and centrifuged at 100,000 g for 80 min. The PM-enriched membrane fraction was collected from the 34/42% sucrose interface, diluted and centrifuged, and the pellet collected.

The PM vesicles were isolated by a two-phase aqueous polymer preparation system [21] with some modifications. The crude microsomes were resuspended in 0.049% phosphate buffer pH 7.8. The PM fraction was isolated by adding the microsomal suspension to a two-phase partition system consisting of 6.3% PEG3350, 6.3% DextranT-500, 8.7% sucrose, 0.014% KCl, 0.049% phosphate buffer, pH 7.8. Three successive rounds of partitioning yielded the final upper phase. This was diluted and centrifuged at 100,000 g for 30 min, and the pellet was collected. Protein concentration was determined by the method of Bradford [22] using BSA as the standard.

PM purity was estimated by assay of the H^+-ATPase activity, which is one of the most commonly used markers for the PM. The PM H^+-ATPase belongs to the P-type characterized by vanadate inhibition, the H^+-ATPases in tonoplast and mitochondria belong to the V-type and F-type identified by nitrate and azide inhibition, respectively [23, 24]. So the degree of contamination by other membranes was characterized using various inhibitors. The H^+-ATPase activity in membrane fractions collected from the 34/42% sucrose interface was inhibited by vanadate by about 70%, and reduced by nitrate and azide by less than 2%, demonstrating minor contaminations of the PM preparations from the 34/42% sucrose interface by other membrane fractions. Similarly, Na_3VO_4, KNO_3 and NaN_3 caused about 75%, 1.3%, and 2.6% inhibition, respectively, in the H^+-ATPase activity of the upper-phase membrane fractions from the two-phase partition method. For the measurement of latent relative to total H^+-ATPase activities, 0.015% Triton X-100 was added to the reaction medium. The latent activity of the PM H^+-ATPase was about 89%. These results suggested an enrichment of sealed and oriented rightside-out PM vesicles in the upper phase.

Assay of H^+-ATPase

ATP hydrolysis assays were performed as described by Qui and Su [25]. Membrane proteins (10–15 μg) were added to 0.5 ml reaction medium containing 25 mM Hepes-Tris (pH 6.5), 3 mM ATP, 50 mM KCl, 1 mM Na_3MoO_4, 0.015% (w/v) Triton X-100, in the presence or absence of 400 μM Na_3VO_4. After 30 min incubation at 37 °C, the reaction was quenched by the addition of 10% (w/v) TCA. H^+-ATPase activity was determined by measuring the release of Pi [26].

Assay of lipid peroxidation

Lipid peroxidation was measured according to the method of Buege and Aust [27]. Briefly, 100 μl of thiobarbituric acid (TBA) solution (8 mg/ml in glacial acetic acid/H_2O 1:1) was added to 20 μl of the sample. This

