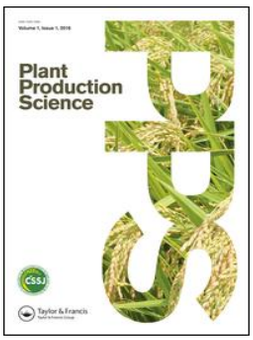

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NADPH Oxidases Act as Key Enzyme on Germination and Seedling Growth in Barley (*Hordeum vulgare* L.)

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Abstract: Reactive oxygen species (ROS) play an important role in seed germination. Although hydrogen peroxide (H_2O_2), a type of ROS, enhances the germination rate of various plant seeds, little is known about the mechanism. NADPH oxidases catalyze the production of superoxide anion (O_2^-) that is one of the ROS and the enzymes regulate plant development. We, therefore, investigated the role of NADPH oxidases in seed germination and seedling growth in barley (*Hordeum vulgare* L.). The production of O_2^- was observed both in embryo and aleurone layers in barley seeds treated with distilled water (DW). However, it was suppressed in seeds treated with diphenylene iodonium (DPI) chloride, NADPH oxidase inhibitor. Moreover, DPI markedly delayed germination and remarkably suppressed α-amylase activity in barley seeds, indicating the importance of NADPH oxidases in germination of barley seeds. The gene expression and the enzyme activity of NADPH oxidases gradually increased after imbibition, and the enzyme activities were closely correlated with seedling growth after imbibition. Besides, DPI markedly suppressed the seedling growth. These results indicated that NADPH oxidases perform a crucial function in germination and seedling growth in barley. These facts clearly reveal that O_2^- produced by NADPH oxidases after imbibition regulates seed germination and seedling growth in barley.

Key words: Barley, Germination, NADPH oxidases, Reactive oxygen species, Seedling growth.

Seed germination and seedling growth are important in seed physiology and agriculture fields. The germination of the barley seed is especially important because of the production of malt for beer and whiskey. Therefore, analysis of germination mechanism of barley is very important in both agriculture and brewing fields. Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) enhanced germination and released residual dormancy of barley seeds (Mabuchi, 1994). In contrast, the exogenously supplied antioxidant, ascorbic acid which acts as an ROS scavenger significantly suppressed germination of barley and wheat (Ishibashi et al., 2006; Ishibashi and Iwaya-Inoue, 2006). Production of hydrogen peroxide at the early imbibition period has been demonstrated in seeds of soybean (Puntarulo et al., 1988), maize (Hite et al., 1999), wheat (Caliskan et al., 1998) and *Zinnia elegance* (Ogawa and Iwabuchi, 2001); ROS produced after imbibition probably regulated seed germination. Indeed, H_2O_2 scavenging regulated germination ability during wheat seed maturation (Ishibashi et al., 2008). However, the mechanism of ROS production involved in seed germination is still not clear.

In developing or germinating seeds, the active mitochondria is one of the major sources of ROS, generating O_2^-, and subsequently H_2O_2 (Noctor et al., 2007). Approximately 2–3% of the oxygen used by the mitochondrial respiration results into O_2^- and hydrogen peroxide production (Puntarulo et al., 1988). However, treatment with exogenous 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 (Ogawa and Iwabuchi, 2001; Oracz et al., 2009). The NADPH oxidases of the plasma membrane, which transfer electrons from cytoplasmic NADPH to oxygen, are also major source of O_2^-, which is subsequently dismutated to H_2O_2 (Cross and Segal, 2004). Plant homologues of the mammalian gp91phox of respiratory burst NADPH oxidase complexes

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Abbreviations: DAT, days after treatment; DPI, diphenylene iodonium; DW, distilled water; NBT, nitroblue tetrazolium; PCR, polymerase chain reaction; ROS, reactive oxygen species.

