

The α -Amylase Induction in Endosperm during Rice Seed Germination Is Caused by Gibberellin Synthesized in Epithelium¹

Miyuki Kaneko, Hironori Itoh, Miyako Ueguchi-Tanaka, Motoyuki Ashikari, and Makoto Matsuoka*

BioScience Center, Nagoya University, Chikusa, Nagoya 464–8601, Japan

We recently isolated two genes (*OsGA3ox1* and *OsGA3ox2*) from rice (*Oryza sativa*) encoding 3β -hydroxylase, which catalyzes the final step of active gibberellin (GA) biosynthesis (H. Itoh, M. Ueguchi-Tanaka, N. Sentoku, H. Kitano, M. Matsuoka, M. Kobayashi [2001] Proc Natl Acad Sci USA 98: 8909–8914). Using these cloned cDNAs, we analyzed the temporal and spatial expression patterns of the 3β -hydroxylase genes and also an α -amylase gene (*RAmy1A*) during rice seed germination to investigate the relationship between GA biosynthesis and α -amylase expression. Northern-blot analyses revealed that *RAmy1A* expression in the embryo occurs before the induction of 3β -hydroxylase expression, whereas in the endosperm, a high level of *RAmy1A* expression occurs 1 to 2 d after the peak of *OsGA3ox2* expression and only in the absence of uniconazol. Based on the analysis of an *OsGA3ox2* null mutant (*d18-Akibare dwarf*), we determined that 3β -hydroxylase produced by *OsGA3ox2* is important for the induction of *RAmy1A* expression and that the *OsGA3ox1* product is not essential for α -amylase induction. The expression of *OsGA3ox2* was localized to the shoot region and epithelium of the embryo, strongly suggesting that active GA biosynthesis occurs in these two regions. The synthesis of active GA in the epithelium is important for α -amylase expression in the endosperm, because an embryonic mutant defective in shoot formation, but which developed epithelium cells, induced α -amylase expression in the endosperm, whereas a mutant defective in epithelium development did not.

During cereal seed germination, α -amylase in the aleurone layer plays an important role in hydrolyzing the endosperm starch into metabolizable sugars, which provide the energy for the growth of roots and shoots (Akazawa and Hara-Mishimura, 1985; Beck and Ziegler, 1989). Previous physiological and biochemical studies have revealed that α -amylase expression in the aleurone layer occurs as follows. First, active gibberellin (GA) biosynthesis commences in the embryo, and the GAs are transported from the embryo to the aleurone layer (Fincher, 1989). Active GAs trigger the expression of α -amylase at the transcriptional level through the induction of a positive transactivating factor for α -amylase transcription (Gubler et al., 1995). Then, α -amylase is secreted from the aleurone layer into the endosperm to catalyze the hydrating reaction of stored starch. Although the process of induction of α -amylase expression in the aleurone layer is well understood, the mechanism of GA biosynthesis in the embryo during cereal seed germination is still unclear. For example, quantitative analysis of GAs using GC-MS has revealed that the level of active GAs is increased in the shoot and

scutella regions during the early stage of barley (*Hordeum vulgare*) seed germination (Lenton et al., 1994), but it is not known which region, shoot or scutella, is important for α -amylase induction.

A set of genes encoding enzymes involved in the GA biosynthetic pathway has been isolated from various plant species (Lange et al., 1994; Sun and Kamiya, 1994; Chiang et al., 1995; Yamaguchi et al., 1996; Lange, 1997; Helliwell et al., 1998; Thomas et al., 1999). We recently isolated two genes encoding GA 3β -hydroxylases, *OsGA3ox1* (*Oryza sativa* GA 3β -hydroxylase 1) and *OsGA3ox2*, from rice (*Oryza sativa*; Itoh et al., 2001). Because GA 3β -hydroxylase catalyzes the final step of the GA biosynthetic pathway and produces bioactive GA₁ and GA₄ from the substrates GA₂₀ and GA₉, tissues or cells that express this enzyme produce the bioactive GAs if the substrates are supplied. *OsGA3ox2* is preferentially expressed in young leaves of rice seedlings at the vegetative stage, and both genes are highly expressed in tapetum cells of the anther (Itoh et al., 2001). These tissues and organs are known to actively synthesize the active GAs (Kobayashi et al., 1988). Therefore, it should be possible to observe where and when active GAs are synthesized in the embryo during rice seed germination through the expression analysis of *OsGA3ox1* and *OsGA3ox2*.