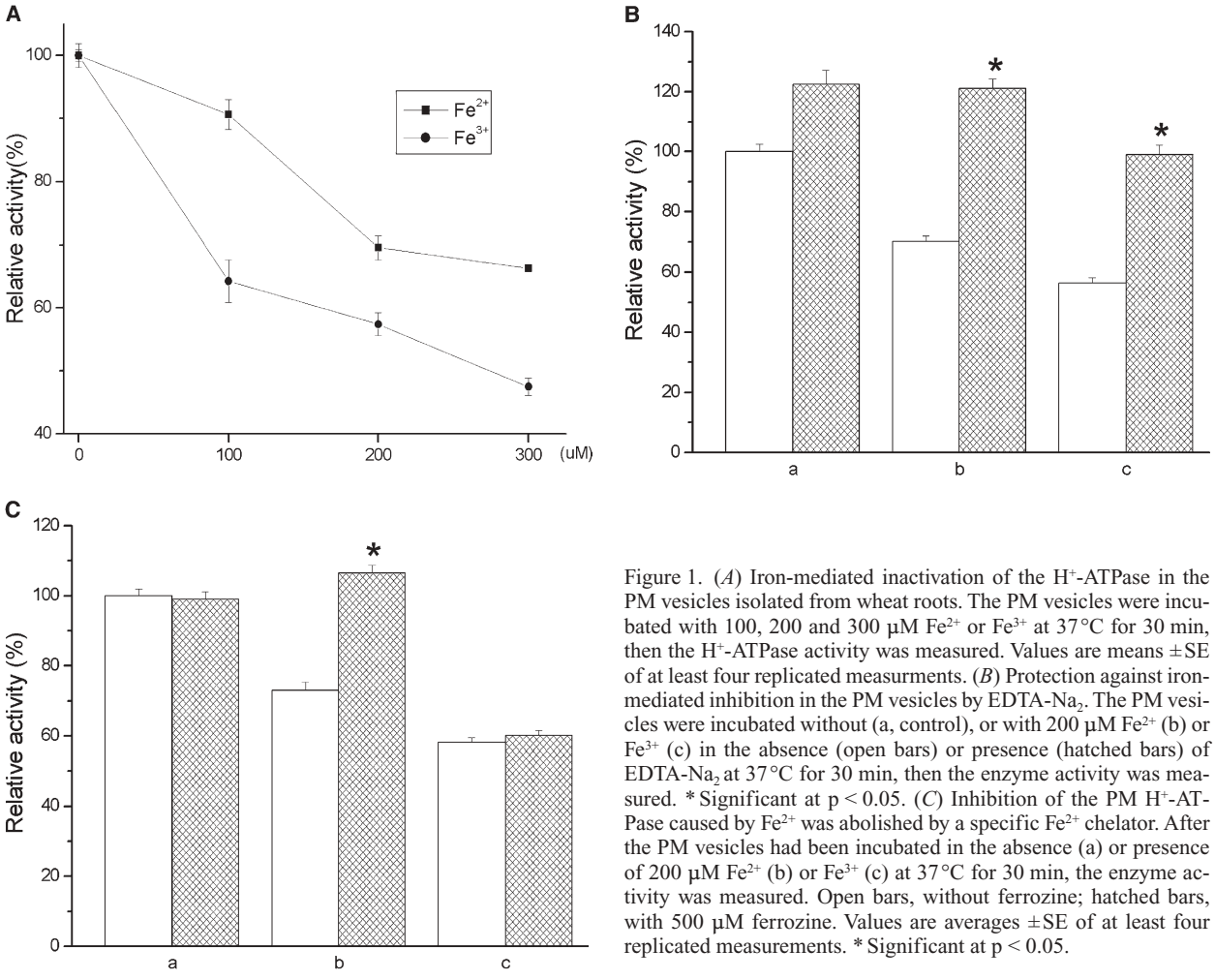

Figure 1. (*A*) Iron-mediated inactivation of the H^+-ATPase in the PM vesicles isolated from wheat roots. The PM vesicles were incubated with 100, 200 and 300 µM Fe^{2+} or Fe^{3+} at 37 °C for 30 min, then the H^+-ATPase activity was measured. Values are means ± SE of at least four replicated measurments. (*B*) Protection against iron-mediated inhibition in the PM vesicles by EDTA-Na_2. The PM vesicles were incubated without (a, control), or with 200 µM Fe^{2+} (b) or Fe^{3+} (c) in the absence (open bars) or presence (hatched bars) of EDTA-Na_2 at 37 °C for 30 min, then the enzyme activity was measured. * Significant at $p < 0.05$. (*C*) Inhibition of the PM H^+-ATPase caused by Fe^{2+} was abolished by a specific Fe^{2+} chelator. After the PM vesicles had been incubated in the absence (a) or presence of 200 µM Fe^{2+} (b) or Fe^{3+} (c) at 37 °C for 30 min, the enzyme activity was measured. Open bars, without ferrozine; hatched bars, with 500 µM ferrozine. Values are averages ± SE of at least four replicated measurements. * Significant at $p < 0.05$.

mixture was heated at 98 °C for 30 min in a dry bath before centrifugation. The absorbance of the supernatant was measured at 532 nm.

Assay of plasma P-SH groups

The amount of plasma P-SH groups was determined according to the method of Jocelyn [28], and expressed as $OD_{412\ nm}$.

Western blotting analysis

SDS-PAGE was performed as described by Laemmli [29]. Membrane proteins (40 µg) were solubilized and separated on a 7.5% acrylamide gel. After electrophoresis, the gel was electrotransferred to a nitrocellulose membrane. The membrane was blocked for 60 min with 5% (w/v) non-fat milk in 0.05% Tween 20, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0. A polyclonal antibody against PM H^+-ATPase was added and incubated overnight, then the secondary antibody coupled to alkaline phosphatase was added for another 1.5 h incubation. The color was developed with a solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP).