have been identified and partially characterized in several plant species, including rice (Groom et al., 1996; Yoshie et al., 2005), *Arabidopsis thaliana* (Keller et al., 1998; Torres et al., 1998), tomato (Amicucci et al., 1999), potato (Yoshioka et al., 2001), tobacco (Yoshioka et al., 2003), and barley (Trujillo et al., 2006; Lightfoot et al., 2008). ROS produced by NADPH oxidases have been shown to play various important roles in cellular signaling and development in plants, such as plant defense response, programmed cell death, abiotic stress, stomatal closure, and root hair development (Baxter-Burrell et al., 2002; Torres et al., 2002; Foreman et al., 2003; Kwak et al., 2003; Yoshioka et al., 2003; Jones et al., 2007). Although the dual role of NADPH oxidase is now quite well documented in plants, there exist few studies on seeds in this area. We, therefore, investigated the role of NADPH oxidases in seed germination and seedling growth in barley.

Materials and Methods

1. Plant material

Hordeum vulgare L. cv. Seijou17 was used as the material. This cultivar was grown in a 30 m^2 plot in an experimental field of Kyushu University, Fukuoka from 2006 to 2007. Irrigation, fertilization and pesticide treatment were performed to ensure optimal plant growth. Ripe kernels were harvested on 1 June, 2007 and stored at 4ºC, in a dehydrated condition.

2. Germination test and seedling growth

Twenty seeds of barley were placed on filter paper in a petri dish (diameter 9 cm). Six milliliters of DW, 10, 50, and 100 mM of H_2O_2 and 1 mM of DPI were applied to each dish. The concentrations of these solutions were determined according to Mabuchi (1994) and Oracz et al. (2009). The petri dishes were incubated at 22ºC in the dark, and the number of germinating seeds was counted daily for 5 d. Seeds were considered as germinated when the radicle had protruded through the seed coat. Using a plastic scale with mm markings, shoot and root lengths were measured daily for 5 d after imbibition. Shoot and root lengths were measured from the tip to the seed. Measurements were rounded to the nearest mm.

3. Localization of O_2^- in seeds

Hand-cut longitudinal sections of seed treated with water for 2 d were incubated in 6 mM nitroblue tetrazolium (NBT) in 10 mM Tris-HCl buffer, pH 7.4 at room temperature for 30 min. O_2^- was visualized as deposits of dark-blue insoluble formazan compounds (Beyer and Fridovich, 1987) using stereo (Stemi DV4, Zeiss) and light microscopes (Escipse, Nikon).

4. Quantitative real-time PCR

Total RNA was extracted from barley seedling (whole plant) by the SDS/phenol/LiCl method (Chirgwin et al., 1979) and cDNA was synthesised from total RNA (1 μg) using Rever TraACE reverse transcriptase (TOYOBO) according to the manufacturer's protocol. cDNA (1 μL) was amplified in a reaction solution containing 10 μL of SYBR Green realtime PCR master mix, 2 μL of plus solution, 0.1 μL each of 50 μM forward and reverse primers, 6.8 μL of water using SYBER Green realtime PCR master mix -plus- (TOYOBO). The amplification was conducted with a real-time PCR machine (MJ Mini, Bio-Rad) as follows: 1 min at 94ºC followed by 40 cycles of 15 s at 94ºC, 30 s at 56ºC, 30 s at 72ºC, and 5 min at 72ºC. A melt curve was obtained from the PCR product at the end

