Metabolic Response to Treatment with Cold, Paraquat, or 3-Amino-1,2,4-triazole in Leaves of Winter Wheat

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We treated leaves of winter wheat (Triticum aestivum L.) with cold, paraquat, or 3-amino-1,2,4-triazole and compared the responses. We assayed the activities of glucose-6-phosphate dehydrogenase, catalase, dehydroascorbate reductase and ascorbate free radical reductase and levels of hydrogen peroxide, glucose-6-phosphate, fructose-6-phosphate, ascorbate, dehydroascorbate, reduced and oxidized glutathione. With any of the three treatments, contents of cellular peroxides and hexose phosphates were raised. The content of ascorbate was lowered markedly by paraquat treatment, which produces active oxygen species, whereas such a decrease did not occur in other two treatments. When the plants were treated with 3-amino-1,2,4-triazole, which is a specific inhibitor of catalase, the content of oxidized glutathione increased severalfold. The glucose-6-phosphate dehydrogenase activity increased with all three treatments, but it decreased after glyphosate treatment, which does not stimulate the formation of peroxides. The activities of catalase and dehydroascorbate reductase were increased by the treatment of cold and paraquat, while 3-amino-1,2,4-triazole did not affect the dehydroascorbate reductase activity. The activity of ascorbate free radical reductase increased after treatment by paraquat only.

Hydrogen peroxide and related active oxygen species are strong oxidants, and a high concentration of hydrogen peroxide in cells causes oxidative damage to cell components. Halliwell and Foyer1 suggest that GSH and glutathione reductase, together with AsA, constitute a system for the removal of hydrogen peroxide.

When cells are exposed to cold, their enzymatic reactions slow, resulting in lowered demands of metabolites such as ATP.2 The lowered demand for ATP in particular results in saturation or overflow of electrons at certain steps of electron transport, and active oxygen species such as hydrogen peroxide tend to be formed. The enhancement of enzymatic activities and the increase in levels of substrates involved in the removal of hydrogen peroxide may be one of the obligatory adjustments during cold acclimation.3-7 Reactions in the ‘AsA-GSH cycle’8-10 and those involved in ASFRD, catalase and peroxidases play roles of such activities.8,9

Metabolic activities change in cells treated with cold. Fatty acids become unsaturated in alfalfa by cold treatment.10 In poplars, G6PDH activity and G6P content are raised in the wintering stage3 and ASFRD, DHARD and ascorbate peroxidase activities are also raised in winter period.4 Peroxide scavenging systems that require GSH and AsA are induced by the first frost in apple trees5,6 and by cold treatment of winter wheat,7 and Zea mays.11 These increases in enzymatic activities and substrate levels suggest an adaptive response of cells to cold environments, and the metabolic organization has been referred to as a wintering stage of metabolism.3

Recently, we have shown an abrupt increase in the level of hydrogen peroxide by cold treatment in leaves of winter wheat.12 When frozen flower buds of apple trees start to thaw, an abrupt increase in the level of hydrogen peroxide occurred, concurrent with a decrease of GSH and G6P.13 Peroxide has been detected in millimolar concentrations in poplars.14 In frozen twigs of poplar, the level of GSH decreases slowly, followed by a decrease in levels of G6P.15 The oxidation of cellular components under frozen conditions has also been reported.16

The injuries occurring in the frozen state may be caused by active oxygen species such as superoxide anion and hydrogen peroxide. Halliwell et al.1,4,16 Asada et al.,17-19 and Sagisaka et al.4,5,7,15,16 showed that plant cells have a set of systems, such as ‘AsA-GSH cycle,’ ascorbate peroxidase, and catalase to dispose of peroxides to prevent oxidative injury. The increase with cold treatment in the enzymatic activities involved in these scavenging systems of peroxide4-7,15,16 suggests that the response to cold is directly related, in large part, to peroxide, and that changes in the levels of hydrogen peroxide is a signal to the plant cells. Cold-inducible proteins in crowns of winter wheat can also be induced by treatment with hydrogen peroxide (Matsuda et al., unpublished data). These results strongly suggest that active oxygen species play an important role in the genetic expression in response to cold stress.

We examined the effect of cold or oxidative stresses imposed artificially on the enzymatic activities and of substrate levels in leaves of winter wheat. Artificial oxidative stress was brought about by treatment with PQ or AT. PQ produces superoxide radicals in chloroplasts20 and AT is a specific inhibitor of catalase.21,22 For comparison, we used another kind of herbicide, glyphosate, which inhibits the reaction catalyzed by 5-enolpyruvyl-3-phosphate synthase23,24 and does not stimulate hydrogen peroxide formation in cells.

