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Rapid Germination of a Barley Mutant Is Correlated with a Rapid Turnover of Abscisic Acid Outside the Embryo

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In our study of the role of abscisic acid (ABA) in controlling the germination of barley grains, we tested a barley mutant line with a gigantum appearance (*Hordeum distichum* cv Quantum) for an ABA-insensitive phenotype by assaying germination in the presence of 10^{-4} M ABA. Dissected embryos of the mutant germinated at least 10 h earlier than did those of the wild type. The half-maximal concentrations of ABA inhibitory for germination were determined to be 5×10^{-4} M for the mutant and 10^{-6} M for the wild type. Expression of an ABA-induced *Rab* gene was studied to determine ABA responsiveness. The ABA concentration required for a half-maximal induction of *Rab* gene expression was 4×10^{-6} M in isolated embryos of both the mutant and wild type. This result suggests that ABA signal transduction pathways were not affected in the mutant. When isolated embryos were allowed to imbibe in water, ABA was released from the mutant and wild-type embryos at the same rate. However, the free ABA level in the incubation medium of the mutant showed a much faster decrease than that of the wild type, as demonstrated by two independent ABA assay methods (high-performance liquid chromatography and enzyme-linked immunosorbent assay). Our results suggest that turnover of ABA outside the embryo is a determining factor in the germination of barley seeds.

The phytohormone ABA plays an important role in development and germination of plant seeds. During early seed development, ABA is involved in embryogenesis, whereas at a later stage of seed development it acts to prevent precocious seed germination (Koornneef et al., 1984; Walker-Simmons, 1987; Groot and Karssen, 1992). ABA-insensitive or ABA-deficient mutants of Arabidopsis are reported to show precocious germination (Karssen et al., 1983, 1987). In cereals, embryos isolated from dormant grains are much more sensitive to exogenous ABA with regard to germination than are embryos isolated from nondormant grains (Walker-Simmons, 1987; Ried and Walker-Simmons, 1990; Corbineau et al., 1991; Van Beckum et al., 1993; Wang et al., 1994). Furthermore, specific ABA-induced mRNAs or proteins in wheat or barley (*Hordeum distichum*) embryos isolated from dormant grains are expressed during a longer period than in embryos isolated from nondormant grains (Walker-Simmons, 1987; Ried and Walker-Simmons, 1990; Wang et al., 1994).

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The inability of dormant barley grains to germinate was found to be due to both a higher endogenous ABA level and a higher ABA sensitivity in these grains as compared with nondormant barley grains (Wang et al., 1995). Our previous studies resulted in a model in which the following factors determined germination of dormant barley: (a) diffusion of ABA out of the embryo, (b) ABA sensitivity of the embryo, and (c) de novo synthesis of ABA (Wang et al., 1995). To test this hypothesis, mutants showing ABA deficiency or an altered sensitivity to ABA were required. However, to our knowledge only one mutant with low basal levels of ABA has been reported for barley (Walker-Simmons et al., 1989).

Here we describe characteristics of a barley mutant with a gigantum appearance (Çağirgan et al., 1995). Embryos isolated from the gigantum barley mutant showed a faster germination than those of the parental line (wild type). We analyzed the responsiveness of this mutant to inhibition of germination by ABA, as well as to specific ABA-induced gene expression. Moreover, the endogenous ABA levels in the isolated embryo were analyzed during germination. We provide evidence that induction of germination requires degradation or conjugation of ABA released from the embryo. This result strengthens the hypothesis that ABA acts outside the embryo during inhibition of germination, as suggested in our previous work (Wang et al., 1995).

MATERIALS AND METHODS

Plant Material

Mature dry grains of barley (*Hordeum distichum* cv Quantum) were treated with 150 Gray of γ-rays, which yielded stable mutant lines, one (M-Q-54) with a gigantum appearance and altered grain yield (Çağirgan et al., 1995). Isolated embryos from the mutant lines were tested for germination on 10^{-4} M ABA. Embryos from M-Q-54 were able to germinate, so this mutant line was chosen to study the role of ABA in the mutant phenotype. After the husk near the embryo was removed embryos were dissected carefully from the grains using a scalpel. Germinated embryos were grown in a phytotron under growth conditions as described previously for nondormant grains (Schuurink et al., 1992). Mature grains were stored at −20°C.

