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Research Paper

Hydrogen peroxide scavenging regulates germination ability during wheat (*Triticum aestivum* L.) seed maturation

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Abbreviations: ABS, absorbance; AsA, ascorbic acid; APX, ascorbate peroxidase; CAT, catalase; DAP, days after pollination; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase

Key words: ascorbic acid, germination, hydorogen peroxide, maturation, wheat seed

Hydrogen peroxide (H_2O_2) promotes seed germination of cereal plants and ascorbic acid which acts as antioxidant suppresses the germination of wheat seeds, but the role of H_2O_2 scavenging on germination during seed maturation has not been demonstrated. We investigated relationship of germination, ascorbate, H_2O_2 scavenging enzymes and sensitivity to ascorbic acid (AsA) maturing seeds of two typical wheat (*Triticum aestivum* L.) cultivars, cvs. Shirogane-Komugi and Norin61. Shirogane-Komugi had marked high germination ability than Norin61 during seed maturation. Although the H_2O_2 content had no difference in the two cultivars, sensitivity to AsA of Norin61 seeds was higher than that of Shirogane-Komugi seeds during seed maturation. The sensitivity to AsA closely correlated with germination characteristic in the two cultivars. Especially, at 28 days after pollination (DAP), sensitivity to AsA in Norin61 seeds was remarkably high. At that stage, no significant differences were observed in endogenous AsA level, ascorbate peroxidase (APX, EC 1.11.1.11) and dehydroascorbate reductase (DHAR, EC 1.8.5.1) activities in the two cultivars. However, catalase (CAT, EC 1.11.1.6) activity and *CAT* mRNA in Norin61 were remarkably higher than in Shirogane-Komugi. Sensitivity to AsA at 35 and 42 DAPs kept high levels in Norin61, and endogenous AsA and CAT activity in the seeds were significantly higher than in Shirogane-Komugi. These results revealed a direct correlation between germination and antioxidant sensitivity during the developmental stages of wheat seeds.

Introduction

Germination and dormancy are an important mechanism in seed physiology and is a complex trait, influenced by a myriad of genetic and environmental factors that interact to maximize the long-term chance of survival of the seed.[1] Abscisic acid (ABA) has been demonstrated to play an important role in regulating seed maturation and germination. During seed maturation, ABA content increases and regulates many key processes including the imposition and maintenance of germination and dormancy.[2] In wheat, the strongest dormancy is associated with a red seed coat color, whereas the lines with white seed coats are nondormant or weakly dormant and therefore are susceptible to preharvest sprouting damage.[3,4] Although Himi et al.[5] reported that the seed color gene might enhance grain dormancy by increasing the sensitivity of embryos to ABA, the dormancy effect conferred by seed coat color was not large, because there was no difference in the amount of inhibitors between red and white-seed bran. Weidner and Paprocka[6] proposed that dormancy of cereal caryopses might be at least partially controlled by the high level of free phenolic acids in seed, through their inhibitory effect on germination and cell division.

It is well known that many phenolic compounds in plant tissues are potential antioxidants: flavonoids, tannins and lignin precursors may work as reactive oxygen species (ROS) scavenging compounds. ROS such as hydroxyl radical (·OH), O_2^- and H_2O_2 in seed physiology are usually considered as toxic molecules.[7] However, exogenously applied H_2O_2 ameliorates seed germination in many plants.[8,9] This has been explained by the fact that the scavenging activity for H_2O_2 is sufficiently high, resulting in the production of O_2 for mitochondrial respiration. In contrast, H_2O_2 promoted seed germination in a dose-dependent manner as did respiratory inhibitors, indicating that H_2O_2 itself possibly promotes seed germination rather than O_2.[10]

Antioxidants which act as ROS scavenger in seed biology play a very important role in the growth processes occurring at early embryogenesis during seed development, participate in the mechanisms underlying radicle protrusion during seed germination and seed aging.[11,12] In plant cells, the most important reducing substrate for H_2O_2 removal is ascorbic acid which acts as an antioxidant.[13] Recently, we have reported that ascorbic acid suppresses the germination of wheat seeds.[14] Ascorbate peroxidase (APX) and catalase (CAT) scavenging H_2O_2 are localized at the site of H_2O_2 generation in plant cells.[15] De Gara et al.[16] have followed the changes in detoxifying enzyme activities during the maturation of *Triticum durum* kernels. Their results show that ascorbic acid, APX and CAT are contained in maturing wheat seeds, whereas the seeds at the end

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of their development do not contain either ascorbate or APX but do still contain CAT. Seed filling is associated with the high potential of the H_2O_2 detoxification machinery, mainly due to APX and CAT activities.