In this study, to elucidate the temporal and spatial pattern of GA synthesis during rice seed germination, we analyzed the expression kinetics and in situ localization of *OsGA3ox1* and *OsGA3ox2* in germinating seeds. Northern analyses showed that the

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* Corresponding author; e-mail makoto@nuagr1.agr.nagoya-u.ac.jp; fax 81–52–789–5226.

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OsGA3ox2 product is important for α -amylase expression, whereas the *OsGA3ox1* product is not. Using mutants defective in shoot formation and/or scutella development, we found that the scutella is essential for α -amylase induction but that the shoot region is not involved in this process.

RESULTS

GA 3 β -Hydroxylase Expression in the Embryo Occurs before α -Amylase Expression in Endosperm

We compared the induction kinetics of the major GA-induced α -amylase gene, *RAmy1A* (O'Neil et al., 1990), to that of the two *OsGA3ox* genes in the embryo during the early germination stage. In the embryo, accumulation of *RAmy1A* mRNA started at 6 to 12 h after the beginning of imbibition with or without uniconazol, which is an inhibitor of GA biosynthesis, and then reached at plateau after 24 h (Fig. 1A, a and b). In contrast to the embryo, accumulation of *RAmy1A* mRNA in the endosperm was hardly detected until 12 h after imbibition with or without the uniconazol treatment (Fig. 1A, a). Up to 48 h after imbibition, *RAmy1A* expression in treated or untreated endosperm reached a similar level to that in the embryo at 24 h after imbibition (Fig. 1A, b). Then, the level of *RAmy1A* mRNA increased more than 5-fold during the following 24 h in the untreated endosperm, whereas it increased by only about 2-fold during the same period in the treated endosperm (Fig. 1A, a and b). This inhibitory effect of uniconazol on α -amylase induction indicates that

α -amylase expression in the endosperm is dependent on de novo-synthesized GA during seed germination.

To further investigate the relationship between α -amylase expression in the endosperm and GA biosynthesis in the embryo, we studied the expression kinetics of the two GA 3 β -hydroxylase genes, *OsGA3ox1* and *OsGA3ox2*, in the embryo during seed germination (Fig. 1B). The expression of *OsGA3ox1* was constitutively seen at low level in this period with or without uniconazol. On the other hand, *OsGA3ox2* expression rapidly increased during the first 12 h to reach a level several times higher than that of dry seeds, or 10 times higher than that of *OsGA3ox1*. During the next 24 h, *OsGA3ox2* expression was reduced to the basal level in untreated seeds but was maintained at a high level in uniconazol-treated seeds (Fig. 1B, a and b). *OsGA3ox2* mRNA levels at 0 and 6 h were not increased by uniconazol. This delayed response is probably because the time needed for uniconazol to inhibit GA biosynthesis after diffusion into the embryos and for the GA catabolism to reduce the amount of bioactive GAs (Fig. 1B, a). The observed rapid increase and high-level expression of *OsGA3ox2* after imbibition suggest that the *OsGA3ox2* product may have a major role in the de novo GA synthesis to induce *RAmy1A* expression. The induction kinetics of *OsGA3ox2* in the embryo seems to be consistent with the GA-dependent induction of *RAmy1A*-expression in the endosperm; the peak of *OsGA3ox2* expression occurred about 50 h before the rapid increase in *RAmy1A* expression. The