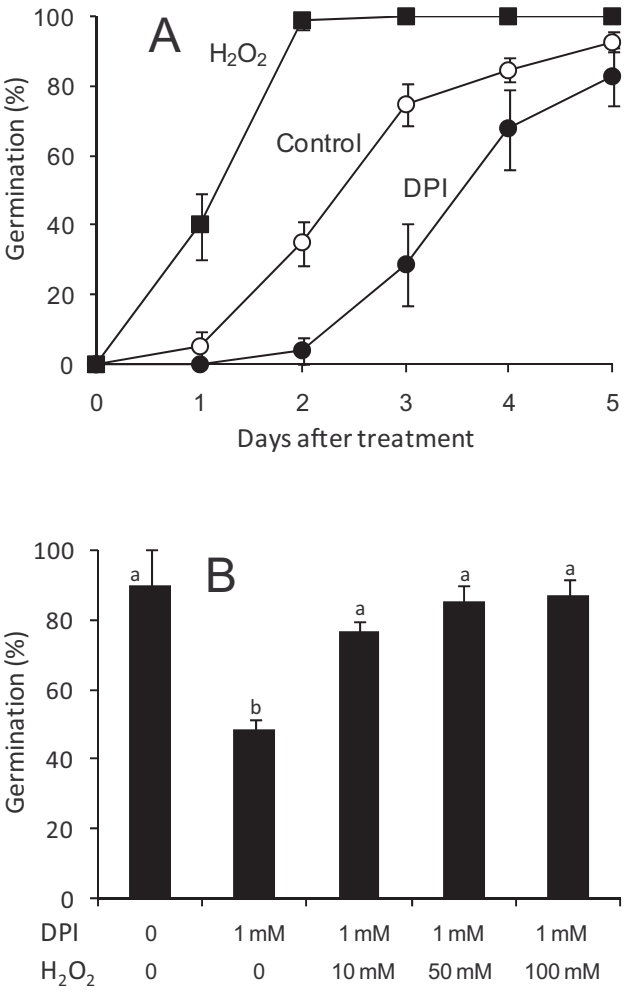

Fig. 1. Effect of H_2O_2 and DPI on germination of barley seeds. (A) Seeds were incubated in distilled water, 100 mM H_2O_2 and 1 mM DPI. The germinating seeds were counted daily for 5 d. Open circles, closed circles and squares indicate control (DW), DPI and H_2O_2 treatments, respectively. (B) Seeds were incubated with solutions containing both DPI (1 mM) and H_2O_2 (10, 50, 100 mM) for 3 d. Different letters indicate significantly difference among treatments at $P<0.05$ by Tukey's test. The experiments of (A) and (B) were performed on independent sample date. The reported values are the means and SD of five replications.

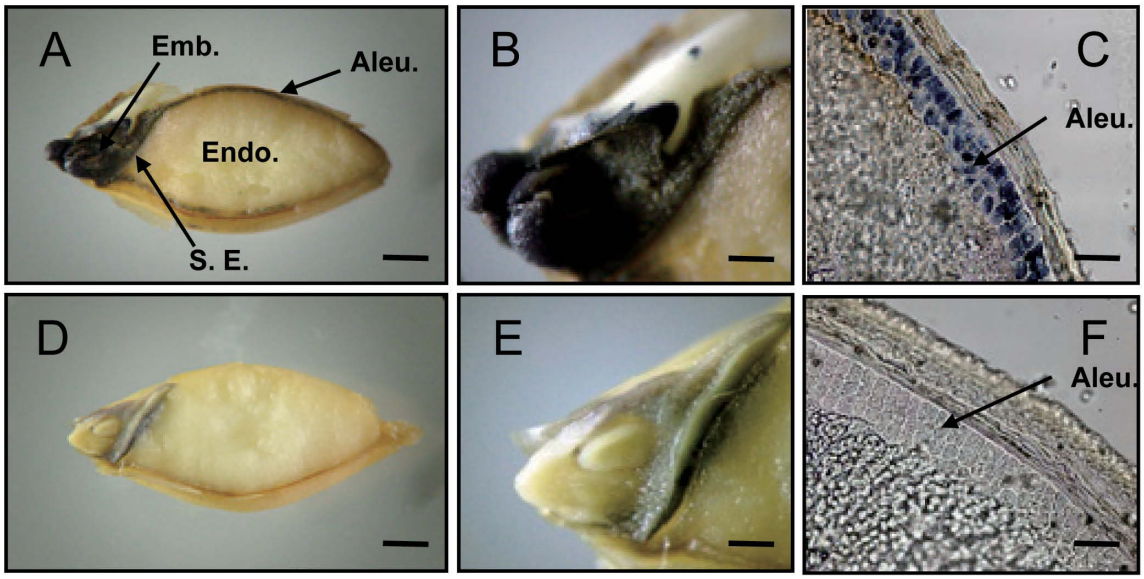

Fig. 2. Localization of O_2^- produced after imbibitions in barley seeds. A, B and C were treated with DW. D, E and F were treated with 1 mM DPI. Emb: embryo, Endo: endosperm, Aleu: aleurone layer, S.E: scutellar epithelial cell. Scale bars are 2 mm (A, D), 0.3 mm (B, E) and 50 μm (C,F), respectively.