Results

Iron mediated inactivation of the PM H^+-ATPase

Figure 1 A shows that iron mediated a remarkable inhibition of H^+-ATPase activity in the PM vesicles isolated from wheat roots. $FeSO_4$ or $FeCl_3$ caused a progressive loss of the H^+-ATPase activity in a concentration-dependent manner. In addition, $FeCl_3$ mediated stronger inhibition of the H^+-ATPase activity than did $FeSO_4$ at the same concentration. However, the inhibitory effects of $FeSO_4$ and $FeCl_3$ could be relieved when the PM vesicles with $FeSO_4$ and $FeCl_3$ were incubated in the presence of EDTA-Na_2, a metal ion chelator (fig. 1 B), indicating that the inhibitory effects of $FeSO_4$ and $FeCl_3$ were due to the action of Fe ions. Because Fe^{2+} can be oxidized to Fe^{3+} when the assay buffer contains oxygen, we next examined whether this transition occurred in our experiments.

When we incubated the PM vesicles with ferrozine, a highly specific chelator for Fe^{2+}, Fe^{2+}-mediated inhibition of the PM H^+-ATPase was completely reversed. However, Fe^{3+}-mediated inhibition was not relieved (fig. 1C). This result indicated that both Fe^{2+} and Fe^{3+} mediated the inhibition of the PM H^+-ATPase and this inhibition was due to the specific action of either Fe^{2+} or Fe^{3+}.

•OH and H_2O_2 might account for Fe^{2+}-mediated inhibition of the PM H^+-ATPase

Iron is a well-known catalyst of the generation of reactive oxygen species (ROS). ROS are known to damage various proteins by inducing oxidative modification, non-enzymatic fragmentation, and aggregation, or indirectly by peroxidation of membrane phospholipids [30, 31]. Therefore, we examined the possible involvement of ROS in the iron-mediated inhibition of H^+-ATPase activity. Figure 2A shows that mannitol (a very effective scavenger of •OH [18]) could relieve Fe^{2+}-mediated inhibition of the PM H^+-ATPase activity, although it did not show any effect on Fe^{3+}-mediated inhibition (fig. 2B). The enzyme activity was decreased to 70% by 200 µM $FeSO_4$, and increased to 113.36% by mannitol. Catalase (CAT), which converts H_2O_2 into water and molecular oxygen [32], also prevented the PM H^+-ATPase inhibition by Fe^{2+}, but it did not abolish Fe^{3+}-mediated inhibition of the H^+-ATPase. When we incubated the PM vesicles with an O_2^- scavenger superoxide dismutase (SOD) in the presence of either Fe^{2+} or Fe^{3+}, the inhibitory effect of iron on H^+-ATPase activity was not reduced. These results indicated that •OH and H_2O_2 might be involved in Fe^{2+}-mediated inhibition of the PM H^+-ATPase.