Abbreviation: GI, germination index.

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Germination Tests

Ten to 15 dissected embryos were transferred to two layers of Whatman no. 1 filter paper in a 9-cm Petri dish containing 2 mL of water with or without ABA. The dishes were incubated at 20°C in the dark and sealed with Parafilm (American National Can, Neenah, WI) to prevent evaporation. Embryos were considered germinated if the leaf shoots and roots were ≥1 mm long. A GI for embryos (Walker-Simmons and Sesing, 1989) was determined that gave maximum values to embryos that germinated first and minimum values to those that germinated later:

$$GI = \frac{(3 \times n_1 + 2 \times n_2 + 1 \times n_3)}{3 \times \text{total embryos}}$$

where n_i represents the number of germinated embryos at i = 1, 2, and 3 d, respectively; 3, 2, and 1 are the values given to the number of embryos germinated during the 1st, 2nd, and 3rd d, respectively. The maximum value of GI is 1.0 and the minimum value is 0.0.

Ten to 20 whole seeds were germinated at 10 and 20°C in the dark to determine the dormancy index, a parameter to determine dormancy levels (Schuurink et al., 1992). The wild-type and mutant grains had a similar dormancy index, indicating a similar level of dormancy (data not shown).

ABA Sensitivity Test

Dissected embryos were first incubated in 2 mL of distilled water for 3 h in polystyrene, 24-well plates (Costar, Cambridge, MA), with each well containing 10 embryos, to remove endogenous ABA. The embryos were then transferred to a new plate, with each well containing 10 embryos and 300 μL of distilled water with or without different ABA concentrations. These plates were incubated for 2 h at 20°C in the dark. During incubation, the plates were sealed to prevent evaporation. After incubation the embryos were briefly dried on Whatman filter paper, and total RNA was extracted (see below) or ABA levels in the embryos and medium were determined. (±)-*cis,trans*-ABA was purchased from Sigma.

RNA Isolation and Analysis

Embryos were ground to powder in liquid nitrogen. Total cellular RNA was isolated and purified as described previously (Wang et al., 1992). Northern blots were made by separating 10 μg of RNA on a glyoxal/DMSO, 1% agarose gel (Sambrook et al., 1989). RNA was transferred to a Genescreen (DuPont) nylon membrane using 10× SSC as a transfer buffer. Blots were hybridized to the *Rab*-16 cDNA probe (Mundy et al., 1990) at 65°C in 1% SDS, 1 M NaCl, and 10% dextran sulfate containing 0.1 mg/mL denatured salmon sperm DNA. Blots were rehybridized with a ribosomal probe to check equal loading of the lanes.

ABA Measurements

ABA extraction was performed as described by Walker-Simmons (1987) with some modifications (Wang et al., 1995). The embryos were ground to powder in liquid nitrogen and vacuum-dried. After dry weight was determined, ABA was extracted from the powder by shaking overnight in 250 μL of methanol containing 100 mg/L butylated hydroxytoluene and 0.5 g/L citric acid monohydrate. After the sample was centrifuged, the supernatant was vacuum-dried and subsequently dissolved in 30 μL of methanol. Samples of the incubation medium were tested for ABA. The ABA concentration was determined by using specific ABA monoclonal antibodies that recognize only free (+)ABA in an ELISA performed according to the instructions of the manufacturer (Idetek, San Bruno, CA).

For analytical UV-HPLC analysis, ABA was separated using a 250- × 4.6-mm C_{18} 5-μm Hypersil ODS (Altech Associates, Deerfield, IL) column and a 50-μL injection loop. The mobile phase consisted of a linear gradient of 0 to 100% methanol in 0.1 M acetic acid, which was delivered at a flow rate of 1 mL/min by a PU-980 pump (Jasco, Tokyo, Japan), equipped with a Jasco LG-980–02 Ternary Gradient Unit. For UV detection, an L-4000 detector (Merck-Hitachi, Tokyo, Japan) was used at 257 nm. The retention time of ABA in this system was 15.7 min. Embryos were soaked in water for 8 h. Medium was collected, further incubated at 25°C for 16 h, and freeze-dried. The pellet was extracted twice with 1 mL of methanol. The samples were concentrated 30 times with respect to the original volume of medium prior to HPLC analysis. An ABA concentration from 10 to 100 μM was used as the standard.