of the amplification by heating from 50ºC to 95ºC. From the melt curve, the optimal temperature for data acquisition was determined. To examine gene expression of *NADPH oxidases* including *NADPH oxidase* families in barley, we used primers with a particular conserved region of *NADPH oxidases* in barley. The expression of *NADPH oxidases* and *HvActin* was analyzed by these primer pairs: NADPH oxidase-forward (5'-GTTTAAAGGAATCATGAAT GAGAT-3'), NADPH oxidase-reverse (5'-GAATTTTGTCG TGCATTTGCCATT-3'), HvActin-forward (5'-GCCGTGCT TTCCCTCTATG-3'), HvActin-reverse (5'-GCTTCTCCTTG ATGTCCCTTA). Relative values of *NADPH oxidases* transcript were calculated by normalizing against the amount of mRNA for a *HvActin* (Trevaskis et al., 2006) following the method of Pfaffl method (2001). The quantitative PCR values were compared with zero time (dry seed) for each time point and expressed as relative levels of expression.

5. Enzyme activity

Alpha-amylase activity was measured using the Amylase HR Reagent (Megazyme International Ireland, Ltd.) according to the manufacturer's instructions. Extracts were diluted to a final concentration of 1:6 with extraction buffer, before 200 μL of the diluted extract were reacted with 200 μL of HR reagent at 40°C for exactly 5 min. The reaction was stopped by adding 3 mL of stop reagent. Spectrophotometric measurement was carried out in 1 cm cuvettes at 410 nm using a spectrophotometer (U-1800, Hitachi). There were five replications of each treatment.

Enzymatic activities of NADPH oxidases were assayed according to the procedure of Van Gestelen et al. (1997) and Sarath et al. (2007). Five seeds were ground into fine powder with liquid nitrogen in a mortar with a pestle, weighed and transferred to 2 mL tubes and kept on ice. One milliliter of cold 10 mM Na-phosphate buffer, pH 6.0 was added to each tube and the contents were mixed and sonicated using a sonicater for 15 s. Homogenates were clarified by centrifugation at 16,000 $\times g$ for 15 min at 4°C in a refrigerated centrifuge. Crude seed homogenates (0.2 mL) were precipitated with acetone (9:1 acetone:homogenate) at −20°C for 15 min. Precipitated proteins were recovered by centrifugation at 12,500 rpm for 10 min at 4°C. Protein pellets were resuspended in buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM $MgCl_2$, 0.25 M sucrose and 0.1% Triton-X100) and used for assay of NADPH oxidases. NADPH-dependent superoxide generation was measured using NBT. NBT is rapidly converted to monoformazan by two molecules of superoxide. This reaction is detected by a spectrophotometer (U-1800, Hitachi) at 530 nm. Monoformazan concentrations were calculated using an extinction coefficient of 12.8 mM^{-1} cm^{-1}. It was confirmed that the NBT reduction rates were strictly linear with time up to 10 to 15 min and were linearly dependent on the protein concentration in the sample. Protein concentration was determined according to the method of Bradford (1976) using bovine serum albmin (BSA) as standard. The results are expressed as μmol mg^{-1} protein.