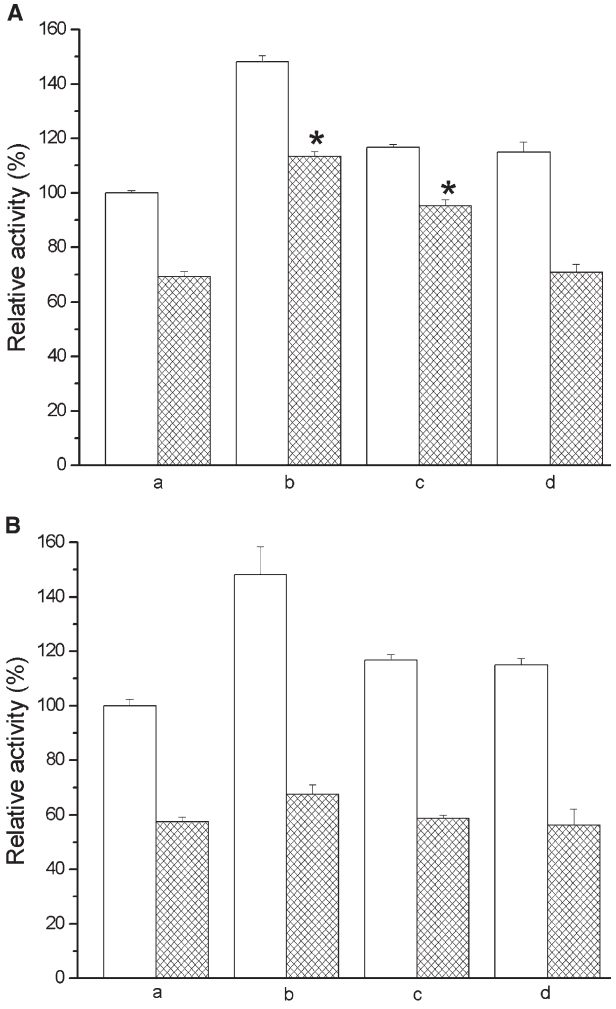

Figure 2. Involvement of ROS in iron-mediated inhibition of the PM H^+-ATPase. (*A*) The PM vesicles were incubated at 37°C for 30 min in the absence (open bars) or presence (hatched bars) of 200 µM Fe^{2+}, and the enzyme activity was measured without scavenger (a; control) with 200 µM mannitol (b), with 10 U catalase (c) or with 50 U SOD. Data represent means ±SE of at least three different experiments. * Significant at $P < 0.05$. (*B*) The PM vesicles were incubated without (open bars) or with (hatched bars) 200 µM Fe^{3+} at 37°C for 30 min, without scavenger (a; control), with 200 µM mannitol (b), 10 U catalase (c) or 50 U SOD. Data represent means ±SE of at least three different experiments.

Lipid peroxidation may not be involved in inhibition of H^+-ATPase activity caused by iron

A further study was performed to investigate the extent of lipid peroxidation caused by iron in the PM vesicles. After the PM vesicles had been preincubated at 37°C for 30 min in the presence or absence of $FeSO_4$ or $FeCl_3$, EDTA-Na_2 was added to end the reaction, and the extent of lipid peroxidation was determinated by measuring TBA-reactive substances (TBARS) in the PM. Figure 3A shows that either Fe^{2+} or Fe^{3+} resulted in an increase in TBARS formation, and that Fe^{3+} mediated a higher level of lipid peroxidation than did Fe^{2+} at the same concentration. Mannitol could reduce TBARS content caused by Fe^{2+} in the PM, but had no effect on the lipid peroxidation caused by Fe^{3+} (fig. 3A). These results seemed to demonstrate that lipid peroxidation was correlated with the changes PM H^+-ATPase activity caused by iron. Butylated hydroxytoluene (BHT), a blocker of lipid peroxidation chain reactions, was used to study the relationship between lipid peroxidation and PM H^+-ATPase inhibition by iron. Butylated hydrotobene (BHT) addition did not reverse iron-mediated inhibition (fig. 3B). This result suggested that iron-mediated inhibition of the PM H^+-ATPase was not mediated via lipid peroxidation.

Thiol oxidation might be involved in the inhibition of H^+-ATPase activity caused by iron

Because ROS can damage proteins by direct oxidation of cysteine residues, we measured the PM protein thiol (P-SH) content in response to iron. Figure 4 shows that a decrease in plasma P-SH content was observed when the PM vesicles were incubated with iron, and that a higher degree of thiol oxidation in PM proteins was caused by Fe^{3+} than by Fe^{2+}.