RESULTS

Selection of Mutant and Phenotype

γ-ray-mutagenized barley was selected for mutant phenotypes with altered plant height and grain yield. One gigantum stable mutant line was obtained. Embryos isolated from grains of the gigantum mutant line (M-Q-54) showed an ABA-insensitive phenotype by germination in the presence of 10^{-4} M ABA (see below). Embryos able to germinate were grown in a phytotron to produce more grains. During growth of this generation, we confirmed that the mutant plants showed the typical phenotype: at an early stage, the plants showed growth retardation and a bushy phenotype as compared with the wild type until the stage in which the spikes were formed. After spike formation, the mutant plants grew to slightly larger sizes, and it is interesting that they produced about 1.4 times more grains (44 ± 3 g/plant grains produced by the parental line and 60 ± 4.5 g/plant grains produced by the mutant line).

Germination and ABA Sensitivity

Embryos from mutant and wild-type grains were used to investigate the nature of the mutation. When isolated embryos were germinated on water, the mutants germinated faster than did embryos of the wild type (Fig. 1). The time required for 50% germination was about 15 h, and 100% germination was reached at 48 h for the mutant. However, at 48 h the wild type reached a level of germination of only 55%.

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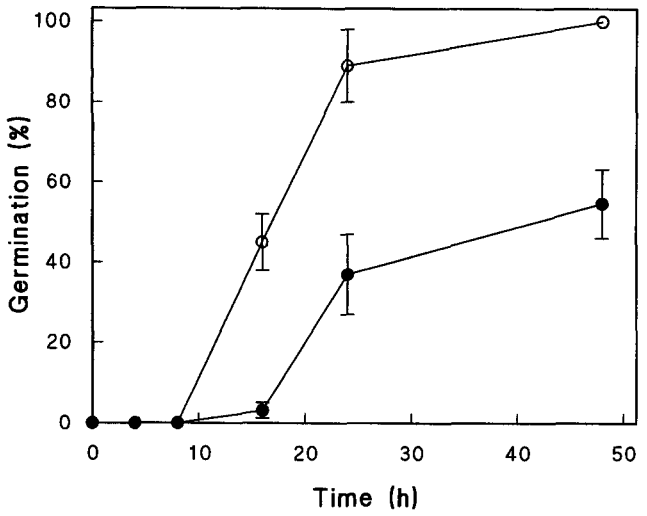


Figure 1. Germination of embryos isolated from wild-type (●) and mutant (○) grains. Isolated embryos were placed in a Petri dish on two layers of Whatman no. 1 filter paper containing 2 mL of water and incubated at 20°C in the dark. Germination percentages were recorded for 3 d. Embryos were considered germinated when the root and shoot were ≥1 mm long. Data are the means ± SD of four independent experiments.

Since mutant embryos were able to germinate in the presence of a high exogenous ABA concentration (10^{-4} M), which normally represents an inhibitory concentration for germination of isolated barley embryos (Van Beckum et al., 1993), we investigated whether the ability to germinate at high ABA concentrations was due to a decreased sensitivity to ABA. Isolated embryos were germinated on various ABA concentrations to determine differences in sensitivity between the mutant and the wild type. Figure 2 shows the effect of ABA on the GI of isolated embryos. Without ABA, the mutant showed a higher GI, which corresponds to its faster germination. With an increasing concentration of added ABA, a dramatic decrease in GI was observed in the isolated embryos from the wild-type grains. In contrast, at ABA concentrations from 10^{-7} to 10^{-5} M, there was hardly any effect on the GI of embryos isolated from the mutant grains. The half-maximal inhibitory ABA concentration was determined to be 10^{-6} M for the wild type, and for the mutant it was 5×10^{-4} M. These findings suggest that the mutant embryos are much less sensitive to added ABA with regard to inhibition of germination. This phenotype may have two possible causes: either ABA is not correctly recognized and the signal not properly transduced or ABA is degraded or conjugated.