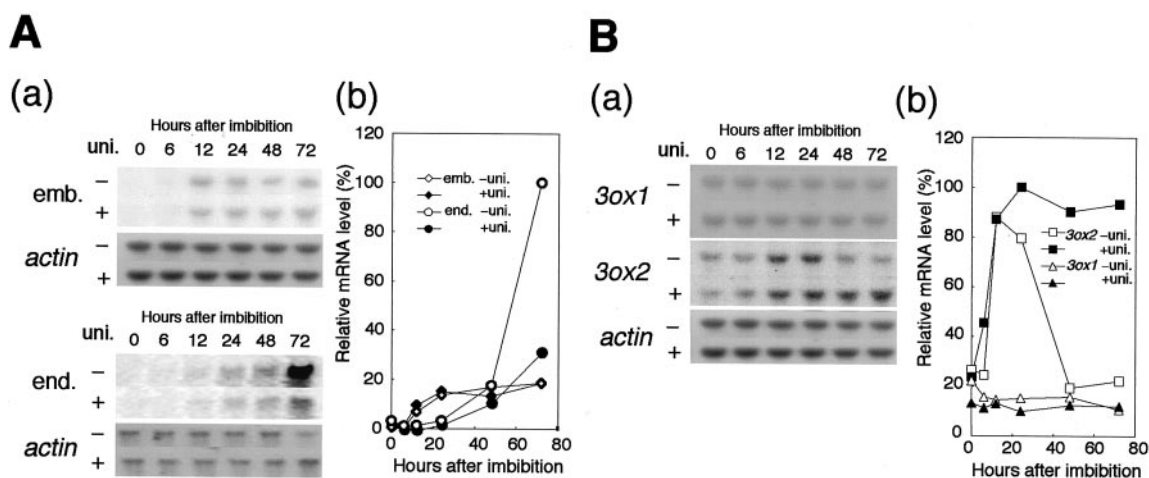


Figure 1. Expression of *RAmy1A* (A), *OsGA3ox1*, and *OsGA3ox2* (B) in germinating rice seeds. A (a), Northern analysis using the *RAmy1A* probe. Seeds were germinated for the indicated hours after imbibition with or without uniconazol (uni. + or -), 5 μ g of total RNA was prepared from the treated and untreated embryo, and 1 μ g of total RNA was prepared from the treated and untreated endosperm. A (b), Relative amount of *RAmy1A* transcript in the embryo (diamonds) and endosperm (circles) in the presence (black symbols) or absence (white symbols) of uniconazol. The level of the *RAmy1A* transcript was normalized against the level of the actin transcript. The normalized values shown here are relative to the expression levels detected in endosperm of untreated seeds at 72 h (100%). B (a), Northern analysis of *OsGA3ox2* and *OsGA3ox1* in embryo. Five micrograms of total RNA was prepared to detect *OsGA3ox2*, and 20 μ g of total RNA was prepared to detect *OsGA3ox1*. B (b), Relative level of the *OsGA3ox1* (triangles) and *OsGA3ox2* (squares) transcripts in the germinating rice embryo treated with (black symbols) or without (white symbols) uniconazol. Normalization of *OsGA3ox* expression is the same as in A.

rapid decrease in *OsGA3ox2* expression in the untreated seeds can be explained by a negative feedback repression of *OsGA3ox2* by GA (see "Discussion").

The *OsGA3ox2* (D18) Product Is Necessary for *RAmy1A* Expression in the Endosperm

The comparative study of the induction kinetics of GA 3β-hydroxylase and α-amylase suggested that 3β-hydroxylase produced by *OsGA3ox2* may be important for the induction of α-amylase expression in the endosperm, whereas the product of *OsGA3ox1* may not importantly contribute to α-amylase induction. To confirm this hypothesis, we compared the pattern of *RAmy1A* expression in the endosperm between the wild-type rice plant, Akibare, and the loss-of-function mutant of *OsGA3ox2*, *d18-Akibare dwarf* (*d18-AD*; Fig. 2). The *d18-AD* mutant was derived from the wild-type cultivar by chemical treatment and has a null allele of *OsGA3ox2* caused by complete deletion of all exon and intron sequences (Itoh et al., 2001).

