Promotion of Seedling Growth of Seeds of Rice (*Oryza sativa* L. cv. Hitomebore) by Treatment with H$_2$O$_2$ before Sowing

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Abstract: High germinability of seeds and establishment of young seedlings in rice (*Oryza sativa* L.) are necessary for direct seeding in paddy fields. We investigated whether germinability and seedling growth were promoted by treatment of rice seeds (cv. Hitomebore) with hydrogen peroxide solution (H$_2$O$_2$) during the imbibition for 24 h. H$_2$O$_2$ treatment with 50 mM H$_2$O$_2$ promoted seed germination, and seedling growth (shoot length, root length and shoot fresh weight) in agar culture under a low temperature condition (18 °C day/14°C night). Seedling growth was promoted by H$_2$O$_2$ treatment not only under the low-temperature condition but also under a normal (23°C day/18 °C night) temperature condition. Furthermore, H$_2$O$_2$ treatment promoted seedling growth under a flooding condition in a greenhouse. These results suggest that H$_2$O$_2$ treatment of rice seeds during the imbibition is advantageous for direct seeding. We discussed the relation between the promotion of the seed germinability and the seedling growth under a low-temperature condition, and the expression of some genes encoding ROS scavenger enzymes induced by H$_2$O$_2$ treatment.

Key words: Ascorbate peroxidase, Growth of young seedling, Hydrogen peroxide treatment, Reactive oxygen species, Rice cultivar.

Recently, some farmers are changing the cultivation system for lowland rice from transplanting to direct seeding in Japan, because the direct seeding saves cost and labor time for rice production. However, low germinability and poor seedling establishment under flooding and low-temperature conditions hinder the adoption of direct seeding, especially in northern Japan (Dakeishi and Fukuoka, 1990). The rapid growth of young seedlings after germination is important for the better establishment of seedlings (Ogiwara and Terashima, 2001). Therefore, it is necessary to develop the cultivation methods that promote the germinability and subsequent growth of seedlings after germination even under flooding and low-temperature conditions.

It is conceivable that the reduction of seedling growth at a low temperature is caused by reactive oxygen species (ROS), such as superoxide radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH.) (Wise and Naylor, 1987; Hodgson et al., 1991; Okuda et al., 1991). Many physiological studies showed that the activities of the enzymes that scavenge ROS in plant cells, such as super oxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathion S-transferase (GST) (Mittler et al., 2002), are related to low-temperature tolerance (Prasad et al., 1994; Saruyama and Tanida, 1995; Tanida, 1996; Sato et al., 2001). Saruyama and Tanida (1995) reported that CAT and APX activities increase in correlation with the low-temperature tolerance of rice seedlings at germination and during subsequent growth. Moreover, overexpression of the genes encoding the scavenger enzymes enhanced the low-temperature tolerance of rice seedlings (Matsumura et al., 2002; Takesawa et al., 2002). The growth of rice seedlings at low temperatures could be promoted by activating the scavenger enzymes.

H$_2$O$_2$ treatment of seeds for breaking the seed dormancy has been well studied (Fontaine et al., 1994; Chien and Lin, 1994; Naredo et al., 1998; Narimanov, 2000; Ogawa and Iwabuchi, 2001). These studies focused on breaking seed dormancy by H$_2$O$_2$ treatment and did not discuss the effect of H$_2$O$_2$ on the subsequent growth of young seedlings except for Narimanov (2000) who observed the promotion of seedling growth by H$_2$O$_2$ treatment of seeds in barley, maize, haricot, vegetable marrow, melon, radish and carrot. However, there are few studies on the promotion of growth of the rice seedlings under flooding and low-temperature conditions by treatment of the rice seeds with H$_2$O$_2$ before sowing.
In this study, we aimed to investigate the effect of H2O2 treatment of seeds before sowing on the early growth of rice seedlings under low-temperature conditions. We also discussed the relationship between the effect of H2O2 treatment of rice seeds and expression of some genes encoding ROS scavenger enzymes.