Rapid germination could be caused by a mutation leading to an altered response to GA. However, mutant embryos germinating on GA_3 did not show a different germination phenotype as compared with the wild type (data not shown). Although aleurone does not produce GA, dose-response experiments on GA-induced α-amylase production in isolated aleurone from both wild type and mutant yielded similar data (not shown). This suggests that the mutant is not altered with regard to GA-induced responses.

ABA Signal Perception and Response

To test ABA perception and signal transduction, *Rab*-16 gene expression in response to ABA was studied. First, the embryos were incubated in water to remove endogenous ABA, thereby reducing the basal level of *Rab* expression (Fig. 3A). During incubation of the embryos over a period of 4 h, *Rab* gene expression first increased and then decreased to an almost nondetectable level (Fig. 3A). The observed increase during the incubation of the embryos may be due to de novo ABA synthesis (although this hardly occurred when nondormant embryos were allowed to imbibe; Wang et al., 1995) or to a slower degradation of ABA.

For dose-response experiments, both mutant and wild-type embryos were first incubated for 3 h in water, which caused *Rab* expression to decrease to almost nondetectable levels (Fig. 3A). Subsequently, these embryos were incubated in the presence of various ABA concentrations for 4 h in the dark at 20°C. The dose-response curve for ABA-induced *Rab* gene expression of isolated embryos is shown in Figure 3B. When the applied ABA concentration was lower than 10^{-7} M, *Rab* gene expression was almost nondetectable in embryos isolated from both wild-type and mutant grains (Fig. 3B). The half-maximal concentration of ABA required for induction of *Rab* gene expression in both mutant and wild-type embryos was 4×10^{-6} M. Since the *Rab* gene expression showed a similar pattern for both wild type and mutant (Fig. 3B), this suggested that the mutation causing the ABA-insensitive character of embryo germination was not due to alterations of ABA perception or signal transduction.

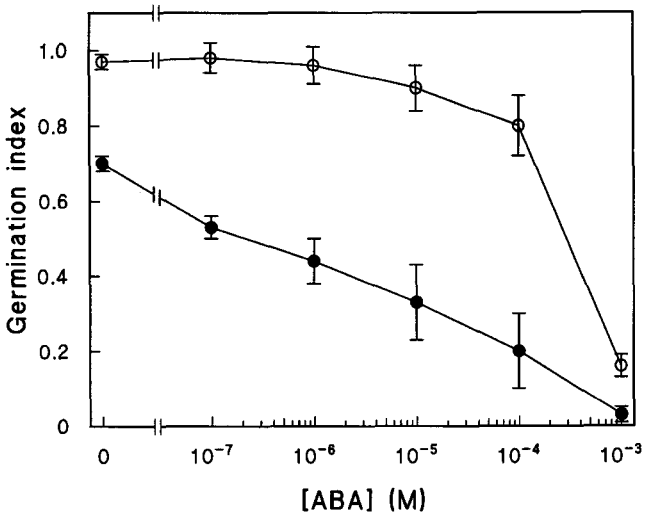


Figure 2. Effect of ABA on the GI of isolated embryos from wild-type (●) and mutant (○) barley grains. Embryos were germinated in the presence of different ABA concentrations. The GI was calculated as described in "Materials and Methods." Data are the means ± SD of four independent experiments.

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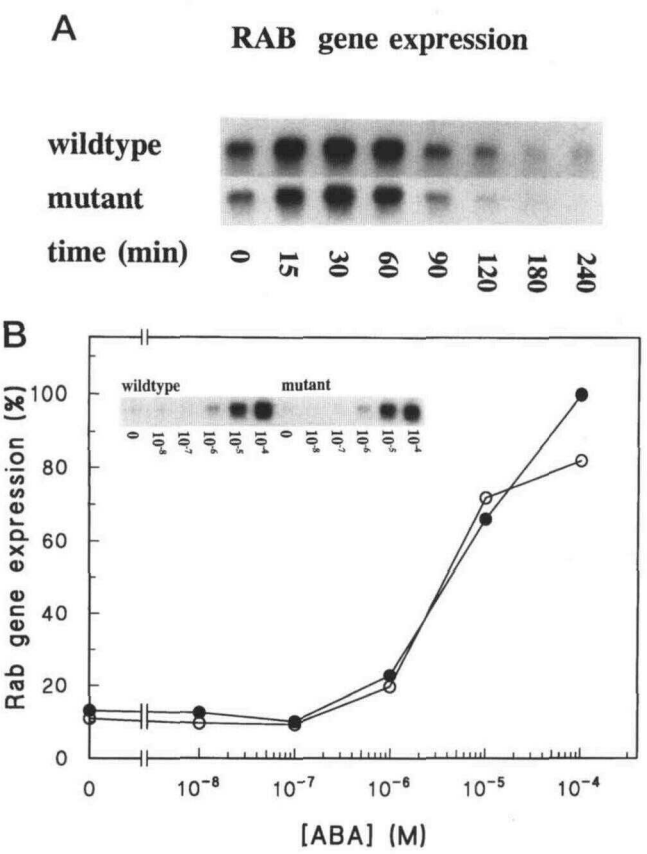