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Abbreviations: AsA, ascorbate; ASFRD, ascorbate free radical reductase; AT, 3-amino-1,2,4-triazole; DHA, dehydroascorbate; DHARD, dehydroascorbate reductase; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; glyphosate, N-(phosphonomethyl) glycine mono-(isopropylammonium) salt; PQ, 1,1'-dimethyl-4,4'-bipyrimidinium dichloride (paraquat).
Materials and Methods

Chemicals. NADP⁺, NADPH, and NADH were products of Oriental Yeast Co., Ltd. (Tokyo, Japan). G6PDH (yeast), phosphoglucomutase (rabbit muscle), phosphoglucoisomerase (yeast), glutathione reductase (yeast), GSH, and GSSG were purchased from Boehringer Mannheim GmbH (Germany). Ascorbate oxidase was a product of Toyo Jozo Co., Ltd. (Shizuoka, Japan). AT, AsA, and PQ (methyl viologen) were from Nakarai Tesque (Kyoto, Japan). DHA and Darco G-60 were purchased from Fluka AG (Buchs, Switzerland) and Atlas Powder Co., respectively. Roundup (a glyphosate herbicide) was obtained commercially. All other chemicals were of analytical grade.

Plant material. Seedlings of winter wheat (Triticum aestivum L. cv. Horoshirikomugi) were grown in moist vermiculite in plastic trays at 28°C during the day and 24°C at night, with the relative humidity of 75%. Illumination was with 10,000 lux for a day of 16 h, and night was 8 h long. Cold, PQ, AT, and glyphosate treatments were carried out 7 d after germination. Primary leaves were used for all assays at 7 to 10 d after germination.

Cold treatments. Cold treatments were done by placing the plants in an adiabatic box at 2°C in the dark.

PQ treatment. A solution containing 50 μM PQ and 0.01% Triton X-100 was sprayed (50 ml/80 seedlings) on the surface of the leaves at room temperature.

AT treatment. A solution containing 30 mM AT and 0.01% Triton X-100 was sprayed (50 ml/80 seedlings) on the surface of the leaves at room temperature.

Glyphosate treatment. Glyphosate herbicide was diluted threefold to 0.8 mM glyphosate and sprayed (50 ml/80 seedlings) on the surface of the leaves at room temperature. After the treatment, the plant material was wilted within 1 d, and the water content decreased steeply. For comparison, the amounts of substrates and the enzymatic activities are expressed per gram of dry weight.

Extraction of substrates. One-half gram of fresh leaves was ground with a mortar and pestle in 2 ml of 5% perchloric acid with 0.6 g of sea sand on ice. Then slurry was centrifuged at 15,000 × g for 5 min. The supernatant was neutralized with solid KHCO₃ (pH 7.5) and centrifuged at 1000 × g for 1 min. The supernatant was used for determination of the substrate concentration.

Determination of substrate content. Hydrogen peroxide, AsA, and DHA were determined as described previously. G6P and F6P were determined enzymatically; 0.5 ml of neutralized extract, 0.5 ml of 0.5 M Tris-HCl (pH 7.7), and 0.76 ml of distilled water were mixed with 25 mg of Darco G-60 and centrifuged at 16,000 × g for 5 min. Then 1.5 ml of the supernatant, 10 μl of 60 mM NADP⁺, and 10 μl of 0.6 M MgCl₂, were mixed, and assays were carried out sequentially by the addition of 1.75 units of G6PDH (for the G6P assay), 2.0 units of phosphoglucomutase (for the G1P assay) and finally 3.5 units of phosphoglucoisomerase (for the F6P assay). The increase in the absorbance at 340 nm was monitored spectrophotometrically (Shimadzu UV-190) at 25°C. The total glutathione content (GSH + GSSG) was assayed by the glutathione reductase-5,5′-dithiobis(2-nitrobenzoic acid) recycling procedure. To determine GSSG, 200 μl portions of supernatant were mixed with 5 μl of 2-vinylpyridine immediately after extraction to mask GSH. The GSSG was calculated from the difference.

For comparison of the amount of GSH and GSSG, the content of GSH is expressed as 2 times the GSH concentration (2GSH).

Extraction of enzymes. Three hundred milligrams of fresh leaves were ground with a mortar and pestle in 2 ml of 50 mM Tris-HCl (pH 7.7) containing 4 μl of 1 M dithiothreitol, 0.1 g of sea sand, and 0.1 g of Polyclay AT on ice. The slurry was centrifuged at 20,000 × g for 5 min. The supernatant was used for measurement of enzymatic activities.