In the wild-type plant, accumulation of the *RAmy1A* transcript in the endosperm occurred at a level about 2.5 times higher than that in the seeds treated with uniconazol (Fig. 2B). *RAmy1A* mRNA did not accumulate to this extent in the *d18-AD* mutant and the uniconazol treatment had no significant effect on the mutant.

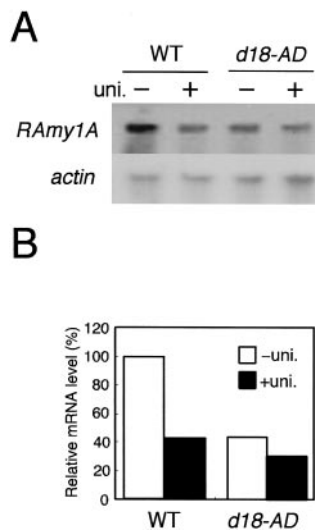


Figure 2. Comparison of the level of *RAmy1A* expression in wild-type (Akibare) and *d18-AD* mutant plants. A, After 72 h of imbibition with or without uniconazol (uni. + or -), total RNA was extracted from the endosperm of wild-type and *d18-AD* plants; 1 μg of total RNA was used for the northern analysis. The blot was re-probed with a radiolabeled actin probe as a loading control. B, Relative amount of *RAmy1A* mRNA. The *RAmy1A* mRNA level was normalized to the corresponding actin transcript level. The values shown are relative to that of the wild type without uniconazol treatment (100%).

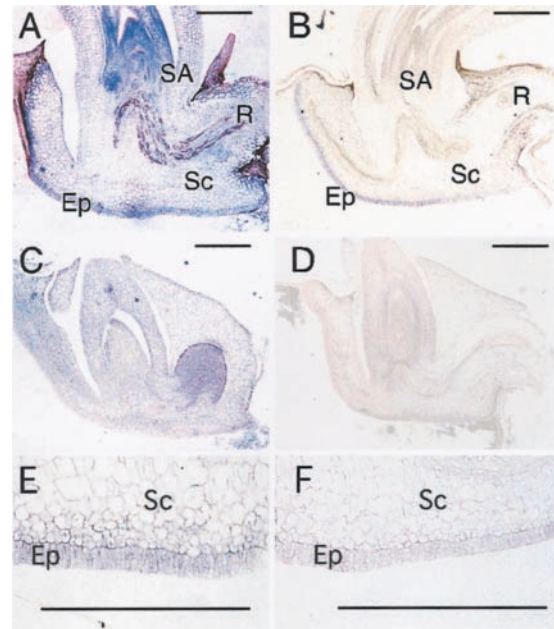


Figure 3. In situ hybridization of *OsGA3ox1* and *OsGA3ox2* transcripts in germinating embryo. Longitudinal sections through rice embryos fixed after 24 h of imbibition were hybridized with digoxigenin-UTP-labeled *OsGA3ox1* (B and F) and *OsGA3ox2* (A and E) antisense probes. E and F, Higher magnification views of epithelium cell layers. The section was hybridized with the sense probe for *OsGA3ox1* (D) or *OsGA3ox2* (C) as control experiment. SA, Shoot apex; R, root; Sc, scutellum; Ep, epithelium. Bar indicates 500 μm.

GA Biosynthesis in the Scutella Epithelium Is Essential for the Induction of α-Amylase Expression

To identify the site of GA biosynthesis, we performed an in situ hybridization study of the *OsGA3ox2* transcript and also of the *OsGA3ox1* in germinating seeds. A strong signal for the *OsGA3ox2* transcript was detected in the shoot apical region, including young leaf primordia and in the epithelial cells facing the endosperm (Fig. 3, A and E). The *OsGA3ox1* transcript was also localized in the epithelium but not in the shoot apical region (Fig. 3, B and F). When we used the sense strands as probes, such specific signal was not seen at all (Fig. 3, C and D). The localized expression of *OsGA3ox2* indicates that there are two regions where the active GA is actively synthesized in germinating rice seeds, namely the shoot apical region and the epithelium.