Figure 3. A, Effect of incubation in water of isolated embryos from wild-type and mutant barley grains on *Rab* gene expression. Ten embryos were placed in 2 mL of water. At the indicated times samples were taken and *Rab* gene expression was determined by northern analysis with an *Rab*-16 rice cDNA probe. B, Dose-response curve of ABA-induced *Rab* gene expression in embryos isolated from the wild type (●) and mutant (○), which were preincubated for 3 h in water before the ABA incubation. Data are those from the autoradiograph presented in the inset. One typical experiment of three is shown.

Rab Gene Expression in Isolated Embryos during Germination

To further investigate the cause of the ABA-insensitive phenotype of the mutant, we studied *Rab* gene expression in both types of embryos during germination in the absence of added ABA. The selected *Rab* gene is specifically induced by ABA, and maintenance of gene expression is dependent on the ABA concentration. Therefore, the expression level of this *Rab* gene is considered to be a specific indicator of the ABA concentration (Van Beckum et al., 1993). During germination of isolated mutant embryos on filter paper, *Rab* gene expression in the embryos showed a rapid decrease (Fig. 4). After 16 h, only a minimal amount of *Rab* gene expression was detectable. This time corresponded to the moment at which the embryos started to germinate (Fig. 1). The wild type showed a much slower decline in *Rab* gene expression. These data point to a more rapid ABA decrease in the embryos of the mutant, as compared with the wild-type embryos.

ABA Turnover

In barley grains, a decrease in the endogenous ABA level during imbibition is correlated with the start of germination (Van Beckum et al., 1993; Wang et al., 1995). Thus, a more rapid germination, together with a disappearance of *Rab* mRNA (Figs. 1 and 4), is likely to be due to a more rapid decline of ABA inside or outside the embryo. First, we determined ABA levels in the isolated embryos from both mutant and wild-type grains during germination by using a specific ELISA (see "Materials and Methods").

ABA levels in the isolated embryos were measured during embryo germination on Whatman filter paper. First, the amount of ABA in dry embryos was determined (Fig. 5). In the wild-type embryos, the ABA amount was 313 ± 41 pg/mg dry weight, whereas in the mutant it was about 422 ± 49 pg/mg dry weight. During germination, the ABA levels in both the wild-type embryos and the mutant embryos initially decreased at a similar rate (Fig. 5). However, the germination of the mutant embryos started earlier than that of the wild-type embryos. Thus, the more rapid germination of mutant embryos could not be explained by lower levels of ABA in the embryo. Declining levels of ABA in the embryo could be due to diffusion of ABA out of the embryo into the filter paper, where it may be rapidly degraded. This could explain the more rapid germination of the mutant embryos. The ABA level in the mutant embryos finally decreased to 55 ± 5.3 pg/mg dry weight (Fig. 5B). At 48 h, the ABA level in the mutant embryos was very low, and a 100% germination rate was observed (Fig. 5b). In the wild-type embryos, a higher level of ABA was measured at 48 h, and the germination rate reached only 55% (Fig. 5A). The high ABA level measured in wild-type embryos at 48 h might be due to the contribution of high ABA levels in nongerminated embryos.