Results

1. Effect of DPI, NADPH oxidases inhibitor, on germination and localization of O_2^- in barley seed

In plants, it is known that ROS promotes seed germination. However, the role of NADPH oxidase which is one of the major sources of ROS in seed germination is still not clear. We, therefore, examined the effect of DPI, NADPH oxidase inhibitor, on barley seed germination. Barley seed treated with distilled water (DW) had the germination rate of 40% at 2 d after treatment (DAT), and were fully germinated at 5 DAT, while seeds treated with 100 mM H_2O_2 as source of ROS had about 40% germination rate at 1 DAT, and fully germinated at 2 DAT (Fig. 1A). These results indicated that seed germination of barley was also accelerated by ROS. In contrast, seeds treated with 1 mM DPI solution had the germination rate of about 5% at 2 DAT, and their germination rate was markedly delayed in comparison with that of DW treatment. Barley seeds treated with solutions containing both DPI and H_2O_2 resulted in a significant reversal of the inhibitory effects observed in the DPI treatment (Fig. 1B).

NADPH oxidase catalyzes the production of O_2^- from oxygen and NADPH (Lambeth, 2004). To analyze O_2^- accumulation in barley seeds, the seeds were stained with 6 mM NBT. When DW-treated seed was incubated with NBT, accumulation of O_2^- was observed as development of dark-blue color in regions of embryo and aleurone layer including scutellar epithelial cells but not in the endosperm (Fig. 2). However, in DPI-treated seeds, accumulation of O_2^- was hardly observed. Although, in DPI-treated seeds, accumulation of O_2^- was observed slightly in embryo, the accumulation was markedly lower than that in DW-treated seeds. These results indicated that the germination rate of barley seed depends on ROS production including O_2^-, and DPI suppressed germination by reducing the O_2^- produced in barley seed.

2. Effect of DPI on α-amylase activity and seedling growth in barley seeds

Alpha-amylase, an endohydrolase, is a key enzyme in seed germination, since cereal α-amylase can degrade amylose and amylopectin in endosperm to low molecular linear oligosaccharides. The α-amylase activity in DW-treated seeds increased during 4 DAT and the level of activity was maintained until 5 DAT (Fig. 3A). Although α-amylase activity in DPI-treated seeds slightly increased during treatment, the activity was markedly lower than that in the control. Additionally, DPI markedly suppressed both shoot and root growth in the time frame of the experiment (Fig. 3B, C). DPI-treated seeds showed only marginal growth if any from 4 to 5 DAT; the lengths of these shoots and roots at 4–5 DTA were only about 3.0 and 0.8 mm, respectively, which were extremely shorter than those in DW treated plants.

