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

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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; Hellwell 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
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 3β-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’Neill 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 3β-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.

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 μg of total RNA was prepared from the treated and untreated embryo, and 1 μg 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 μg 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.

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 primodia 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.
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 primodia (Satoh 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...
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 tissue 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 imbition, 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 *shl2, orl1*, and *adm78* 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 μM uniconazol in 0.2% (v/v) ethanol for *adm* 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.

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 μg 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× 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× Denhardt’s solution, 0.5% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg/mL denatured salmon sperm DNA. Filters were washed with 2× SSC and 0.1% (w/v) SDS for 20 min at 65°C and 0.2× 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 reprobed 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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LITERATURE CITED