Assay of enzymatic activity. Reaction mixtures for assays of enzymatic activities were as follows. All assays were carried out at 25°C. G6PDH: 50 mM Tris-HCl (pH 7.7), 4 mM MgCl₂, 0.4 mM NADP⁺, 0.7 mM G6P, and 50 μl of the test enzyme solution in a total volume of 1.5 ml. The reaction was started by the addition of G6P and the increase in absorbance at 340 nm was monitored. Catalase: 50 mM potassium phosphate buffer, pH 6.9, 11.6 mM H₂O₂, and 5 μl of the test enzyme solution in a total volume of 1.5 ml. The reaction was started by the addition of the test enzyme and the decrease in absorbance at 240 nm was monitored. An extinction coefficient of 0.0436 mM⁻¹ cm⁻¹ was used.

DHAR: 50 mM potassium phosphate buffer (pH 6.1), 1.7 mM GSH, 1 mM NADPH, 0.6 units of GSR, 3.3 mM DHA, and 20 μl of the test enzyme solution in a total volume of 1.5 ml. The reaction was started by the addition of DHA and the decrease in absorbance at 340 nm was monitored.

ASDR: 50 mM potassium phosphate buffer (pH 7.5), 1 mM NADH, 1 mM AsA, 1 unit of ascorbate oxidase and 20 μl of the test enzyme solution in a total volume of 1.5 ml. The reaction was started by the addition of ascorbate oxidase and the decrease in absorbance at 340 nm was monitored.

Results

Changes in appearance of the plants

Four days after PQ treatment, the whole leaf had browned. Three days after AT treatment, the basal part of leaves of the plant had browned. Within a day of glyphosate treatment, the leaf had wilted. The plants looked unchanged after the cold treatment.

Changes in hydrogen peroxide levels

One day after PQ treatment, the contents of hydrogen peroxide had increased twofold (0.6 μmol per gram fresh weight) compared with the control (Fig. 1). The hydrogen peroxide level increased after AT treatment, as well. The high level of hydrogen peroxide declined within 3 d of treatment. Cold treatment increased the hydrogen peroxide level severalfold within a few minutes, and the level later decreased to slightly above the control level (Fig. 1).

Changes in substrate levels

After treatment with cold, or PQ, the contents of G6P and F6P increased twofold compared with control (e.g., 0.25 and 0.12 μmol per gram fresh weight, respectively, in the cold-treated plants) within 1 d (Figs. 2A and 2B). The same pattern of change in contents of the sugar phosphates was observed with AT treatment (Figs. 2A and 2B). In contrast to the above three treatments, the levels of G6P and F6P were decreased by glyphosate treatment (Figs. 3A and 3B). The ratio of [F6P]/[G6P] was about 0.32 to 0.42 in the control plants, and tended to increase after treatment with cold, PQ, and glyphosate.

![Graph](http://example.com/graph.png)

Fig. 1. Changes with Oxidative Treatment in the Hydrogen Peroxide Level in Leaves of Winter Wheat.

Each treatment started at 0 h when plants were 7 days old. The first leaf was measured. •, control; ▲, cold; ■, PQ; □, AT.
The AsA level decreased to about half of the control level within 2 d of PQ treatment (0.95 μmol per gram fresh weight) (Fig. 2C). The AsA level changed slightly after cold or AT treatment (Fig. 2C). The DHA level decreased steeply within 1 d of PQ or AT treatment (Fig. 2D).

The contents of GSH remained similar levels after cold, PQ, or AT treatment (Fig. 2E), while the GSSG level increased twofold (about 0.1 μmol per gram fresh weight) and 3.6 times (0.18 μmol per gram fresh weight) of the control within 1 d of treatments with PQ and AT, respectively (Fig. 2F). After that, the content of GSSG remained similar levels (PQ treatment) or continued to increase (AT treatment) (Fig. 2F).

**Changes in enzymatic activities**

The G6PDH activity increased after cold, PQ, or AT treatment (0.39, 0.74, and 0.50 μmol/min per gram fresh weight, respectively; Fig. 4A), but in leaves treated with glyphosate, this activity decreased apparently (Fig. 5). The activity of NAD⁺ specific glyceraldehyde-3-phosphate dehydrogenase increased after cold or AT treatment, but not after PQ treatment (data not shown).

Of the enzymatic activities studied here, catalase was a highest (1 to 2 mmol/min per gram fresh weight) and this activity increased much after cold or PQ treatment (Fig. 4B). Within 1 d of AT treatment, catalase was completely inhibited (Fig. 4B). DHARD activity increased after cold or PQ treatment, but AT treatment did not change the
activity (Fig. 4C). ASFRD activity increased greatly after PQ treatment (Fig. 4D). ASFRD activity decreased gradually after AT treatment (Fig. 4D). The activities of catalase, DHARD, and ASFRD did not change after glyphosate treatment (data not shown).

The degree of changes in substrate levels and enzymatic activities caused by cold, PQ, and AT treatments is summarized in Table I.