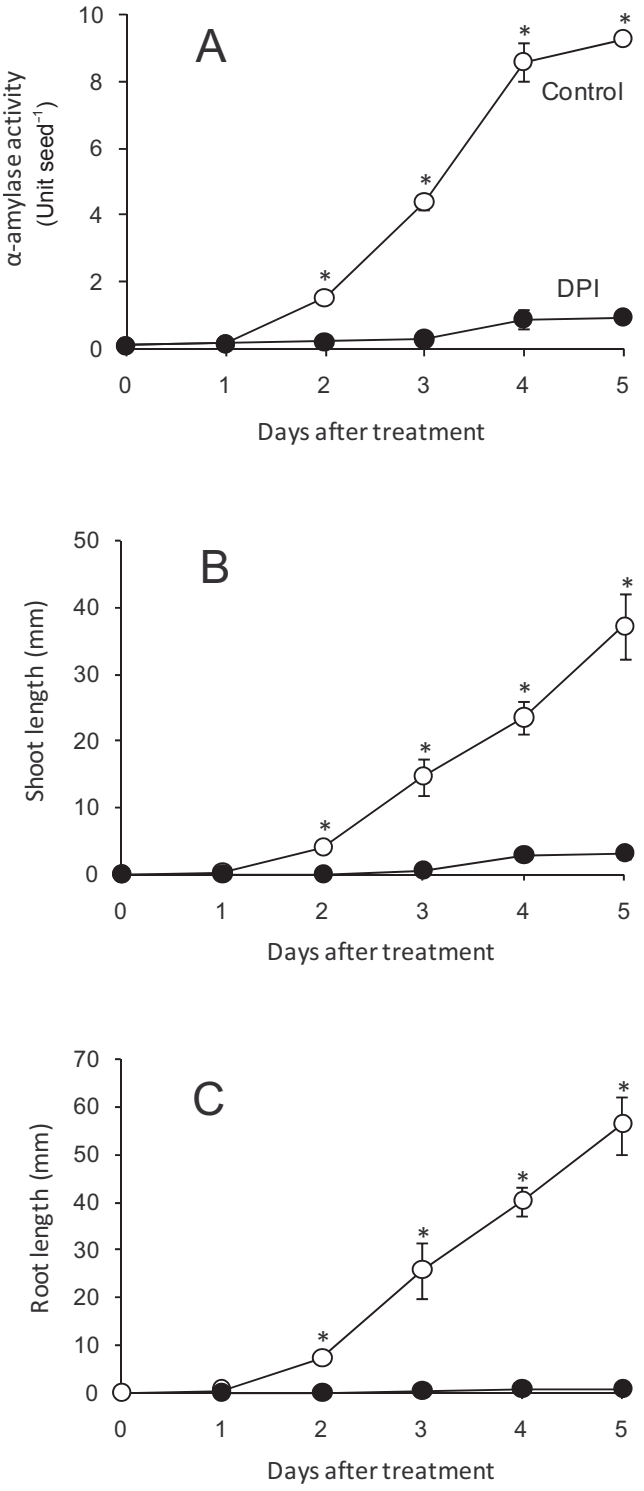

Fig. 3. Effects of DPI on alpha-amylase activity (A), length of shoot (B) and root (C). Open and closed circles show control (DW) and DPI treatments, respectively. An asterisk indicates statistical significance at the 5% level (Student's *t*-test). The reported values are the means and SD of five replications.

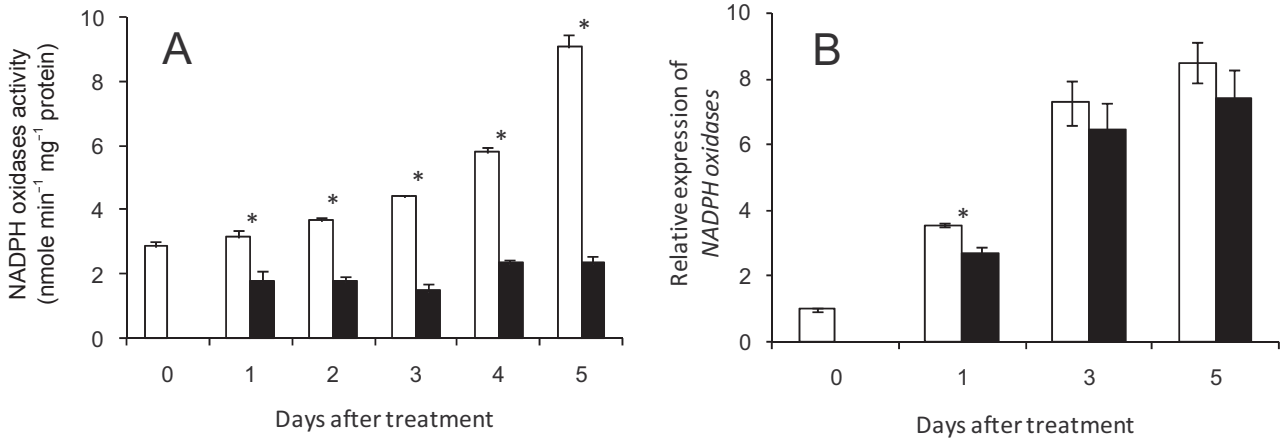

Fig. 4. Enzyme activities and gene expression of *NADPH oxidases* in barley seeds after imbibition. The gene expression of *NADPH oxidases* was measured by quantitative real-time PCR, and the relative level of expression were represented in comparison with zero time (dry seed) for each time point. Activities of NADPH oxidases are the means and SD of five and three replications, respectively (A). Relative gene expression of *NADPH oxidases* are the means and SD of five and three replications, respectively (B). White and black bars show DW and DPI treatment, respectively. An asterisk indicates statistical significance at the 5% level (Student's *t*-test).

