



Gibberellins are not required for rice germination under anoxia

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Key words: α -amylase, anaerobiosis, anoxia, gibberellins, *Oryza sativa*, rice, sugar sensing

Abstract

Production of α -amylase during the germination of rice grains is thought to play an important role for tolerance to anoxia of these cereal grains. Under aerobic conditions α -amylases production is enhanced in response to gibberellins produced by the embryos, but the role of these hormones is less clear under anoxia. In this paper we analysed α -amylase gene expression in a rice mutant (Tan-ginbozu) severely impaired in gibberellin biosynthesis. Expression of α -amylase genes others than the gibberellin-induced *Amy1A* gene is observed. The expression of the *Amy3D* gene, which does not require gibberellins to be induced, is high under anoxia in the Tan-ginbozu mutant suggesting that germination under anoxia can proceed thanks to the activity of the α -amylase isoform encoded by the *Amy3D* gene. *Amy3D* gene expression is repressed in the presence of high levels of soluble carbohydrates, indicating that the anaerobic expression of this gene can be triggered by a lower carbohydrate content of rice grains kept under anoxia. Germination under anoxia of Tan-ginbozu grains can proceed even in absence of exogenously-added gibberellic acid. Overall, results indicate that gibberellins are not required for the anaerobic germination of rice grains.

Abbreviations: GA – gibberellin; GA₃ – gibberellic acid

Introduction

Tolerance to anaerobiosis varies drastically among plant species (Vartapetian and Jackson, 1997). The vast majority of cereals fail to germinate under anoxia, but rice grains represent a remarkable exception to this rule. Rice grains under anoxia show coleoptile elongation, while roots fail to grow (Alpi and Beevers, 1983). Carbohydrate availability is thought to be important under anoxia, to provide substrate for the fermentative pathway, but cereal grains store starch as a reserve carbohydrate, requiring its degradation to simple sugars before glycolysis and fermentation can proceed (Perata et al., 1998). Several enzymes are needed for starch degradation (α -amylase, β -amylase, α -glucosidase and debranching enzyme) but

only α -amylase is considered to play a major role in starch degradation (Dunn, 1974; Sun and Henson, 1991). Remarkably, α -amylase is produced in rice grains under anoxia (Perata et al., 1992), while it is not in the anoxia-intolerant cereals (wheat, barley) (Guglieminetti et al., 1995b). Starch is not degraded in the absence of α -amylase, and anoxia-intolerant cereals such as wheat and barley suffer soon from sugar starvation, leading to their death (Perata et al., 1996).

The ability to produce α -amylase under anoxia thus appears to play a role in anoxia tolerance, and this is apparently not restricted to cereals. Indeed, Arpagaus and Braendle (2000) demonstrated that α -amylase plays an important role for carbohydrate metabolism also in the anoxia stress tolerant rhizomes of *Acorus calamus* L..

The molecular regulation of α -amylase in anoxic rice grains is unknown at present. Gibberellins (GA)