Materials and Methods

1. Plant material and growth

Non-dormant rice seeds (Oryza sativa L. cv. Hitomebore) selected by specific gravity (>1.13) were used in this study. Lowland rice cultivar "Hitomebore" is one of leading cultivars in the northern region of Japan. Rice seeds were surface-sterilized with 70 % ethanol for 30 s, and then imbibed in sterilized water (control) or solutions of H2O2 at concentrations of 5, 10, 50, 100, 500 or 1,000 mM (treatment) at 29 °C for 24 hr. We examined the effect of H2O2 treatment on the seed germination and seedling growth on agar medium in an incubator and on soil culture in a greenhouse. In the agar culture, twenty seeds were sown on a 0.5 % agar-bed (bed volume was about 100 ml) in each polycarbonate pot (Agripot, Kirin Brewery Co.) with three replicates. A quarter-strength of Murashige and Skoog basic salts (Murashige and Skoog, 1962) was added to the agar bed. The seeds were embedded 5 mm deep from the surface of agar-bed. After sowing, distilled water was added to cover the surface of agar-bed. The pots were placed in an incubator controlled at a normal temperature (23/18 °C; day/night) for 10 d or at a low temperature (18/14 °C; day/night) for 15 d. We chose ten seedlings randomly from each pot and measured shoot length, root length, and shoot fresh weight of the seedlings.

Under the low-temperature condition, the germinated seeds, which had a radicle or plumule at least 2 mm in length, were counted every 8 h in each pot and then the mean percentages of germination determined. Based on mean values of three replications, we evaluated the time from the sowing to 50% germination using Richards function (Hara, 1999). We set the incubator for the low-temperature condition at 18/14° C because many studies showed that the physiologically critical temperature for early seedling growth of rice is around 17°C (Ogiwara and Terashima, 2001).

2. Reverse transcription (RT) - PCR analysis

The RNA sample (total RNA) was prepared by the hot phenol/SDS method (Shirzadegan et al., 1991) from whole seeds. Rice seeds on agar beds were sampled after a 24-h treatment with H2O2 or distilled water (control) at 29°C, and one and three days after sowing under low-temperature and flooded conditions. First-strand cDNA was synthesized from 10 µg of.

Table 1. Sequence of primers for ROS scavengers (APX, CAT and GST) and genes for enzymes related to cellular oxygen level (AOX and ADH) used for RT-PCR.

| gene name | Accession No | Primer sequence (5'-3') | Reference |
|-----------|--------------|-------------------------|-----------|
| APXa      | D45423       | ACCCGGACGGCATGGTAAGAACATAC | Sato et al. 2001 |
| (ascorbate peroxidase) | | ACTAGAATACTCTATACGAATCCGG | |
| CATb      | D64013       | GGTGGTGCTATGTTGCCGACTTCAG | Iwamoto et al. 2000 |
| (catalase) | | AGTTCACAGATATAGCATGCCGACTTCAG | |
| CATc      | D86611       | CGATCAGGCTGCAATGCTTCAG  | Iwamoto et al. 2000 |
| (catalase) | | GGTACAGATTACATGATGGTG | |
| GST       | AF402792     | ATGTTCCAGACATGTTACATTCAC | Takesawa et al. 2002 |
| (glutathion S-transferase) | | AGCAAGGGCGGAGATGATGCTAC | |
| AOX1a     | AB007452     | CACCTGTAGACCTCTAGTAGGAG | Abe et al. 1997 |
| (alternative oxidase) | | TGATGCCAAATGCCGACGTA | |
| ADH1      | X16296       | TTGTTGCACTGAAATTCTGGAACC | |
| (alcohol dehydrogenase) | | CCAACACCATAACCTGAAAA | |
total RNA using the First-Strand cDNA Synthesis kit (Amersham Bioscience, Tokyo, Japan), and was used as a template for PCR amplification with specific primers for APXα, CATβ, CATε, GST, AOX1α, and ADH1 shown in Table 1. The tubulin-specific primers were used to amplify the β-tubulin gene as a positive control of constitutive expression.

3. Northern blot analysis
Ten µg each of total RNA was subjected to Northern blot analysis. A part of the APX gene was amplified using primer APXα and labeled with digoxigenin using a PCR DIG probe synthesis kit. Hybridization, washing and detection were performed according to the manufacturer’s instructions for the DIG DNA labeling and detection kit (Roche Diagnostics, Quebec, Canada).