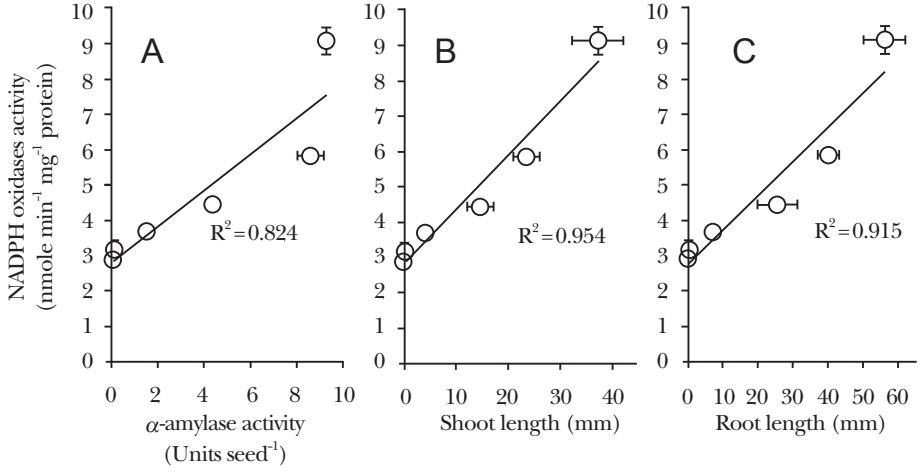

Fig. 5. Relationship between NADPH oxidases activity and α-amylase activity (A), shoot (B) and root (C) lengths.

3. Gene expression of *NADPH* oxidases in barley seeds and relationship between NADPH oxidase activity and seedling growth in barley

The enzyme activity of NADPH oxidases in seedlings of control also increased gradually during 4 DAT and remarkably at 5 DAT, while the increase in NADPH oxidase activity was suppressed in the seed treated with DPI (Fig. 4A). The quantitative analysis of gene expression of *NADPH oxidases* in seedling after imbibition is shown in Fig. 4B. To examine gene expression of *NADPH oxidases*, we used a set of primers designed from particular conserved region of *NADPH oxidases*. The gene expression of *NADPH oxidases* in barley seeds treated with DW increased about 3.5-fold compared with that of dry seeds during 1 DAT, then about over 8-fold during 3 to 5 DAT (Fig. 4B). The gene expression in seeds treated with DPI also increased as well as DW treatment after imbibitions. In addition, the enzyme activity of NADPH oxidases was closely correlated with α-amylase activity, shoot and root lengths after imbibition (R^2=0.824, R^2=0.954 and R^2=0.915, respectively) (Fig. 5).

Discussion

The present study shows that NADPH oxidases are important for germination and seedling growth of barley. This is illustrated by the use of an NADPH oxidases inhibitor, DPI. Since DPI can inhibit flavin-containing enzymes (Foreman et al., 2003), it is possible that proteins other than NADPH oxidases are also targets for DPI inhibition. However, our data on NADPH oxidases activity

and localization of O_2^- using NBT staining showed that DPI apparently suppressed ROS production due to inhibition of the NADPH oxidases (Figs. 2, 4A). Furthermore, the gene expression of *NADPH oxidases* was hardly suppressed by DPI treatment (Fig. 4B). These results show that DPI suppresses the NADPH oxidases activity but not the gene expression of *NADPH oxidase*.