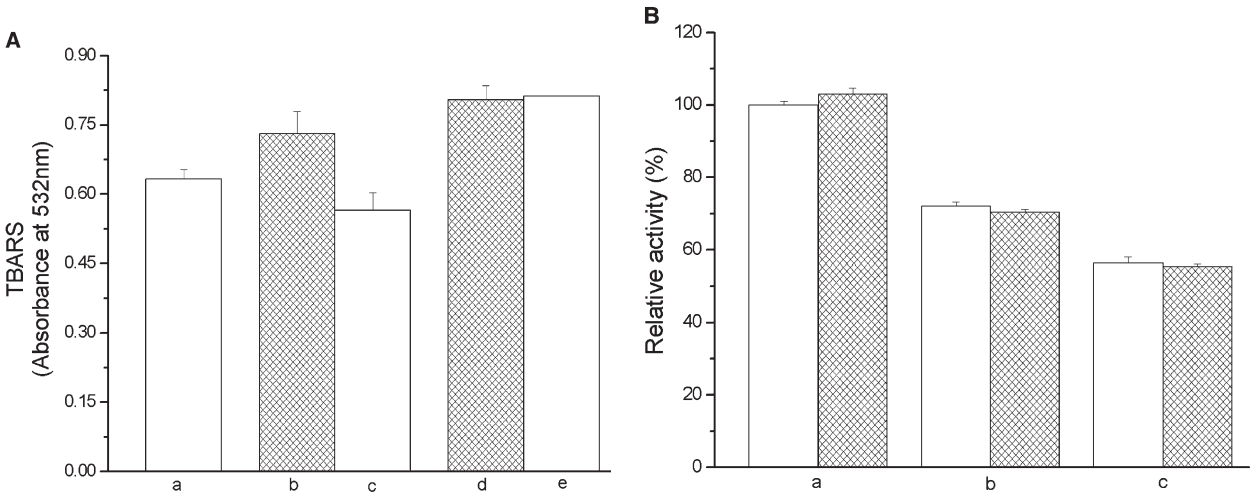

Figure 3. (*A*) Effects of iron on lipid peroxidation in the PM isolated from wheat roots. PM vesicles were preincubated at 37 °C for 30 min without treatment (a), or with 200 µM Fe^{2+} (b, c) or Fe^{3+} (d, e), and then TBARS were measured. a, b, d, without mannitol; c, e, 200 µM mannitol. (*B*) Effect of BHT on H^+-ATPase inhibition caused by iron. After incubation of the PM vesicles without treatment (a), with 200 µM Fe^{2+} (b), or Fe^{3+} (c) at 37 °C for 30 min, the enzyme activity was measured. Open bars, without BHT; hatched bars, 100 µM BHT. Data represent means ± SE of at least four replicated measurements.

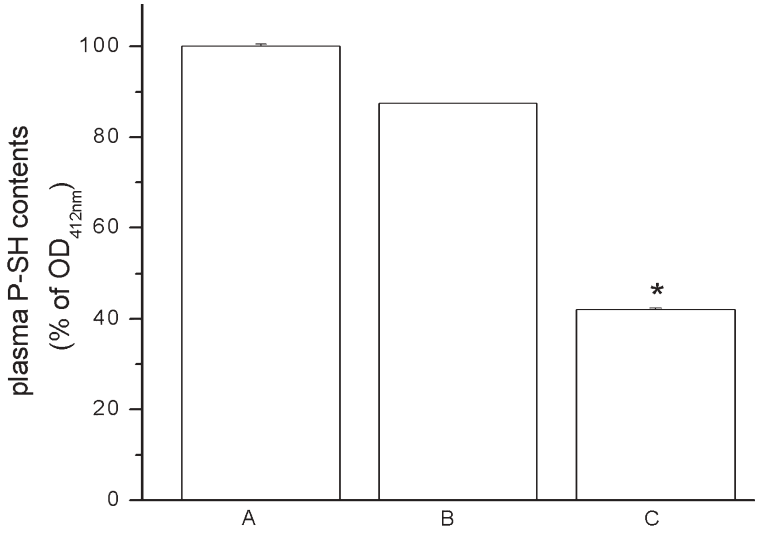

Figure 4. Iron-mediated changes in plasma P-SH contents. After incubation of the PM vesicles treated with 200 µM Fe^{2+} or Fe^{3+} at 37 °C for 30 min, EDTA-Na_2 was added to end the reaction and the amount of -SH groups was measured. A, control; B, Fe^{2+}; C, Fe^{3+}. Values are means ± SE of three replicates. * Significant at $p < 0.05$ to the control.

Thiol groups are considered to be an important factor for the function of some proteins, and thiol oxidation often causes loss of protein function [33]. Thus, we investigated the effects of thiol redox state on PM H^+-ATPase activity. Figure 5 shows that DTT (a thiol group reducing agent) enhanced PM H^+-ATPase activity. The enzyme activity was increased by 164.42% when DTT was added to the PM vesicles. The PM H^+-ATPase activity was inhibited by about 5, 20, and 35% in the presence of, respectively, 0.5, 1, and 2 mM N-etylmaleimide (NEM), a thiol alkylation reagent (fig. 6A). Oxidized glutathione (GSSG), which induces oxidation of thiol groups, resulted in a progressive loss of PM H^+-ATPase activity in a concentration-dependent manner (fig. 6B). Figure 5 shows that PM H^+-ATPase activity was inhibited by Fe^{2+} and Fe^{3+} by about 30% or 40% in the absence of DTT, respectively, and restored to 180 or 220%, respectively, by the addition of 5 mM DTT. This result suggested that DTT could protect against iron-mediated inhibition of the PM H^+-ATPase. To study whether the thiol modification occurred in the apoplasmic or cytoplasmic face of the PM, we obtained rightside-out PM by two-phase partition