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play a crucial role in the induction of α -amylase genes in germinating, aerobic cereal grains. Rice embryoless half-grains kept under aerobic conditions rapidly respond to exogenous gibberellic acid (GA_3) showing rapid induction of α -amylase mRNA accumulation (Perata et al., 1993). Under anoxic conditions α -amylase mRNA is produced in response to exogenous gibberellic acid (GA_3), but with great delay when compared to aerobic conditions (Loreti et al., 2002b). However, the production of the α -amylase protein is unaffected by anoxia as demonstrated by immunoblot analysis (Guglielminetti et al., 1995b; Perata et al., 1997a), possibly because α -amylase genes not regulated by gibberellins are active under anoxia (Hwang et al., 1999; Loreti et al., 2002b). Indeed, among several genes belong to the rice α -amylase gene family (Huang et al., 1992; Mitsui and Ito, 1997). *Amy1A*, *Amy 3B/C*, *Amy3D*, and *Amy3E* are expressed in germinating rice grains under either aerobic or anaerobic conditions, whereas *Amy1B*, *Amy1C*, *Amy2A*, and *Amy3A* are not detectable, when anoxic (Hwang et al., 1999). *Amy1A* is the gene showing the higher expression level in rice grains germinating under aerobic conditions, while under anoxia other α -amylase genes, besides *Amy1A*, are expressed at comparable levels, namely *Amy 3B/C*, *Amy3D*, and *Amy3E* (Hwang et al., 1999). *Amy1A* induction by gibberellins and repression by abscisic acid is well described under aerobic conditions (Itoh et al., 1995; Loreti et al., 2002a), while very little is known about the mechanisms regulating other α -amylase genes in rice. The *Amy3D* and *Amy3E* do not show a GARE (Gibberellin Acid Response Element) in their promoter (Hwang et al., 1999), but absence of GA-response in the embryoless half-grains, to our knowledge, have not been reported. The *Amy3C* promoter contains a sequence able to bind GA-induced proteins extracted from rice aleurones, thus suggesting that this gene might be GA-modulated (Yu et al., 1992). Direct evidence for the GA-responsiveness of *Amy3C*, *Amy3D* and *Amy3E* in rice aleurones is however missing. *Amy3D* and *Amy3E* are sugar-repressed in rice embryos (Yu et al., 1996), and sugar repression of *Amy3D* transcription in anoxic rice aleurones has also been observed (Loreti et al., 2002b).

Rice grains under anoxia are able to respond to gibberellins in terms of *Amy1A* induction (Loreti et al., 2002b), while anoxia-intolerant cereals fail to respond to gibberellins under anoxic condition (Perata et al., 1992). This is suggestive of gibberellins and

GA-responsiveness as a pre-requisite for α -amylase production under anoxia and subsequent germination.

In this paper we investigated the importance of gibberellins in rice germination under anoxia, taking advantage of a GA-deficient mutant (Tan-ginbozu) in which one of the steps in the GA-biosynthetic pathway is genetically blocked. The level of the major gibberellin produced during rice grain germination (GA_1) is strongly reduced in the shoots of 10-days old mutant's seedlings (Kobayashi et al., 1989). No data is available about the GA content in Tan-ginbozu grains during the first days of germination, but Mitsunaga and Yamaguchi (1993) demonstrated that the production of α -amylase is drastically reduced in Tan-ginbozu grains germinating in the absence of exogenous gibberellins, while wild-type rice grains do not require exogenous GAs for α -amylase production (Mitsunaga et al., 1994), thus indirectly indicating the absence of enough GAs to trigger α -amylase induction in this mutant. We investigated the expression pattern of various α -amylase genes in Tan-ginbozu grains kept under aerobic or anoxic conditions in the presence or absence of exogenous gibberellins. We also tested the effects of gibberellins on anoxic germination of this rice mutant. The results obtained suggest that rice grain germination under anoxia does not require gibberellins.

Materials and methods

Plant material

Rice grains (*Oryza sativa* L.) cvs. Tan-ginbozu and M202 were used. Grains were germinated (27 °C) in petri dishes containing filter paper moistened with sterile water under aerobic conditions or in an anaerobic incubator for the time indicated in figures (Forma Scientific, Anaerobic System Model 1025). Germinated grains were dissected into embryo and embryoless grains (aleurone as the only living tissue; Fincher, 1989) prior to RNA extraction. Embryoless half-grains used in the GA_3 -induction experiments were obtained by dissection of the grains into two parts, 1/3 of the intact grain containing the embryo, and 2/3 of the grain lacking the embryo (embryoless). Incubation of embryoless half-grains was carried out in test tubes, each containing four embryoless half-grains and 500 μ L of 5 mM $CaCl_2$ containing 5 μ g of chloramphenicol. Incubation was carried out at 27 °C with vigorous shaking. When used, 1 μ M GA_3 , or 100 mM gluc-