In the present study, we examined the role of antioxidant in regulating germination during wheat seed maturation. The aims of the present work were (1) to study the influence of endogenous and exogenous ascorbate in germination during wheat seed maturation, and (2) to understand in more detail the relationship between H_2O_2 scavenging efficiency and germination ability of developing and maturing wheat seeds.

Results

Germination ability during seed maturation of the two cultivars. The water contents in the wheat seeds of both cultivars were the highest at 14 DAP and decreased gradually until 42 DAP (Fig. 1A). From 3 to 35 DAP, water content did not differ significantly between the two cultivars. According to the classification of wheat seed development,[20] these seeds appear to have reached harvest-maturity at 40 DAP.

The germination rate of whole seeds in the two cultivars during maturation is shown in Figure 1B. At 28 DAP, none of the seeds of Norin61 germinated, while aproximately 40% of Shirogane-Komugi germinated. In both cultivars, the highest germination rate was observed at 35 DAP. The germination rates of Shirogane-Komugi were higher than those of Norin61 from 21 to 42 DAP. This tendency was closely consistent with results for the past two years (data not shown). In Shirogane-Komugi, although the germination rate of hydrogen peroxide-treated whole seeds was the same as that of DW-treated seeds at 28 DAP, the germination rate of H_2O_2-treated whole seeds at 35 and 42 DAPs was almost 100% compared with about 65% for DW-treated whole seeds (Fig. 1B and C). On the other hand, H_2O_2-treated whole seeds in Norin61 had germinated at the same rate as DW-treated whole seeds at 35 DAP and the ratio was about two times that of DW-treated whole seeds at 42 DAP. These results indicated that H_2O_2 promoted germination during wheat seed maturation.

Ascorbic acid sensitivity during seed maturation of two cultivars. The germination rate of the embryo-half seeds of two cultivars is shown in Figure 2. Kawakami et al.[21] measured ABA sensitivity during the seed maturation of wheat mutants by using embryo-half grains. In both cultivars, the most of DW-treated embryo-half seeds had germinated at 21 DAP. Although the most of Shirogane-Komugi embryo-half seeds treated by AsA germinated, the germination rate of Norin61 was 18% at 28 DAP and was about 80% at 35 and 42 DAPs. Ascorbic acid suppressed specifically Norin61 during the seed maturation. Especially, at 28 DAP, germination of Norin61 embryo-half seeds treated by AsA was suppressed by about 80% as compared with DW-treated embryo-half seeds, while that of Shirogane-Komugi was only negligibly suppressed.

Hydrogen peroxide and ascorbate contents during seed maturation of two cultivars. The H_2O_2 contents in the wheat seeds of both cultivars were the highest at 28 DAP and decreased gradually until 42 DAP (Fig. 3). The H_2O_2 contents during the seed maturation did not differ significantly between the two cultivars. The total ascorbate content (AsA + DHA) in the whole seeds of both cultivars was the highest at 21 DAP (Fig. 4). In Shirogane-Komugi, ascorbic