It is known that ROS plays a key role in the release of dormancy and the completion of germination (Bailly et al., 2008). Treating seeds with H_2O_2 has been shown to promote germination in several species such as rice (Narado et al., 1998), *Zinnia elegans* (Ogawa and Iwabuchi, 2001) and wheat (Wahid et al., 2007). Our data also showed that treatment with H_2O_2 as an exogenous source of ROS significantly promoted seed germination in barley (Fig. 1). The disproportion of H_2O_2 resulting in an increased O_2 level is considered to enhance the oxidative respiration, which can be the reason why the promotion of seed germination was observed. However, respiratory inhibitors promoted the germination of *Z. elegans*, sunflower and barley seeds (Ogawa and Iwabuchi, 2001; Oracz et al., 2009; unpublished), suggesting that such promotion effects are not mainly attributable to the increased O_2 level and that the oxidative respiration is not a rate-limiting step for the seed germination. DPI treatment delayed seed germination and suppressed the accumulation of O_2^- generated in embryo and aleurone layer of barley seed (Figs. 1A, 2). Furthermore, the inhibitory effect of DPI on barley seed germination was reversed by H_2O_2 (Fig. 1B). These results indicated that seed germination in barley was regulated by ROS including H_2O_2 and O_2^- produced by NADPH oxidase after imbibition. The seed germination and dormancy is complicatedly accounted for by control of plant hormones such as abscisic acid (ABA), gibberellins (GA), ethylene, auxin or brassinosteroids (Feurtado et al., 2007). ROS could play pleiotropic and essential roles in the transduction of the hormone signal since the interaction between hormones and ROS in many developmentally controlled processes in plants has been reported (Kwak et al., 2006). Recently, it has been reported that ROS could be a ubiquitous signal involved in dormancy alleviation and facilitate the shift from a dormant to a non-dormant status in seeds (Bailly, 2004; Oracz et al., 2007; El-Maarouf-Bouteau et al., 2008). In this study, DPI treatment markedly suppressed induction of α-amylase activity after imbibitions (Fig. 3A), and production of O_2^- in barley seeds after imbibition, which were detected in the embryo and aleurone layer including scutellar epithelial cells (Fig. 2A). These results indicated that NADPH oxidases act as the sources of ROS production in barley seeds and it might relate to the mechanism of germination and dormancy in seeds regulated by GA and ABA.

There is evidence showing the participation of ROS in controlling rapid cell growth (Carol and Dolan, 2006) and abiotic stress (Yamane et al., 2009). NADPH oxidases as source of ROS have been shown to play versatile and important roles in signaling and development in plants. It has been reported that the root hair cells of *A. thaliana* are regulated by the elevation of the concentration of cytoplasmic Ca^{2+} and the localized production of ROS by the NADPH oxidase (Wymer et al., 1997; Hepler et al., 2001; Foreman et al., 2003; Carol et al., 2005). The production of ROS such as both O_2^- has been detected in the expansion zone of maize leaf blades (Rodriguez et al., 2002). The growth of segments excised from the expansion zone was inhibited by DPI. Indeed, in our results, DPI treatment suppressed shoot and root growth in barley (Fig. 3B, C). Additionally, DPI also markedly suppressed induction of α-amylase activity (Fig. 3A). These results suggested that the suppression of shoot and root growth by DPI in barley seedling was attributed not only to direct inhibition of NADPH oxidase but also to insufficinent supply of nutrition caused by inhibition of α-amylase expression. Interestingly, in switch-grass, treatment with DPI inhibited seed germination and root growth, but not coleoptile growth (Sarath et al., 2007). Further studies are needed to elucidate the mechanism of growth suppressed by DPI. In barley seeds treated with DW, gene expression and enzyme activity of NADPH oxidases are enhanced after imbibition (Fig. 4A, B). Also, the NADPH oxidases gene expression and enzyme activity indicated high correlation with α-amylase activity, shoot and root growth after imbibition (Fig. 5). These results indicated that NADPH oxidases assume a key role in seedling growth in barley.

In conclusion, NADPH oxidases control seed germination and seedling growth in barley through ROS production. ROS plays a dual role in seed physiology as an actor of cellular signaling pathway and a toxic role (Bailly, 2004). Recently, Bailly et al. (2008) have proposed the concept of an "oxidative window" for germination. This window is arrangement of critical levels of ROS on germination. Within the oxidative window ROS play a role in cell signaling on seed germination, on the other hand, upper level of oxidative window is harmful on seed germination because of leading to oxidative damage. NADPH oxidase may play in seed physiology as an actor in "oxidative window" of barley seeds.

Acknowledgements

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