ose was added. The amount of glucose resulting from starch degradation is low during the first 2 days of aerobic and anaerobic germination (Guglielminetti et al., 1995a), and indeed glucose concentration in the incubation media was found to be lower than 10 mM at the end of the 2-day long incubation of half-grain, a concentration unable to negatively affect α -amylase expression. The amount of ethanol produced by half-grains under anoxia was also low (between 0.5 and 1 mM after 2 days of incubation; Loreti et al., 2002), and the pH of the external medium was not modified by the different treatments.

Chemicals

The commercially available compounds were purchased from Sigma.

Gene specific probes

The gene-specific probes for the detection of *Amy1A*, *Amy3B/C*, *Amy3D*, and *Amy3E* mRNAs were prepared by PCR labelling as described by Hwang et al. (1999). The primers, as described by Hwang et al. (1999), were designed to amplify the 3' untranslated region of the genes. The *Amy3B/C* probe is unable to distinguish between the *Amy3B* and the *Amy3C* transcripts. Primer extension experiments performed using germinating rice grains indicated that *Amy3C* contribute 75% of the RNA detected by the *Amy3B/C* probe (Sutliff et al., 1991). The probe for rRNA was a rice rRNA probe.

RNA isolation and gel blots

RNA extraction was performed using the aurintricarboxylic acid method as previously described (Perata et al., 1997b). The amount of RNA loaded in electrophoresis was 20 μ g. RNA was electrophoresed on 1% agarose-formaldehyde gels, and blotted on nylon membrane (BrightStar-Plus[®], Ambion) by using the procedure suggested by the manufacturer. Membranes were pre-hybridised and hybridised using the NorthernMax[®] kit (Ambion). Equal loading was checked by reprobing with a rRNA cDNA probe (not shown).

Results

Gibberellins affect α -amylase gene expression under aerobic but not anoxic conditions

Tan-ginbozu grains were germinated in the presence/absence of exogenous GA₃ and RNA gel blots hybridised with gene-specific α -amylase probes. Both the embryo and aleurone tissue produced α -amylase during germination (Loreti et al., 2002b).

Figure 1A shows the pattern of expression of α -amylase genes in the aleurone tissue from aerobic seedlings, indicating that all the α -amylase genes were positively affected by exogenous GA₃. The *Amy1A* gene was readily induced by GA₃, while its expression was delayed in the absence of this hormone. *Amy3B/C* transcript level was low in the absence of GA₃, but addition of the hormone enhanced its expression. The expression of *Amy3D* in the aleurone (Figure 1A) was slightly affected by GA₃, showing a transient expression pattern peaking at day 1 followed by a rise in expression late during germination (Day 7, Figure 1A). *Amy3E* was expressed at a very low level in the absence of GA₃.

Under anoxia only *Amy3B/C* and *Amy3D* were expressed at high level in the aleurone (Figure 1B), with *Amy3D* not influenced by GA₃ and the expression of *Amy3B/C* slightly anticipated in the presence of the hormone. The expression of *Amy3D* (Figure 1) was much higher than that normally expressed in the 'wild-type' cultivar M202 (data not shown; Hwang et al., 1999).

Expression of α -amylase genes were also investigated in embryos from germinating seedlings (Figure 2). *Amy1A* and *Amy3E* were not expressed in embryos, regardless of oxygen or GA₃ availability (Figure 2A, B). Expression of α -amylase genes in the aerobic embryo of germinating Tan-ginbozu grains was restricted to the *Amy3D* gene, with GA₃ reducing slightly the expression level of this gene (Figure 2A). Anoxia enhanced the expression of *Amy3D* and *Amy3B/C*, and expression during anoxia was slightly enhanced by GA₃ (Figure 2B).