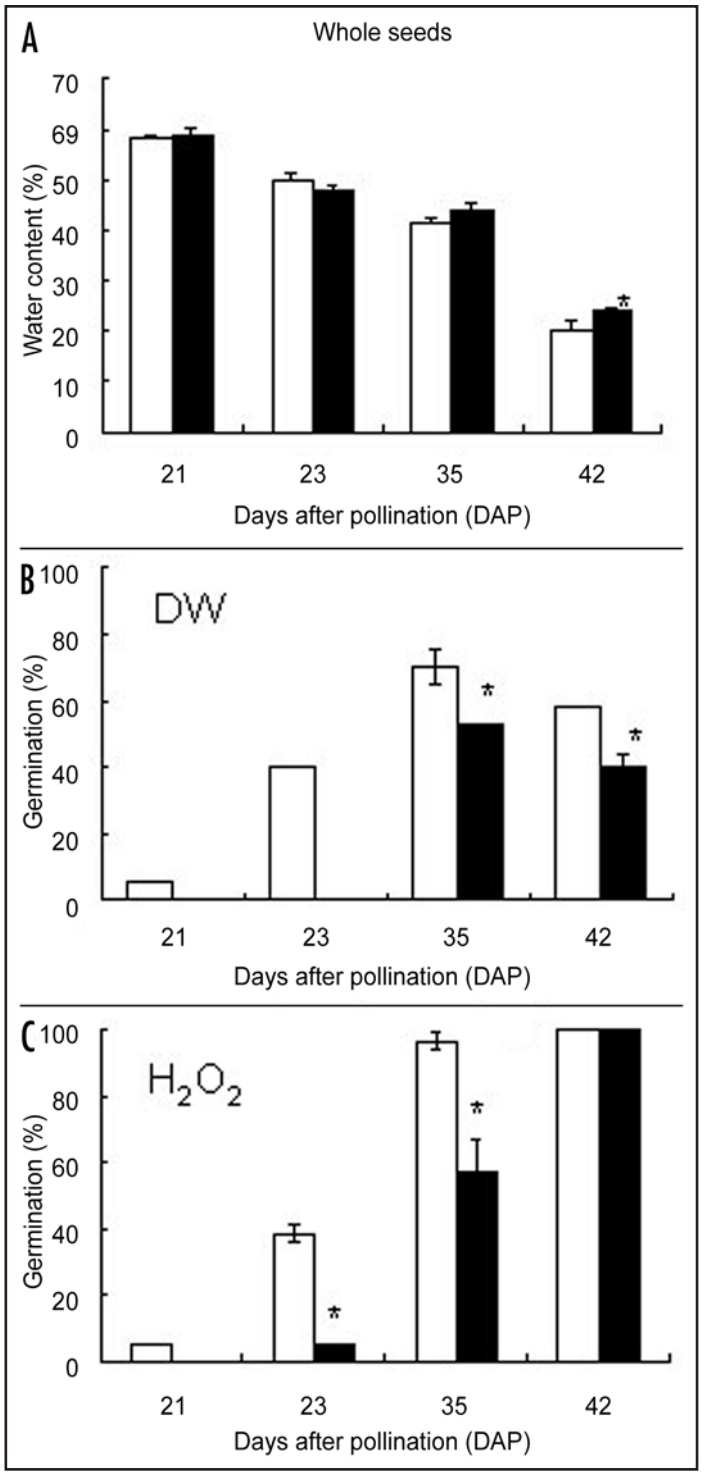

Figure 1. Water content (A), germination ratio of wheat seeds treated by DW (B) (control) and H_2O_2 (C) in Shirogane-Komugi and Norin61 cultivars during the development and maturation processes. White bars indicate Shirogane-Komugi and black bars indicate Norin61. The reported values are the means and S. D. of five replications. An asterisk indicates statistical significance at the 5% level (Student's *t*-test).

acid content was high until 21 DAP, markedly decreasing at 28 DAP and then disappearing. On the other hand, the ascorbate contents of Norin61 was highest at 14 DAP, decreased markedly at 28 DAP, and then remained at a constant value until 42 DAP. Although AsA