Results and Discussion
1. Effects of H₂O₂ treatment of rice seeds on seedling growth
Imbibition of seeds in 5, 50 and 100 mM H₂O₂ solution for 24 h significantly increased the shoot fresh weight of the seedling, which was 31.3 ± 1.0 (S.E.), 32.9 ± 0.8 and 33.3 ± 0.8 mg, respectively, under a low-temperature condition as compared with the control (imbibed in distilled water) in which the shoot fresh weight were 25.6 ± 0.7 mg (Fig. 1). On the contrary, treatment with a higher concentration, 500 and 1,000 mM, of H₂O₂ decreased the shoot fresh weight, which was 14.7 ± 2.1 and 2.6 ± 1.0 mg, respectively (Fig. 1). These results showed that the treatment of seeds with H₂O₂ at 5 to 100 mM before sowing promoted the seedling growth. Thus, we chose the treatment with 50 mM H₂O₂ in the following experiments.

The time from the sowing to 50% germination was 57.1 h in the seeds treated with 50 mM H₂O₂, while that was 85.9 h in the seeds treated with distilled water (Fig. 2). In agar culture in a growth chamber, shoot length, root length and shoot fresh weight of the seedlings were increased by the treatment of seeds with 50 mM H₂O₂ before sowing not only under the low but also under the normal temperature conditions (Table 2). The relative shoot length (RSL), root length (RRL) and shoot fresh weight (RFW) (relative to the control without H₂O₂ treatment) under the low-temperature condition was 110, 240 and 120 %, respectively (Table 2). RSL, RRL and RFW under the normal temperature condition were 148, 342 and 207 %, respectively (Table 2). A similar result was observed in soil culture in a greenhouse: RSL and RFW under the low-temperature condition for 7 d were 138 and 137 %, respectively (Table 3). RSL and RFW under the low-temperature condition for 14 d were 148 and 136 %, respectively (Table 3). In addition, we examined the rate of seedling growth that was calculated by subtracting shoot length or shoot fresh weight of seedlings grown for 7 days from that of seedlings grown for 14 days. The growth rate of seedlings from the seeds treated with H₂O₂ was 13.3 mm in shoot length while that of control seedlings was 8.5 mm. In fresh weight, the growth rate of seedlings from the seeds treated with H₂O₂ was 8.0 mg while that of control seedlings was 5.9 mg. These results showed that H₂O₂ treatment enhanced germination and seedling growth after germination not only under the normal temperature condition but also under the low-temperature condition.

H₂O₂ treatment was generally used for breaking seed dormancy of rice (Naredo et al., 1998). In this study, we showed that H₂O₂ treatment of seeds affected the germination rate and the subsequent growth after
germination under the low-temperature condition. H₂O₂ treatment, which increases shoot length, root length and shoot fresh weight may be useful for direct seeding because rapid growth of seedling is important for the better establishment of seedlings in direct seeding (Ogiwara and Terashima, 2001). It is notable that H₂O₂ treatment increased the root length over two times in both low- and normal temperature conditions. However, Inoue et al. (1997) reported that there were no relationships between root length and seedling establishment in the experiment using seven varieties (four Japanese, one Portuguese, one Chinese, and one Hungarian) in a flooded paddy field. Thus it must be examined whether this treatment is particularly useful in paddy fields under a low-temperature condition in further studies.

2. Effects of H₂O₂ treatment on the expression of genes for ROS scavenger enzymes (APX, CAT and GST) and for enzymes related to the cellular oxygen level (AOX and ADH)

We showed that H₂O₂ treatment increased the germination rate and seedling growth under a low-temperature condition (Fig. 2). Fontaine et al. (1994) suggested that seed germination in barley was increased by H₂O₂ treatment due to O₂ generation from H₂O₂ because, in barley, H₂O₂ scavenging activity was high and O₂ was essential for mitochondrial respiration during germination. To investigate the effect of oxygen that may be generated by H₂O₂ pretreatment, we examined the expression levels of two genes, alternative oxidase (AOX1a) and alcohol dehydrogenase (ADH), which indicate the cellular oxygen level. Under aerobic conditions, the transcript level of AOX1a increases while that of ADH decreases (Tuji et al., 2000). In the present experiment, the transcript level of AOX1a at 24 hr after H₂O₂ treatment slightly higher than that in the control, but the transcripts of ADH were observed in H₂O₂ treatment as well as in the control (Fig. 3A). These results show that rice seeds were still under anaerobic conditions. We conclude that O₂ generated from H₂O₂ cannot increase the germinability and seedling growth. Therefore, we infer the existence of other mechanisms for the promotion of the germinability and seedling growth by H₂O₂ treatment.