Interaction between GA₃ and glucose on expression of α -amylase genes

To gain further insights on the regulation of α -amylase gene expression, we tested the effects of GA₃ and glucose on the mRNA levels of the α -amylase genes in anaerobic embryoless half-grains (i.e., aleurone tis-

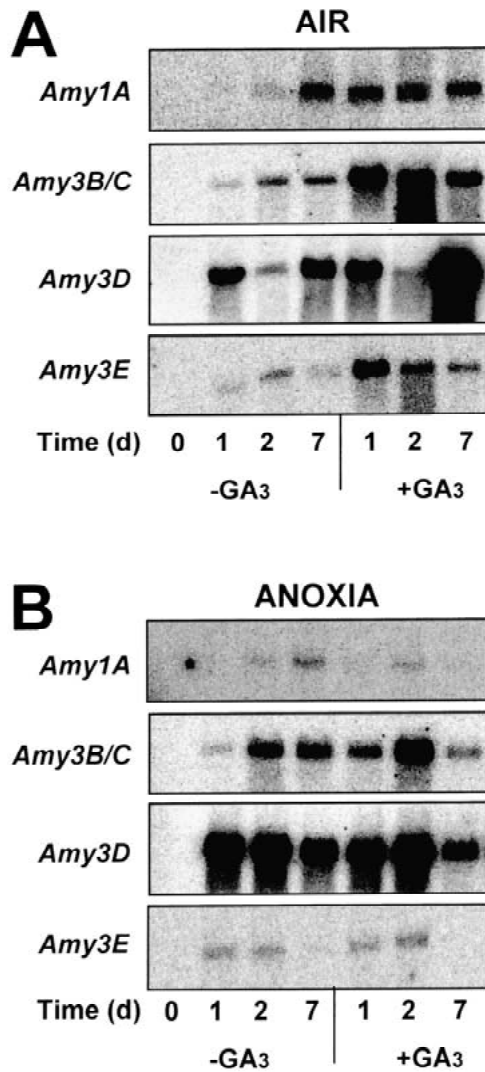


Figure 1. Pattern of expression of α -amylase genes in aleurones from germinating rice grains. Rice grains (Tan-ginbozu mutant) were germinated under aerobic (A) or anoxic (B) conditions, in the presence/absence of exogenous $1 \mu\text{M}$ GA₃ for 0, 1, 2, and 7 days. The embryo and endosperm-side (aleurone) of the seedling were separated and RNA was extracted. RNA from aleurones was electrophoresed and Northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (not shown). The results reported are from a representative experiment.

sue). The Tan-ginbozu mutant, as well as the rice cultivar M202 were used. In both genotypes *Amy3D* was expressed independently of GA₃ (Figure 3), while all the other α -amylase genes studied required GA₃ to be induced. Glucose repressed *Amy3D* expression, but addition of GA₃ counteracted the repressive effect of the sugar. Glucose did not influenced the expression of *Amy1A*, *Amy3B/C* and *Amy3E*. No differences were