We also examined which gene, *OsGA3ox1* or *OsGA3ox2*, was dominantly expressed in epithelium by northern analysis (Fig. 4), because the in situ analysis demonstrated that both genes expressed in epithelium. Total RNA was isolated from the embryo removed from the shoot region. The expression pattern of each gene agreed well with the previous results using embryo including the shoot region as shown in Figure 1B, and the amount of *OsGA3ox2* was much higher than that of *OsGA3ox1* at 0.5 or 1 d after imbibition.

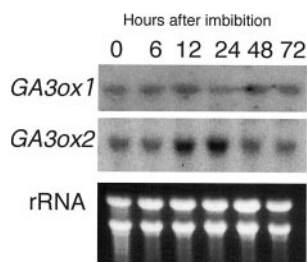


Figure 4. Level of *OsGA3ox1* and *OsGA3ox2* expression in the shoot-removed embryo. Total RNAs were hybridized with *OsGA3ox1* (upper panel) and *OsGA3ox2* (lower panel) probes. Seeds were germinated for the indicated hours after imbibition. Ten micrograms of total RNA from shoot-removed embryo was loaded on the gel and stained with ethidium bromide (rRNA).

The above results indicate that the shoot apex and epithelial layer where *OsGA3ox2* expression occurs is responsible for the induction of α -amylase in endosperm. Nagato et al. (1989) isolated many rice embryonic mutants, and some of these mutants cannot form shoot organs. By using these embryonic mutants, we investigated whether both organ and tissue are required for the α -amylase induction in endosperm. We used three mutants defective in the formation of shoots (Fig. 5A). *shootless2* (*shl2*) develops almost all of the embryonic organs, such as scutellum with epithelium, root, and vascular tissues, but does not form shoot-related tissues, such as the shoot apical meristem, coleoptile, epiblast, and leaf primordia (Sato et al., 1999). *organless1* (*orl1*) forms a scutellum-like organ with the palisade cells facing toward the endosperm, but lacks almost all other organs. The expression of *RAmy1A* in the palisade cells in *orl1* occurs the same as in the wild-type epithelium (Nagato et al., 1989), indicating that the *orl1* palisade cells have epithelium-like characteristics at least in terms of α -amylase expression. The *organ deficient mutant 78*, *odm78*, cannot develop any organized organs and only forms small globular embryo with vacuolated cells, although the development of the endosperm occurs normally (Hong et al., 1995). *RAmy1A* expression in the endosperm was observed in the *shl2* and *orl1* mutants but not in *odm78* (Fig. 5, B and C). The level of the *RAmy1A* transcript detected in *shl2* was similar to that found in the wild-type plant, but in *orl1* it was about 40% of the level in the wild type. The similar level of *RAmy1A* expression in the wild-type and *shl2* plants demonstrates that the shoot apical region plays only a small role in α -amylase induction in the endosperm. In contrast to the shoot apical region, the epithelium is essential for α -amylase induction. *odm78* seeds defective in epithelium formation did not induce *RAmy1A* expression at all.