Extracellular ABA Acts as an Inhibitor for Germination

When embryos are soaked on Whatman filter paper, it is difficult to determine the total amount of ABA in the system, since ABA diffused out of the embryo is difficult to detect in the filter paper. To overcome this complication we used a different imbibition system (Wang et al., 1995). Isolated embryos from both wild type and mutant were

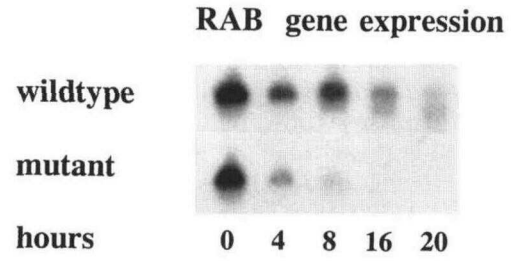


Figure 4. *Rab* gene expression during germination of isolated embryos. Isolated embryos from the wild type and mutant were germinated on filter paper in a Petri dish. *Rab* gene expression during germination was determined by northern analysis with the *Rab*-16 rice cDNA probe. One typical experiment of three independent experiments is presented.

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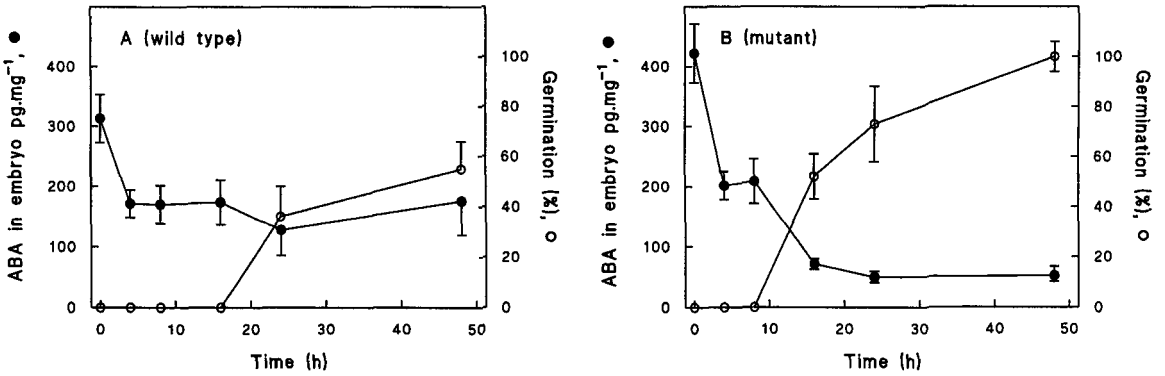


Figure 5. ABA in isolated embryos during germination. Embryos isolated from wild type (A) and mutant (B) were germinated on Whatman no. 1 filter paper. ABA was measured in embryos during germination. Data are the means ± SD of three independent experiments. Closed symbols, ABA in embryos; open symbols, germination percentages.

incubated in a 24-well plate, with each well containing 10 embryos in 300 μL water. After different incubation periods, the ABA levels in both the embryos and the incubation medium were analyzed. Figure 6 shows a fast decrease in ABA level in the embryos from both wild type and mutant. The rate of decrease in ABA level is similar to that which we previously reported (Wang et al., 1995). However, there is a clear difference between wild type and mutant when the amounts of ABA accumulating in the incubation medium are compared (Fig. 6, cf. A and B). The ABA levels at 6 h were determined to be 41 ± 1.2 nM for the wild type and 36 ± 1.4 nM for the mutant. These levels are consistent with the inhibitory concentrations for the wild type, but not for the mutant (Fig. 2). A much faster decrease in ABA level was observed in the incubation medium of mutant embryos than in that of wild-type embryos. Since the decrease in ABA level in the medium is not accompanied by an increase in ABA level in the embryos (Fig. 6), we may conclude that the decrease in ABA level is due to turnover or conjugation of ABA.

A decrease in ABA level in the incubation medium is correlated with the observed start of germination. The mutant embryos started to germinate at 6 h, whereas the wild-type embryos started at 18 h (Figs. 1 and 6). These observations indicate that the inhibitory effect of ABA on germination requires a relatively high ABA level outside the embryo.