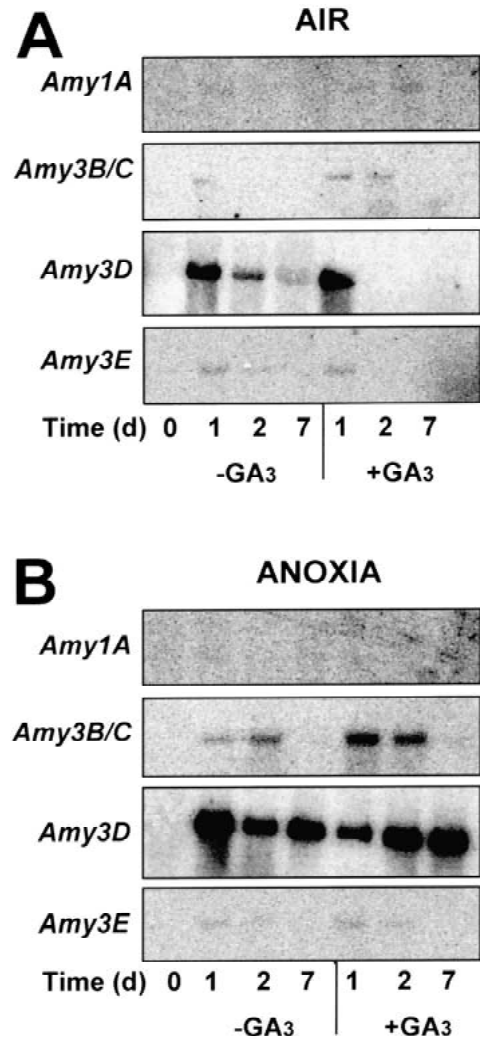


Figure 2. Pattern of expression of α -amylase genes in embryos from germinating rice grains. Rice grains (Tan-ginbozu mutant) were germinated under aerobic (A) or anoxic (B) conditions, in the presence/absence of $1 \mu\text{M}$ exogenous GA₃ for 0, 1, 2, and 7 days. The embryo and endosperm-side (aleurone) of the seedling were separated and RNA was extracted. RNA from embryos was electrophoresed and Northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe (not shown). The results reported are from a representative experiment.

observed between Tan-ginbozu and the M202 cultivar (Figure 3).

Rice germination under anoxia does not require gibberellins

Gibberellin deficiency, together with minimal α -amylase production, in Tan-ginbozu did not inhibit aerobic germination, but reduced sheath length of the

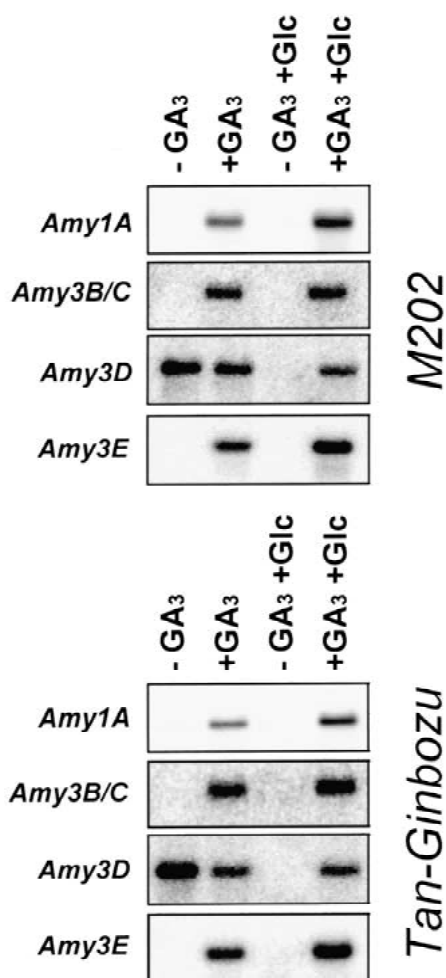


Figure 3. Effects of $1 \mu\text{M}$ gibberellic acid and 100 mM glucose on α -amylase mRNA accumulation in anaerobic embryoless half-grains from Tan-ginbozu mutant and wild-type cultivar (M202). Northern blot analysis of *Amy* mRNA accumulation in embryoless half-grains incubated for 2 days in presence/absence of $1 \mu\text{M}$ GA_3 and, when used, in presence of 100 mM glucose under aerobic conditions. RNA from embryos was electrophoresed and northern analysis carried out using gene specific probes. Equal loading was checked by reprobing with a rRNA probe. The results reported are from a representative experiment.

second leaf of seedlings (Mitsunaga et al., 1993). We tested whether the gibberellin-deficient mutant Tan-ginbozu was able to germinate under anoxia in the presence/absence of exogenous gibberellins. The time-course of coleoptile elongation in anoxia (Figure 4) was the same between gibberellin-treated and control seedlings, and was remarkably similar to that of several wild-type rice cultivars (data not shown). These results indicate that rice germination under anoxia does not require gibberellins.