DISCUSSION

The high-level expression of *RAmy1A* occurred in endosperm without treatment of uniconazol,

whereas such high-level expression was not observed in endosperm treated with uniconazol or in the embryo (Fig. 1A). The fact that the uniconazol treatment had no effect on the *RAmy1A* expression in the embryo (Fig. 1A) indicates that α -amylase expression in the embryo does not depend on de novo-synthesized GAs but may depend on pre-existing GAs synthesized during seed maturation, as occurs in wheat (*Triticum aestivum*) seeds (Lenton et al., 1994). In contrast to this situation, the induction of *RAmy1A* expression in endosperm was caused by the de novo-synthesized active GA (Fig. 1A). Based on the following evidence, we have concluded that the GA

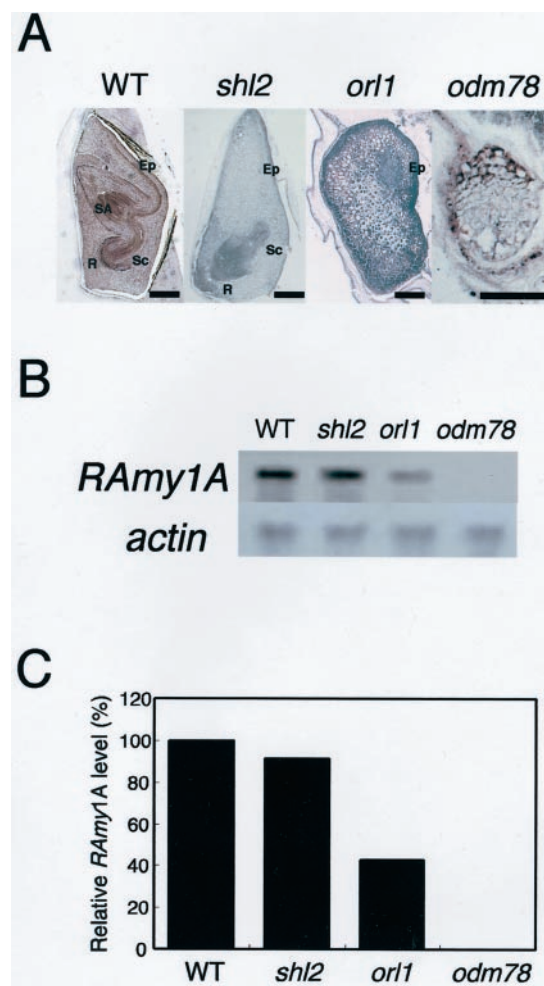


Figure 5. *RAmy1A* expression in three types of embryonic organ-deficient mutants. A, Phenotypes of the mature mutant embryos. Each embryo is shown with the embryo-epithelium boundary to the right side. The characteristic phenotype of each mutant is described in the text. SA, Shoot apex; R, root; Sc, scutellum; Ep, epithelium. Bar indicates 500 μ m. B, *RAmy1A* expression in the mutant endosperm. After 96 h of imbibition, total RNA was extracted from the endosperm; 1 μ g of total RNA was used for the northern analysis. The lower panel shows a loading control probed with actin cDNA. C, Relative levels of *RAmy1A* expression in B. The *RAmy1A* expression levels were normalized against the level of expression of the actin gene. The *RAmy1A* level in the wild type was set at 100%.

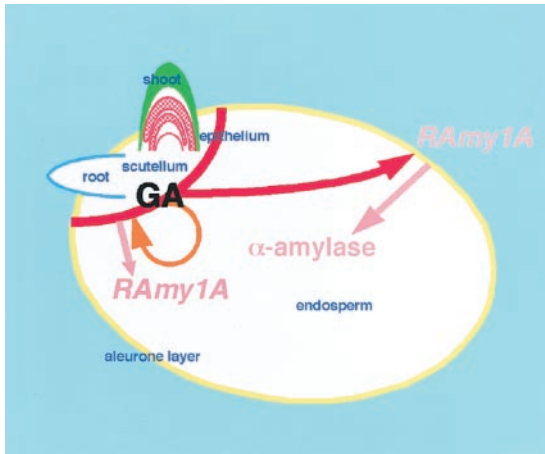


Figure 6. Schematic illustration of the sites of GA synthesis and *RAMy1A* expression during germination. The sites of GA biosynthesis, the shoot apical region and epithelium, during seed germination are shown in red, and the transport of GA for *RAMy1A* induction is indicated by the red arrow. The expression of *RAMy1A* in the aleurone layer (yellow) is triggered by the transported GA and induces the α -amylase activity in the endosperm. The pre-existing GA in the embryo induced the expression of *RAMy1A* in the epithelium (orange circular arrow).