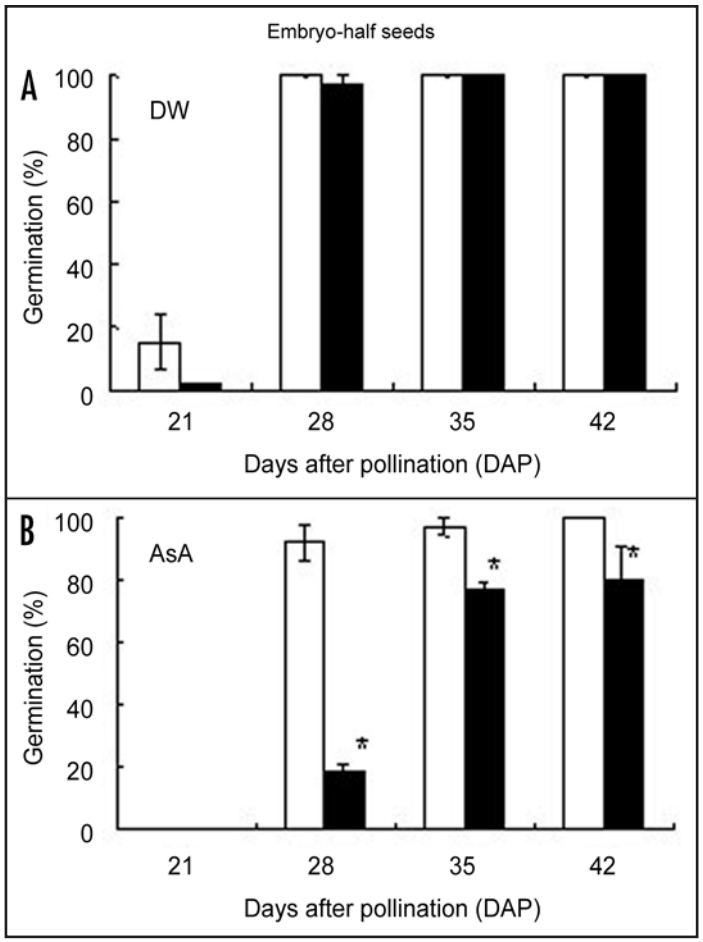

Figure 2. Sensitivity of ascorbic acid in Shirogane-Komugi and Norin61 embryo-half seeds during development and maturation processes. (A) DW (control), (B) AsA (ascorbic acid). White bars indicate Shirogane-Komugi and black bars indicate Norin61. The reported values are the means and S. D. of five replications. An asterisk indicates statistical significance at the 5% level (Student's *t*-test).

content in Shirogane-Komugi at 21 DAP was higher than that of Norin61, AsA content in Shirogane-Komugi and Norin61 at 28 DAP were 9.8 ± 0.31 and 10.7 ± 3.34 nmol/kernel, respectively, and AsA/DHA ratio in Sirogane-Komugi and Norin61 at 28 DAP were 0.41 ± 0.05 and 0.61 ± 0.17, respectively. AsA content and AsA/DHA ratio at 28 DAP were indicated no significant difference in the two cultivars.

Activity of antioxidant enzymes and CAT gene expression during seed maturation. In both cultivars, when determined on a per kernel basis, no significant differences were detected for APX during the first 35 DAP, and the H_2O_2 scavenging enzyme markedly decreased until it was no longer detectable in the mature kernels (Fig. 5A). DHAR activity acts as AsA recycling enzyme and the activity of DHAR in Shirogane-Komugi increased from 21 to 28 DAP, remained constant for the following week, and then decreased to values similar to that of 21 DAP while that in Norin61 increased at 35 DAP and markedly decreased at 42 DAP (Fig. 5B). CAT activity which has global H_2O_2 removal capability of kernel cells during maturation was also measured (Fig. 5C). CAT activity in both cultivars during maturation was the highest at 28 DAP, and then gradually decreased. However, CAT activity in Norin61 during maturation was significantly higher

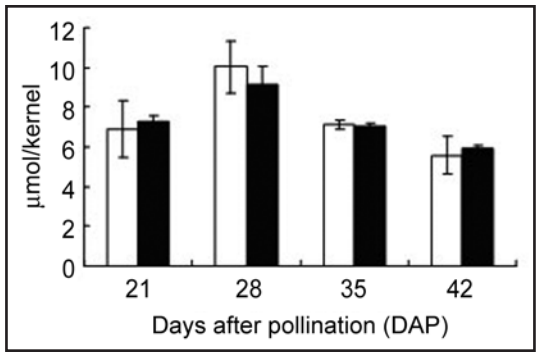

Figure 3. Contents of hydrogen peroxide in Shirogane-Komugi and Norin61 seeds during development and maturation processes. White bars indicate Shirogane-Komugi and black bars indicate Norin61. The reported values are the means and S.D. of five replications.