**Table 1. Changes Caused by Cold, PQ, or AT Treatment in the Substrate Levels and Enzymatic Activities**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Treatment</th>
<th>Activity</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>G6P</td>
<td>+ + + +</td>
<td>G6PDH</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>F6P</td>
<td>+ + + +</td>
<td>CATALASE</td>
<td>+ + + + +</td>
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<tr>
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<td>NC</td>
<td>NC</td>
<td>NC</td>
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<td>NC</td>
<td>NC</td>
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<tr>
<td>GSH</td>
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<td>NC</td>
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<tr>
<td>GSSG</td>
<td>NC</td>
<td>+ + + +</td>
<td>+ + + +</td>
</tr>
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++, increased to a level less than twofold of the control; ++, increased to a level more than twofold of the control; +++, increased to a level more than threefold of the control.

-, decreased to a level more than half of the control; --, decreased to a level less than half of the control. NC, not changed.

**Discussion**

Hydrogen peroxide contents in leaves of winter wheat increased by cold, PQ and AT treatment (Fig. 1). To protect cells from oxidative stresses, scavenging systems of peroxides may be induced in the cell.

G6P plays a very important role to keep cells in a reduced state because it produces NADPH by the reaction with G6PDH. The increase in the ratio of [F6P]/[G6P] by cold and PQ treatment suggests that to supply G6P, F6P synthesis proceeds from fructan, a major storage carbohydrate of wheat. The G6P level and G6PDH activity increased under conditions in which activation of the system scavenging hydrogen peroxide was necessary for cell survival.

The increase in the activities of catalase, DHARD, and ASFRD caused by cold and oxidative treatments suggested that these enzymes are induced so that they can scavenge peroxide. Cold-inducible proteins were synthesized about 1 d after cold treatment (Matsuda et al., unpublished data). Thus, cold causes dose- and time-dependent increases in enzymatic activity such as that of G6PDH. Induction of mRNAs in response to cold treatment was seen at 11 h and was complete by 24 h at 0°C (Matsuda et al., unpublished data). The mechanisms of dose- and time-dependent regulation of synthesis of proteins in response to cold and oxidative stresses in higher plant is poorly understood.

PQ decreased the AsA level and increased the GSSG level, possibly because in chloroplasts, superoxide anion oxidizes AsA and yields ascorbate free radical, which is converted back to AsA by ASFRD. Hydrogen peroxide formed from superoxide anions is also eliminated by AsA yielding ascorbate free radical, although the rate of reaction is lower than that of superoxide anions. Some of the hydrogen peroxide in cytosol is presumably eliminated by catalase or by nonenzymatic reactions at the expense of GSH. Some AsA is oxidized by the above reactions to DHA, which is reduced to AsA by DHARD, causing an increased GSSG level.

Stems of winter wheat contain low levels of glutathione peroxidase (0.06 μmol/min per gram fresh weight), and the rate of the nonenzymatic reaction at pH 7.0 with 1.5 mM GSH with DHA is fast. Therefore, when catalase in the cytosol is inhibited by AT, it seems likely that most of hydrogen peroxide in the cytosol would be eliminated nonenzymatically by AsA and GSH, yielding DHA and GSSG. The DHA formed is reduced by GSH, resulting in the accumulation of GSSG. That under the conditions in which consumption of GSH and the resultant accumulation of GSSG are taking place, the level of GSH does not change during PQ or AT treatment suggests that some GSH may be newly synthesized to scavenge hydrogen peroxide that was accumulated in the cytosol. This means that there are some regulatory mechanisms to maintain the concentration of such reducing materials in the cell. In control plants, the activity of glutathione reductase was about 0.2 μmol/min per gram fresh weight, and this activity was not changed by cold, PQ, or AT treatment (data not shown).

The lack of change in glutathione reductase activity, together with results mentioned above, also suggest that AsA and GSH, enzymatically and nonenzymatically, seem to be responsible for the scavenging of hydrogen peroxide as shown earlier by Halliwell et al.1,8,9

An abrupt increase in hydrogen peroxide occurred not only leaves but stems of winter wheat by cold treatment, and when stems of the plant was treated with KCN (0.1 mM) at 28°C, the hydrogen peroxide level also increased (Okuda et al., unpublished data). These results suggested that much of the hydrogen peroxide is generated from mitochondria, which seem likely to be formed by function to dispose of a large amount of hydrogen peroxide.

Our results show that when cells are treated with PQ, the scavenging system uses mainly AsA followed by GSH. In the case of AT, the system uses AsA and GSH, and GSH plays a major source of reductant to scavenge hydrogen peroxide. The scavenging systems that prevent oxidative damage may be different in the chloroplasts and cytosol or mitochondria.

**References**

Metabolic Response to Oxidative Stresses