3β -hydroxylase encoded by *OsGA3ox2* mainly catalyzes the active GA synthesis to induce the *RAMy1A* expression in endosperm. First, the dramatic increase in *RAMy1A* expression in endosperm occurred after the rapid induction of the *OsGA3ox2* expression in embryo (Fig. 1). Second, the level of *RAMy1A* expression in the loss-of-function mutant of *OsGA3ox2* (*d18-AD*) was almost the same as that of uniconazol-treated seeds, whereas the level in the nontreated wild-type endosperm was about 2.5 times higher than that of treated endosperm (Fig. 2).

The expression kinetics of *OsGA3ox2* is also consistent with a previous finding that the increase in the level of GA_1 during rice seed germination starts 1 d after imbibition (Choi et al., 1996), whereas the peak of the *OsGA3ox2* expression was from 12 to 24 h after imbibition. The correlation between the *OsGA3ox2* expression pattern reported here and the previous observation confirms that the *OsGA3ox2* product mainly contributes to the de novo GA synthesis in germinating seeds. In contrast to the rapid increment of *OsGA3ox2* expression at the early time course, the expression of *OsGA3ox2* was then suppressed to basal level during next 24 h (Fig. 1B). This *OsGA3ox2* suppression may be regulated by the negative feedback mechanism by GA. It is known that the expression of some 3β -hydroxylase genes in various plants is negatively regulated by bioactive GAs at the transcriptional level (Chiang et al., 1995; Lester et al., 1997; Martin et al., 1997; Ait-Ali et al., 1999). In fact, *OsGA3ox2* expression is suppressed in seedlings by treatment with GA_3 , whereas the expression of *OsGA3ox1* is not affected by this treatment (Itoh et al., 2001). The maintenance of a high level of *OsGA3ox2*

in the uniconazol-treated seeds can be also explained by the same mechanism, that is, the treatment with uniconazol inhibits active GA biosynthesis even under the high level of expression of *OsGA3ox2*.

Although the *OsGA3ox2* product is important for the *RAMy1A* expression in endosperm, the fact that some *RAMy1A* mRNA was detected in *d18-AD* also indicates that some other factor(s) contributes to the *RAMy1A* mRNA induction. There are two possible explanations for this pattern of *RAMy1A* mRNA accumulation. One is that the product of *OsGA3ox1*, which is constitutively expressed at a low level in the embryo, functions to induce *RAMy1A* expression. However, this hypothesis does not explain why the wild-type and *d18-AD* seeds treated with uniconazol accumulated the *RAMy1A* transcript at a similar level to that in the untreated *d18-AD* seeds (Fig. 2). Uniconazol blocks early GA biosynthesis by inhibiting *ent*-kaurene oxidase. If the *OsGA3ox1* product functions to produce active GAs in the mutant, the *d18-AD* seeds treated with uniconazol should show a low level of α -amylase expression relative to that in the untreated seeds. The other possibility is that pre-existing active GAs trigger the accumulation of the *RAMy1A* transcript. This hypothesis seems more plausible than the former because it would also explain why some *RAMy1A* expression was detected in the uniconazol-treated seeds.

In situ hybridization analysis revealed that the *OsGA3ox2* transcript was localized in the shoot apical region and in the epithelial cells (epithelium; Fig. 3). The *OsGA3ox2* expression in young leaf primordia is consistent with our previous finding that *OsGA3ox2* expression occurs around the shoot apex of vegetative seedlings (Sakamoto et al., 2001), and also with the quantitative analysis that the active GA content is preferentially higher in young leaves (Choi et al., 1995). Based on their biochemical and molecular studies, Appleford and Lenton (1997) proposed that the scutellum tissue may be important for de novo GA biosynthesis in wheat seeds. Our observations confirm the importance of the scutellum for induction of α -amylase expression in the endosperm.