than that in Shirogane-Komugi throughout seed maturation. Unlike APX, CAT activity was still present in the dehydrated kernel, and there was significant varietal difference in two cultivars. Based on these results, *CAT* gene expression during seed maturation was also measured (Fig. 6). *CAT* gene expression was also remarkably high at 28 DAP in Norin61. This result clearly revealed a similar tendency in CAT activity and its gene expression.

Discussion

Water contents during maturation did not show a significant difference between the two cultivars (Fig. 1A). However, the seeds of Norin61 were significantly more germination ability than those of Shirogane-Komugi from 21 to 42 DAP (Fig. 1B). These results indicated that the two cultivars have a physiological difference in the germination characteristics during seed maturation.

H_2O_2 enhanced the germination rate in wheat, rice, and barley[22] and is produced at the early imbibition period in wheat,[23] soybean,[24] radish,[25] maize,[26] sunflower,[27] and tomato[28] seeds. Indeed, in the present study, H_2O_2 enhanced the germination rate and the influence of this promotion was significantly different during maturation in wheat cultivars (Fig. 1C). Phenolic compounds which act as an antioxidant suppressed seed germination.[10] Recently, we have reported that ascorbic acid acts as an antioxidant scavenging H_2O_2 and suppresses the germination of mature wheat seeds.[14] Therefore, we examined sensitivity to ascorbic acid in developing and maturing seeds of wheat cultivars having different germination characteristics (Fig. 2).

ROS generation is known to occur during the dehydration of various plant tissues.[29] In wheat leaves, drought-induced H_2O_2 accumulation correlated with a decrease in soil water content, and leaf H_2O_2 content increased even though CAT activity doubled under severe drought conditions.[30] It has also been demonstrated that the desiccation of developing sunflower seeds is associated with an increase in CAT activity.[31] In the present study, water contents of seeds in the two cultivars during maturation did not show significant difference (Fig. 1A), and H_2O_2 content of the seeds had no significant difference in the two cultivars (Fig. 3).On the other hand, sensitivity to AsA of developing seeds was higher in Norin61 than in Shirogane-Komugi. Additionally, the sensitivity to AsA was closely associated with germination characteristic in the two cultivars. This

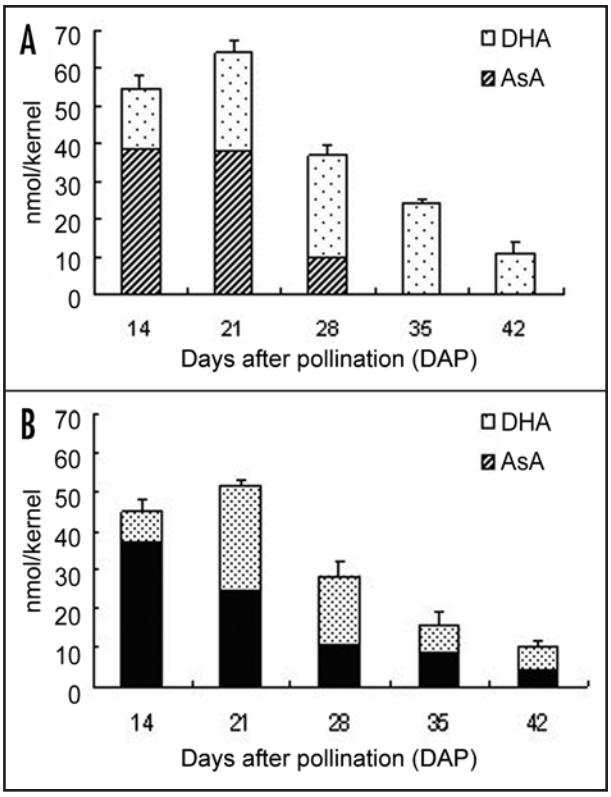

Figure 4. Contents of ascorbate and dehydroascorbate in Shirogane-Komugi and Norin61 seeds during development and maturation processes. (A) Shirogane-Komugi, (B) Norin 61. The reported values are the means and S.D. of five replications.

poses the question as to why significant differences exist in wheat cultivars affected by ascorbic acid.