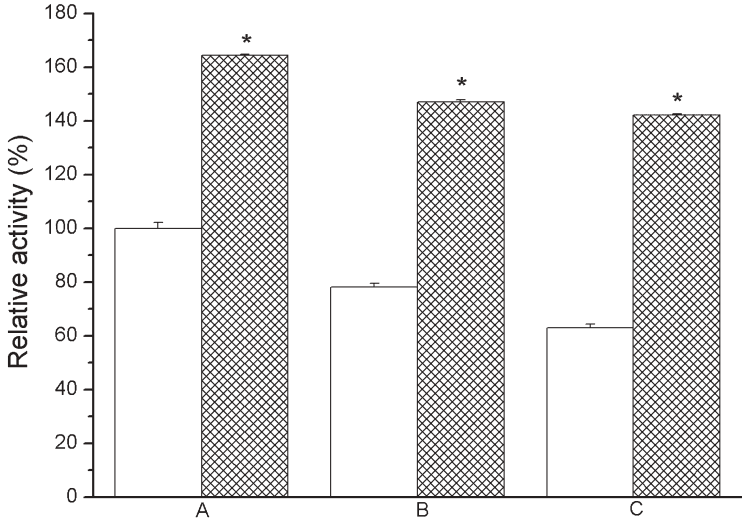

Figure 5. Protection by DTT against iron-mediated inhibition of the PM H+-ATPase. PM vesicles were incubated at 37°C for 30 min in the absence (A) or presence of 200 µM Fe^{2+} (B) or Fe^{3+} (C), and the enzyme activity was measured. Open bars, without DTT, hatched bars, 5 mM DTT. Data represent means ± SE of at least three experiments. * Significant at $p < 0.05$ compared with respective control.