ABA Turnover Occurs Outside of the Embryo

A rapid decrease in ABA outside the embryos would suggest that metabolic activity is responsible for the turnover of ABA into inactive compounds. There are two possibilities: either ABA is quickly degraded within the embryo after re-uptake and, therefore, we could not observe the re-uptake in the embryo, or degradation of ABA occurs outside the embryo. The latter possibility was tested first. The embryos were allowed to imbibe (10 embryos per 300 μL water). After 8 h the embryos were removed from the incubation medium. ABA levels in the incubation medium from both soaked wild-type and mutant embryos were measured. After an additional 16 h of incubation (without embryos), the medium from the wild-type embryos showed only a slight decrease in ABA, whereas in the medium from the mutant embryos the ABA level decreased 36.9% (Fig. 7). The latter could be confirmed by HPLC analysis of medium from mutant embryos. In both ELISA and HPLC assays, a similar amount of ABA incubated for 16 h in water did not diminish. These results clearly demonstrate a more rapid ABA turnover outside

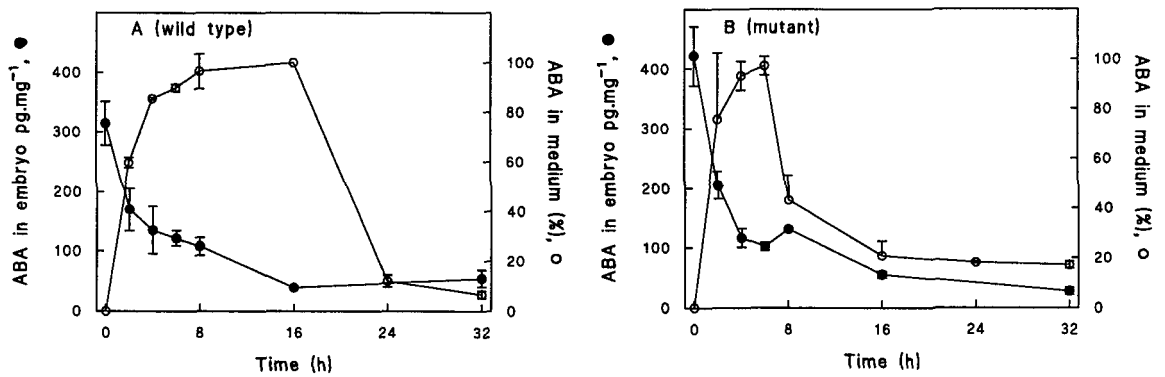


Figure 6. ABA in isolated embryos (●) and in the incubation medium (○) of wild type (A) and mutant (B) during germination in 300 μL of water. The data of ABA in the medium were calculated from the data point 8 h for wild type and 6 h for mutant (100%). Data are the means ± SD of three independent experiments.

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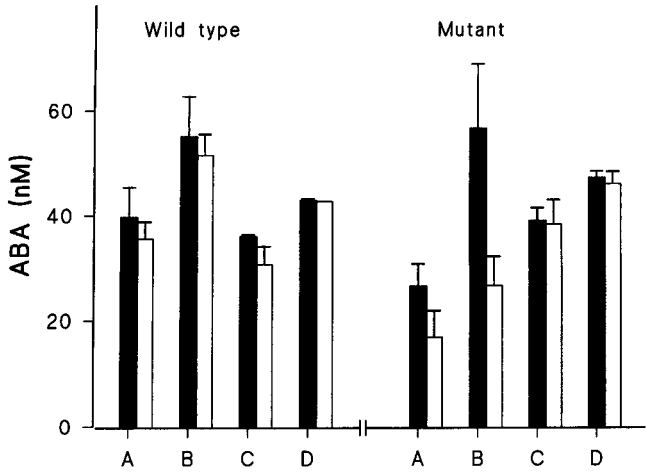


Figure 7. ABA concentrations in the incubation medium of wild-type and mutant barley embryos. Embryos were incubated in water at 25°C in the dark and were removed from the incubation medium after 8 h (time = 0). Samples of the medium were then treated as follows. A, Medium untreated; B, medium plus 500 pg of (+)ABA; C, medium boiled for 5 min; and D, medium boiled for 5 min plus 500 pg of (+)ABA. ABA levels in the medium were measured at time = 0 and after further incubation for 16 h. Solid bars, time = 0; open bars, time = 16 h. Data are the means ± SD of four experiments.

mutant embryos. In addition, when mutant embryos were germinated in a well together with wild-type embryos, the mixture showed the same germination rate as mutant embryos alone (data not shown).