De Tullio and Arrigoni[32] showed that the ascorbic acid system functions dynamically in seeds. The strategies for ascorbic acid production and utilization may vary according to developmental and functional stages in seeds, while ascorbic acid function in seeds is also likely to be related to its action as a specific cosubstrate required for the activity of dioxygenases involved in the synthesis of ethylene, gibberellins, and abscisic acid, respectively. Hydrogen peroxide is eliminated by ascorbate peroxidase (APX) and catalase (CAT).[33,34] These enzymes rapidly destroy the vast majority of H_2O_2 produced by metabolism.[13] We inferred that the difference of ascorbic acid sensitivity in the two cultivars is caused by a difference in the amount of endogenous ascorbate or a difference of APX or CAT activity.

Ascorbate contents at 28 DAP was not significantly different in the two cultivars, while ascorbate contents at 35 and 42 DAP in Norin61 were higher than in Shirogane-Komugi (Fig. 4). These results indicated that AsA synthesis ability at 35 and 42 DAPs in Norin61 were higher than in Shirogane-Komugi because there were no differences in endogenous H_2O_2 content, APX and DHAR activities (Figs. 3, 5A and B) and the difference of sensitivity to ascorbic acid at 35 and 42 DAPs was contributed by endogenous ascorbic acid in seeds. However, the difference of sensitivity to AsA at 28 DAP could not prove by endogenous AsA only. It has been widely reported that APX plays a key role in removing the toxic H_2O_2 in plant cells,[35,36,37] the correct temporal expression of APX has to be an important factor for seed germination.[38] APX activity in the