Our other approach using rice embryonic organ-deficient mutants has clearly demonstrated that the epithelial cells are essential to induce the *RAMy1A* expression in endosperm, whereas the shoot apex region, which is the other site for the *OsGA3ox2* expression, is not important for the *RAMy1A* expression (Fig. 5). The biological activity of the scutellum or epithelium may directly influence the level of expression of α -amylase, because the *orl1* mutant with a partially developed scutellum induced about one-half the level of *RAMy1A* transcript relative to that of the wild-type plant.

Taking all of the above observations into consideration, we have modeled the relationship between GA biosynthesis in the embryo and the α -amylase expression in the endosperm during rice seed germina-

tion as shown in Figure 6. At the early stage of imbibition, pre-existing GA in the embryo triggers the expression of α -amylase in the embryo without de novo synthesis of GA (orange arrow). Then, *OsGA3ox2* expression is induced in the epithelium cells, and the product synthesizes the active GA (red bold line at the epithelium layer). *OsGA3ox2* expression also occurs in the shoot apical region (red hatch) but the product in this region does not contribute to α -amylase induction. The GAs produced in the epithelium layer are mainly transported to the aleurone (red arrow), where they induce the production of α -amylase. Endosperm starch is gradually hydrolyzed by α -amylase to supply energy for germination.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Several types of rice (*Oryza sativa*) seeds were used in this study. Taichung 65 (T65) is the original wild-type line for the *shl2*, *orl1*, and *odm78* mutants, and Akibare is the original wild-type line for *d18-AD*. Mature dry seeds were sterilized with 30% (v/v) sodium hypochloride and then rinsed several times with sterilized water. The seeds were incubated on filter paper wetted with water in the presence or absence of 10 μ M uniconazol in 0.2% (v/v) ethanol for *odm* for 72 h at 30°C. Total RNA was extracted separately from the embryo and endosperm. For in situ hybridization, the sterilized seeds were incubated on 1% (w/v) agar for 24 h at 30°C.

In Situ Hybridization

Plant materials were fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight at 4°C, dehydrated through a graded ethanol series followed by a *t*-butanol series (Sass, 1958), and finally embedded in Paraplast Plus (Sherwood Medical, St. Louis). Microtome sections (8–10 μ m thick) were mounted on glass slides treated with silane. Digoxigenin-labeled RNA probes were prepared from the 3'-terminal halves of cDNA clones of *OsGA3ox1* and *OsGA3ox2* (Itoh et al., 2001). Hybridization and immunological detection of the hybridized probes were performed according to the method of Kouchi and Hata (1993).

Northern-Blot Analysis

Total RNA from the embryo and endosperm was extracted by the standard method (Sambrook et al., 1989). Five micrograms of total RNA from the embryo and 1 μ g of total RNA from the endosperm were electrophoresed in a 1% (w/v) agarose/formaldehyde gel and then transferred to Hybond N⁺ membrane (Amersham, Buckinghamshire, UK) with 20 \times SSC. Twenty micrograms of total RNA from the embryo was electrophoresed to detect *OsGA3ox1*. Hybridization was performed at 65°C in sodium chloride/sodium phosphate/EDTA, 5 \times Denhardt's solution, 0.5% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg mL⁻¹

denatured salmon sperm DNA. Filters were washed with 2 \times SSC and 0.1% (w/v) SDS for 20 min at 65°C and 0.2 \times SSC and 0.1% (w/v) SDS for 5 min at room temperature.

Quantification of mRNA

To determine the level of hybridization, the membranes were exposed to a Imaging Plate (Fuji Photo Film, Tokyo) and analyzed on a BAS2000 by using Image Gauge (version 3.41) software (Fuji Photo Film). After autoradiography, the membranes were washed and reprobred with a radio-labeled actin fragment from rice. The highest level of the ratio of target gene to actin mRNA was set at 100.

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