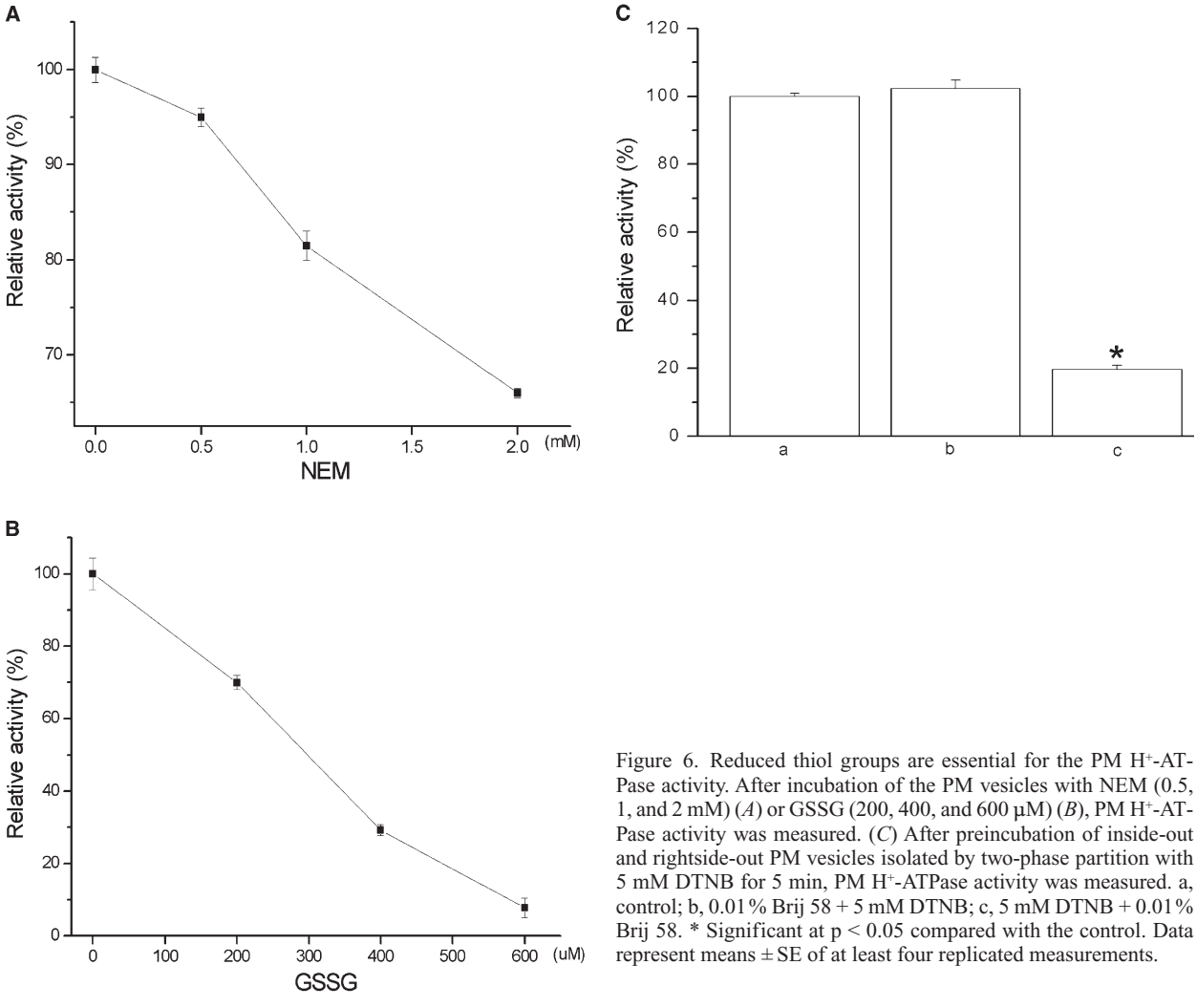

Figure 6. Reduced thiol groups are essential for the PM H+-ATPase activity. After incubation of the PM vesicles with NEM (0.5, 1, and 2 mM) (*A*) or GSSG (200, 400, and 600 µM) (*B*), PM H+-ATPase activity was measured. (*C*) After preincubation of inside-out and rightside-out PM vesicles isolated by two-phase partition with 5 mM DTNB for 5 min, PM H+-ATPase activity was measured. a, control; b, 0.01% Brij 58 + 5 mM DTNB; c, 5 mM DTNB + 0.01% Brij 58. * Significant at $p < 0.05$ compared with the control. Data represent means ± SE of at least four replicated measurements.

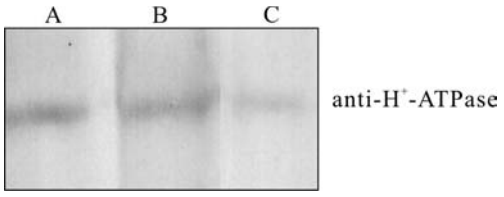

Figure 7. Western blotting analysis. The PM vesicles were treated with 200 μM Fe^{2+} or Fe^{3+} for 30 min, then EDTA-Na_2 was added to end the reaction. Isolated total proteins were separated by SDS-PAGE and the H^+-ATPase contents were immunodetected with the specific antibody against PM H^+-ATPase. Lane A, control; lane B, Fe^{2+}; lane C, Fe^{3+}.

isolation procedures, and inside-out PM by the addition of 0.01% Brij 58. Incubation of the rightside-out PM vesicles with 5,5′-dithio-bis(2-nitro benzoic acid (DTNB)), which alkylates thiol groups and does not penetrate PM, strongly inhibited the PM H^+-ATPase activity. However, incubation of DTNB with inside-out PM did not affect the PM H^+-ATPase (fig. 6C). These results showed that thiol groups are essential for PM H^+-ATPase activity and that the thiol oxidation occurred on the apoplastic face of the PM.

Western blotting analysis

Because ROS will induce non-specific fragmentation or trigger the degradation of protein, we examined the changes in the amount of PM H^+-ATPase under 200 μM Fe^{2+} or Fe^{3+} treatment by Western blotting analysis of PM proteins with an antibody against PM H^+-ATPase (fig. 7). After the PM vesicles were incubated with Fe^{2+} or Fe^{3+}, the amount of the H^+-ATPase was decreased remarkably in the presence of Fe^{3+}. Only slightly decrease of the PM H^+-ATPase was observed in the presence of Fe^{2+}.

Discussion

In the present work, we studied the mechanism of iron-mediated inhibition of H^+-ATPase activity in the PM vesicles isolated from wheat roots. Our results showed that a significant inhibition of H^+-ATPase activity occurred when the PM was incubated with $FeSO_4$ or $FeCl_3$. $FeCl_3$ mediated a higher decrease in PM H^+-ATPase activity than did $FeSO_4$ (fig. 1). The addition of metal ion chelators, such as EDTA-Na_2, to the reaction medium containing iron completely prevented inhibition of PM H^+-ATPase by Fe^{2+} or Fe^{3+}. However, ferrozine (a specific Fe^{2+} chelator) only reversed Fe^{2+}-mediated inhibition of the enzyme, but did not abolish Fe^{3+}-mediated inhibition (fig. 1). These results indicated that the inhibitory effects on PM H^+-ATPase were due to the specific action of Fe^{2+} or Fe^{3+}.