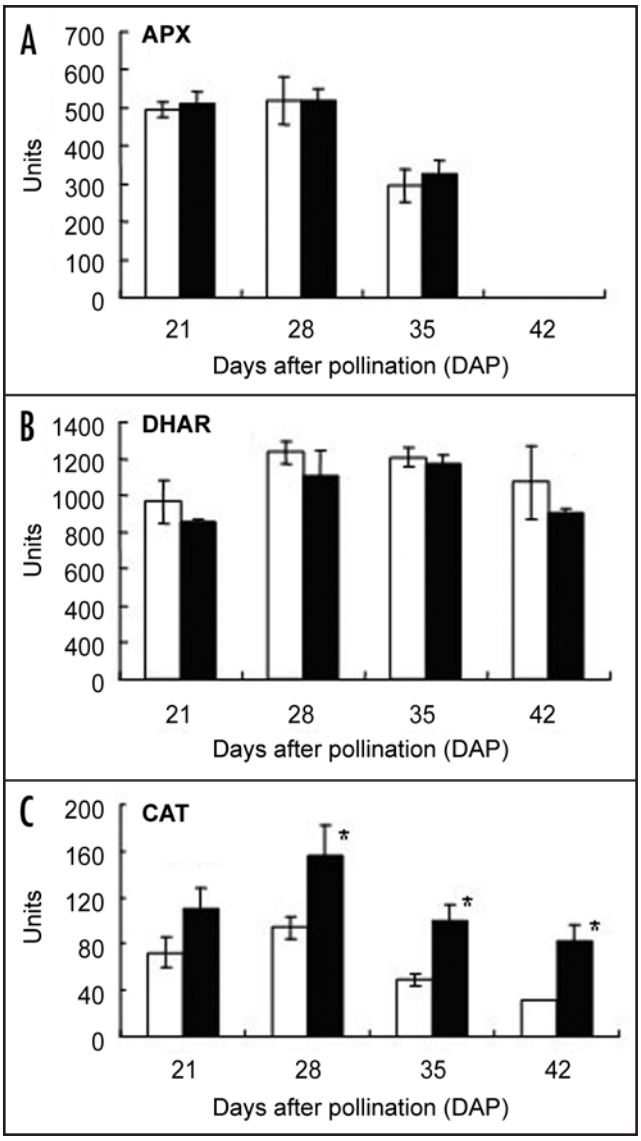

Figure 5. Antioxidant enzymes in Shirogane-Komugi and Norin61 seeds during development and maturation processes. (A) APX (ascorbate peroxidase), (B) DHAR (dehydroascobate reductase), (C) CAT (catalase). White bars indicate Shirogane-Komugi and black bars indicate Norin61. The reported values are the means and S.D. of five replications. 1 unit = 1 nmol ascorbate oxidized min-1 kernel-1 (APX); 1 nmol DHA reduced min-1 kernel-1 (DHAR); 1 nmol H2O2 dismutated min-1 kernel-1. An asterisk indicates statistical significance at the 5% level (Student's t-test).

two cultivar seeds disappeared at the end of their development and did not show any significant differences during seed maturation (Fig. 5A). In addition, DHAR activity was also not significantly different in the two wheat cultivars (Fig. 5B). CAT performs important roles in seed germination. In sunflower seeds, a quite close relationship between CAT activity and germination rate has (previously reported in ref. 39). Maize *CAT* genes have response elements that allow for fine tuning of their expression by ABA and GA.[40,41] CAT activity in Norin61 was higher than that in Shirogane-Komugi during seed maturation (Fig. 5C). Especially, at 28 DAP, CAT activity of Norin61 seed was remarkably high. In addition, the mRNA level of the *CAT* gene was also highest from 28 to 42 DAP in Norin61 (Fig. 6). These results indicated that the difference of

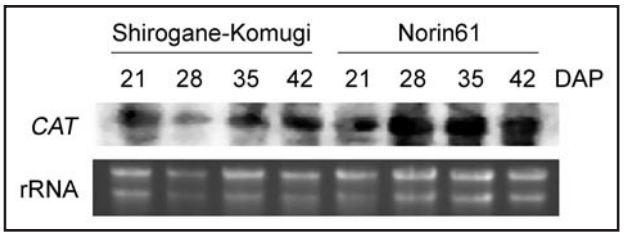

Figure 6. Northern blots of *CAT* gene expression in Shirogane-Komugi and Norin61 seeds during development and maturation processes.

sensitivity to ascorbic acid at 28 DAP was closely contributed by CAT activity in seeds. In the present study, it was indicated that the difference of ascorbic acid sensitivity in the two cultivars was caused by a difference in endogenous ascorbate content, CAT activity, and its gene expression. Namely, sensitivity to AsA in Norin61 was higher than that of Shirogane-Komugi because H_2O_2 scavenging ability was high in Norin61 (Figs. 2–6). This indicates that germination during seed maturation in wheat seeds is closely related to H_2O_2 scavenging ability during maturation.

Walker-Simmons[42] showed that ABA sensitivity disappeared in parallel with the loss of dormancy during seed development and after ripening, and was a key factor of seed dormancy rather than the ABA content of the wheat embryos. Recently, it was reported interfere with ABA metabolism and seed dormancy in arabidopsis and barley.[43] mRNAs encoding superoxide dismutase (SOD), CAT and APX were almost undetectable in aleurone layers 24 h after incubation in gibberellin (GA).[44] For aleurone layers incubated in ABA, however, the amounts of these mRNA increase. Western blotting and enzyme activity assays confirm that GA but not ABA reduced the amount and activity of ROS scavenging enzymes. Seed germination and dormancy may be possibly influenced by ROS scavenging ability controlled by ABA metabolism.

In this paper we showed a direct correlation between germination ability and antioxidant sensitivity during the developmental stages of wheat seeds. Therefore, it was indicated that H_2O_2 scavenging substances and enzymes are related to wheat seed germination ability during maturation, and H_2O_2 scavenging was one of the important factors related to premature germination of seeds.