We examined the expression of genes for ROS scavengers by RT-PCR to analyze the mechanism of their induction by H₂O₂ treatment. The transcripts of APXa and CATc were increased by H₂O₂ treatment of seeds, but those of CATb and GST were not (Fig. 3A). Northern blot analysis ensured that expression of APXa was strongly induced from 0 to 3 d after H₂O₂ treatment (Fig. 3B). These results clearly showed that H₂O₂ treatment of seed could induce the expression of APXa after imbibition.

The expression level of rice cytosolic APX was significantly increased by oxidative stress, when the

| Table 2. Effects of treatment of rice seeds with H₂O₂ before sowing on shoot length, root length and shoot fresh weight of seedling under low and normal temperature conditions. |
|---|---|---|---|---|---|---|---|
| Temperature (°C) day/night | H₂O₂ solution (mM) | Shoot length (mm) | RSL (%) | Root length (mm) | RRL (%) | Fresh weight (mg) | RFW (%) |
|---|---|---|---|---|---|---|---|
| 23/18 | 0 | 24.7 ± 1.2 | 100 | 5.3 ± 0.9 | 100 | 9.0 ± 0.1 | 100 |
| | 50 | 36.6 ± 3.4** | 148 | 18.1 ± 3.0** | 342 | 18.6 ± 1.0** | 207 |
| 18/14 | 0 | 38.4 ± 0.8 | 100 | 6.3 ± 0.8 | 100 | 15.3 ± 1.8 | 100 |
| | 50 | 42.1 ± 3.5 | 110 | 15.1 ± 1.9** | 240 | 18.3 ± 2.0** | 120 |

Each value is the mean ± standard error (n = 3). **, Significantly different from the control without H₂O₂ treatment at P < 0.01. RSL, relative of shoot length; RRL, relative root length; RFW, relative shoot fresh weight (relative to the control without H₂O₂ treatment).

| Table 3. Effects of treatment of rice seeds with H₂O₂ before sowing on shoot length and shoot fresh weight of seedling in a greenhouse. |
|---|---|---|---|---|---|---|
| Day | H₂O₂ solution (mM) | Shoot length (mm) | RSL (%) | Growth rate (14-7) (mm) | Shoot Fresh weight (mg) | RFW (%) |
|---|---|---|---|---|---|---|
| 7 | 0 | 7.0 ± 0.3 | 100 | 1.1 ± 0.1 | 100 |
| | 50 | 9.6 ± 0.3** | 138 | 1.5 ± 0.1** | 137 |
| 14 | 0 | 15.5 ± 0.7 | 100 | 8.5 | 7.1 ± 0.3 | 100 |
| | 50 | 22.9 ± 0.6** | 148 | 13.3 | 9.6 ± 0.2** | 136 |

Each value is the mean ± standard error (n = 30). **, Significantly different from the control without H₂O₂ treatment at P < 0.05.
suspension-cultured germinated rice embryos were treated with 1 mM H₂O₂ (Motira et al., 1999). In the present study, the H₂O₂ applied to the seeds during imbibition might mainly induce APX gene, not CAT. Sato et al. (2001) reported that rice seedlings, when kept at 42 °C for 24 hr before sowing under a low-temperature condition with flooding are shown as '1' and '3', respectively. RNA was extracted from whole seeds. (A) RT-PCR analysis using specific primer for each gene described in Table 1. (B) Expression of APXα shown by northern blot analysis. The bottom panel (rRNA) shows the ethidium bromide-stained RNA gel as the loading control.

CAT activity was decreased and SOD activity was not significantly influenced by heat stress (Sato et al., 2001). Overproduction of APX in the chloroplast stroma of cotton leaves resulted in enhanced scavenging of H₂O₂ and the resistance to chilling temperatures (Kornyeyev et al., 2003). Therefore, expression of APX gene after germination, which was induced by H₂O₂ treatment during imbibition, might be related to the promotion of seedling growth under a low-temperature condition observed in this study, presumably with decreasing damage of plant to scavenge ROS. Further studies are needed to elucidate the nature of this mechanism.

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