Iron is a well-known catalyst for the formation of ROS [30]. To reveal the involvement of various ROS species in the inhibition of PM H^+-ATPase activity, we incubated various ROS scavengers with the PM in the presence of iron. The effects of mannitol, SOD, and CAT on iron-mediated inhibition of the PM H^+-ATPase (fig. 2), suggested that •OH and H_2O_2 might be involved in Fe^{2+}-mediated inhibition. Because SOD did not show any effect on iron-mediated inhibition of PM H^+-ATPase, $O_2^{\cdot-}$ might not participate in the inhibition of activity. Since these ROS species are short lived [31], they must be generated during the reaction and are not from the PM preparation. A previous report showed that ROS, generated in situ at the site of iron binding to the protein, react with neighboring amino residues [18]. This might be the case in our experimental system. Ferrous ions can activate H_2O_2 to generate hydroxyl radicals (•OH) by the Fenton reaction [34]. This free radical is among the most reactive species generated by the Haber-Weiss reaction [35] and can trigger oxidative stresses and lead to lipid peroxidation.

Taking into consideration that ROS could cause oxidative damage to the PM, we investigated iron-mediated lipid peroxidation by measuring TBARS. Both Fe^{2+} and Fe^{3+} caused significantly oxidative damage to the PM vesicles, and Fe^{3+} caused a higher level of lipid peroxidation than Fe^{2+} (fig. 3). The oxidative damage on the PM caused by Fe^{2+} could be reversed by adding mannitol to the PM vesicles. However, mannitol did not affect the extent of Fe^{3+}-mediated lipid peroxidation. This result implied that •OH might account for the Fe^{2+}-mediated peroxidation of membrane phospholipid. Membrane proteins depend on the membrane structure, and oxidative damage to the membrane might affect the activity of membrane proteins. Lipid peroxidation caused by iron was correlated with iron-mediated inhibition of PM H^+-ATPase activity, suggesting that the inhibition to the enzyme was a consequence of oxidative damage to the PM mediated by iron. However, BHT, which blocks the lipid peroxidation chain reaction, did not reduce iron-mediated inhibition of PM H^+-ATPase activity (fig. 3). This result suggested that inhibition of the PM H^+-ATPase by iron might not be a consequence of lipid peroxidation.

Thiol groups are considered to be an important factor for the function of some proteins, and thiol oxidation often causes loss of protein action [33]. We found that iron caused a significant decrease in the amount of PM P-SH (fig. 4). Reduced thiol groups are essential for H^+-ATPase, such as tonoplast H^+-ATPase [36], activity. Investigating the effect of thiol reagents on PM H^+-ATPase activity, we found that DTT (a thiol-reducing agent) and mannitol, which can protect thiol groups against oxidative damage, remarkably enhanced PM H^+-ATPase activity. Alkylation of thiol groups with NEM resulted in a progressive decrease in PM H^+-ATPase activity with increasing NEM concentrations (fig. 6). GSSG, which might cause thiol oxidation, also mediated a progressive decrease in PM H^+-ATPase in a concentration-dependent

manner (fig. 6). These results demonstrated that reduced thiol groups are essential to maintain PM H+-ATPase activity. Oxidative damage mediated not only lipid peroxidation but also protein oxidation. Our results showed that DTT could abolish the Fe^{2+}- or Fe^{3+}-mediated inhibition of the PM H+-ATPase (fig. 5), implying that oxidation of protein sulfhydryl groups caused by iron might result in the inactivation of PM H+-ATPase activity. Investigation of alkylation of thiol groups with DTNB, an impermeable membrane thiol-modifying reagent, indicated that the protein- reducing thiol groups at the apoplast side of the PM are essential for PM H+-ATPase activity (fig. 6). Oxidative stress may occur when Fe^{2+} or Fe^{3+} reaches the apoplast side of the PM.

Western blotting analysis showed that the PM H+-ATPase content was reduced after the PM vesicles were treated with Fe^{2+} or Fe^{3+}. Thiol oxidation might lead to the formation of inter/intra molecular disulfide bridges, resulting in the formation of high molecular-weight aggregates of the H+-ATPase, and explaining the disappearance of the H+-ATPase band in the Western blot analysis.

In conclusion, our results showed that iron mediated a significant inhibition of PM H+-ATPase activity in a concentration-dependent manner, and that the thiol oxidation caused by Fe^{2+} or Fe^{3+} might be responsible for the decrease in H+-ATPase activity. We also showed that •OH and H_2O_2 might be involved in Fe^{2+}- mediated inhibition of the PM H+-ATPase.

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