Materials and Methods

Plant growth. Common wheat (*Triticum aestivum* L.) cultivars, Shirogane-Komugi and Norin61, were grown in the experimental fields of Kyushu University at Fukuoka in 2004–2005 on $30m^2$ plots. Ear flowering started on 14 April 2005 and the two cultivars were protected against rain under a transparent vinyl roof. Irrigation, fertilization, and plant protection were performed to ensure optimal plant growth. The seeds were sampled at the primary and secondary florets of the central spikelets at 7 d intervals after anthesis and stored at -80°C for analysis.

Germination was determined in five replications of twenty fresh seeds or half seeds containing embryos (embryo-half seeds) incubated in a Petri dish (9 cm in diameter) containing filter paper moistened with 6 ml of distilled water (DW), 10 mM H_2O_2, and 50 mM aqueous solution of ascorbic acid at 15°C for 7 d in a dark condition.

Water contents. Dry seed weight was measured after the incubation of 20 seeds dried at 90°C for 20 h. Water content (%) of seeds was calculated in terms of (fresh weight - dry weight) / fresh weight.

Analysis of ascorbate and hydrogen peroxide. The seeds (1 g) were homogenized with 8 ml of 5% (w/v) meta-phosphoric acid in liquid N_2. The homogenate was centrifuged at 20000 g for 15 min at 4°C, and the supernatant was used for analysis of ascorbate contents. The AsA and DHA content were determined according to the method (described in ref. 17). The H_2O_2 content was measured colorimetrically (described in ref. 18). H_2O_2 was extracted by homogenizing wheat seeds with phosphate buffer (50 mM, pH 6.5) containing 1 mM hydroxylamine. The homogenate was centrifuged at 6000 *g* for 25min. To determine H_2O_2 content, the extracted solution was mixed with 0.1% titanium sulphate in 20% (v/v) H_2SO_4. The mixture was then centrifuged at 6000 g for 25 min. The absorbance was measured at 410 nm.

Enzyme activity. The seeds (1 g) were ground in liquid N_2 and then resuspended in 4ml of a medium [50 mM Tris-HCl (pH 7.8), 0.05% (w/v) cysteine, and 0.1 % (w/v) BSA], just as the last trace of liquid N_2 disappeared. The thawed homogenate was then ground and centrifuged at 20000 g for 15 min at 4°C. The supernatant was used for spectrophotometric analysis.

Ascorbate peroxidase (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) and DHA reductase (glutathione: dehydroascorbate oxidoreductase, EC 1.8.5.1) were assayed according to the method (described in ref. 19).

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity assay was performed according to the method described by De Gara et al. by monitoring the decrease of ABS_{240nm} of the reaction mixture as H_2O_2 dismutation, which consisted of 0.1 M phosphate buffer, pH 7.0, 50-100 μg protein, and 18 mM H_2O_2 (extinction coefficient 23.5 mM^{-1} cm^{-1}). Enzyme activities were calculated on a per-seed basis.

Northern blot analysis. Total RNA was isolated from seeds during maturation (21 DAP to 42 DAP) using the sodium dodecyl sulfate (SDS)-phenol lithium chloride method. PCR products of wheat *CAT* ORF (NCBI accession no. D86327) were amplified from cDNA, and digoxigenin (DIG) labelling of the PCR products was carried out using a nonisotopic DIG-labelling kit (Roche Diagnostic). Total RNA (1 μg) was denatured in a mixture of 70% (v/v) formamide and 8% (v/v) formaldehyde, and separated on a 1.5% agarose gel containing 1.8% (v/v) formaldehyde. After electrophoresis, RNAs were transferred to a Hybond-N^+ membrane (Amersham Biosciences). Prehybridization of the membrane was carried out at 65°C for 1 h in 0.3 M phosphate buffer containing 7% SDS, and hybridization was then carried out by incubating the membrane in the same buffer with DIG-labelled probes at 65°C for over 15 h. Membranes were washed in 2x SSC containing 0.1% SDS (15 min), and then in 0.1x SSC containing 0.1% SDS (15 min) at 65°C. After incubation of the blots with anti-DIG antibody-horseradish peroxidase at 37°C for 1 h, DIG-epitopes on membranes were detected by Fluorchem (Alpha Innotech) using an ECL kit (Amersham Biosciences).

Acknowledgements

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