Extra ABA was added to the incubation medium after the embryos were removed, as mentioned above. The ABA level in the medium from the mutant again showed a considerable decrease after an additional 16 h of incubation, whereas in the wild-type medium hardly any decrease was observed (Fig. 7). There was no decrease in ABA level observed following the addition of ABA to boiled medium from the mutant.

DISCUSSION

Our previous work demonstrated that a decrease in endogenous ABA in the embryo was required for triggering the germination of barley grains. It has been hypothesized that germination of barley requires one or more of the following factors: (a) an increase in the ability of ABA to diffuse out of the embryo, (b) a reduction in the ABA sensitivity of the embryo, and/or (c) inhibition of de novo ABA synthesis (Wang et al., 1995). The results from the present study add a fourth factor to the hypothesis: increased ability to degrade extracellular ABA.

Here we have described a barley mutant that is almost insensitive to ABA with regard to embryo germination. The mutant embryos germinate faster than do wild-type embryos. The altered sensitivity of the mutant to ABA appeared to be due to a fast decrease in ABA levels in the embryo rather than to changes in the ABA signal transduction pathway(s). The rapid germination of the mutant embryos was not due to a lower ABA level in the dry embryo, since the ABA level in the mutant embryos was even slightly higher than that in the wild type. Also, the difference in the germination of embryos in both wild type and mutant was not due to a different dormancy level, as was determined by germination of whole mutant grains at 10 and at 20°C. A rapid decline of active ABA from the mutant embryos could point to a more rapid diffusion of ABA out of the mutant embryos, thus providing the ability to germinate earlier. However, the ABA levels in both the wild-type and mutant embryos showed a similar rate of decrease during germination, whereas the germination of the mutant embryos started earlier (Figs. 1 and 5). This indicates that a decrease in endogenous ABA in the embryo is not sufficient for triggering germination.

A decrease in the ABA level in the embryo was accompanied by an increase in ABA in the incubation medium (Fig. 6). The subsequent decrease in ABA in the medium could not be explained by re-uptake into the embryo, since the ABA level in the embryo remained low. However, both by specific ABA ELISA and by HPLC analysis we showed that the ABA level in the incubation medium from mutant embryos decreased much more rapidly than in the wild-type incubation medium. The fast decrease in the medium from the mutant embryos corresponded to the early start of germination of the embryos (Figs. 5 and 6). This finding suggests that the inhibitory action of ABA occurs at the outside of the embryo; thus, an ABA-perception site might be located at the cell surface. This is consistent with our previous report in which it was shown that a higher ABA level in the incubation medium of dormant embryos was correlated with a slower germination (Wang et al., 1995). In addition, it was reported for barley aleurone protoplasts and *Commelina* guard cells that ABA is likely to act extracellularly (Anderson et al., 1994; Gilroy and Jones, 1994).

During incubation of embryos in water, an increase in *Rab* expression was observed in both wild type and mutant, followed by a decrease. Since the induction and maintenance of *Rab* gene expression is dependent on the amount of ABA (Van Beckum et al., 1993), the initial increase in *Rab* gene expression could be due to an increase in ABA level inside or outside of the embryo caused either by de novo ABA synthesis in the embryo or by diffusion of ABA out of the embryos, as described by Slovik et al. (1992) in a model of ABA diffusion. It is also possible that enzyme(s) involved in ABA degradation are secreted from the embryo and are immediately diluted in the incubation medium in the first period of incubation, since the ABA turnover occurred outside of the embryos. Consequently, an ABA peak was observed in the incubation medium. However, when embryos were soaked in water no initial increase in ABA in the embryo was observed (Fig. 6). In contrast, an increase in ABA level was observed outside of the embryos in the incubation medium. Thus, an initial increase in *Rab* gene expression in the embryos (Fig. 3A) is correlated with an increase in ABA level in the incubation medium (Fig. 6). This observation suggests that the action of ABA on the induction of *Rab* gene expression is likely to occur from the outside of the embryo. In whole grains, the ABA-degrading

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enzymes will be located near the embryo, resulting in an ABA gradient in the grain.

Our data strongly suggest that the ABA turnover can occur in vitro outside of the embryo tissue, which, to our knowledge, has not been demonstrated before. The nature of the mutation is likely to be in the enzymes involved in the degradation/conjugation pathway of ABA.

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