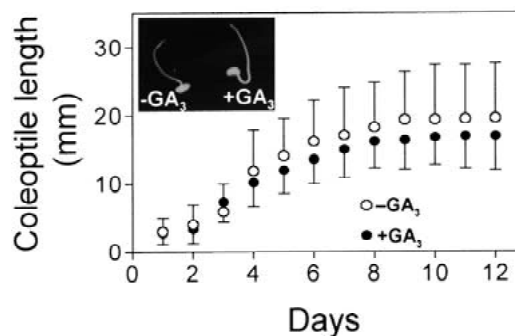


Figure 4. Effects of gibberellic acid on the germination of rice grains (Tan-ginbozu mutant) under anoxia. Rice grains were sown under anoxia in the presence/absence of $1 \mu\text{M}$ GA_3 and coleoptile length recorded every day. The inset shows 12-day-old rice seedlings germinated under anoxia in the presence/absence of $1 \mu\text{M}$ GA_3 .

Discussion

Availability of readily-fermentable carbohydrates is likely a requisite for successful germination under anoxia (Perata et al., 1998). As far as cereals are concerned this assumption is supported by the successful induction of α -amylase observed in anoxic rice grains when compared to anoxia-intolerant cereals such as wheat, oat, and barley (Perata et al., 1992; 1993). Response to gibberellins is thought to play a crucial role in the ability of cereals to induce α -amylase and, consequently, to degrade the starchy reserves stored in the endosperm (Bethke et al., 1997). Under anoxia, the model proposed postulates that gibberellins can induce α -amylase in rice, while anoxia-intolerant cereals fail to produce α -amylase because they are unable to respond to the hormone (Perata et al., 1998). Although some evidences (see Perata et al., 1998) have been presented supporting this model, additional considerations are needed. Firstly during germination under anoxia gibberellins cannot be synthesized *de novo* since membrane-bound monooxygenases that convert entkaurene to GA_{12} require NADPH and oxygen (Hedden and Kamiya, 2000). Even if the hypothesis of the possible existence of stored gibberellins (or precursors) in the dry grain of rice is considered, this proposal would imply that release from the stored pool is anoxia-dependent, since aerobic GA-dependent α -amylase gene expression required exogenous GA_3 when GAs biosynthesis was blocked using inhibitors (Mitsunaga and Yamaguchi 1993). Recent evidence (Hwang et al., 1999) suggests that, besides the well-characterized GA-dependent expression of *Amy1A*, other α -amylase

genes, not necessarily GA-modulated, are expressed under anoxia in rice (*Amy3B/C*, *Amy3D*, and *Amy3E*).

The germination of rice grain under anoxia could be a gibberellin-independent process, which is supported by the following evidences: (i) the GA-deficient rice mutant used in this study germinates successfully under anoxia (Figure 4), despite the extremely low level of *Amy1A* transcript (Figures 1 and 2) and (ii) the expression of *Amy3D*, whose expression does not require gibberellins, predominates over that of *Amy3B/C* under anoxia (Figures 1 and 2). The expression of *Amy3B/C* requires exogenous GA₃ in embryoless half-grains (Figure 3), but the gene is also expressed in the aleurone of intact germinating grains of the Tan-ginbozu mutant under aerobic conditions without addition of GA₃, although adding GA₃ enhances *Amy3B/C* expression (Figure 1). Interestingly, all the GA-modulated genes (*Amy1A*, *Amy3B/C* and *Amy3E*) are expressed in the mutant aleurone under aerobic conditions, although with low expression levels and a delayed timing of appearance when compared to the GA₃-treated grains (Figure 1). This leads to the suggestion that either gibberellins accelerate the process of α -amylase gene transcription rather than being an absolute requirement for this process to occur, or that the very limited amount of GA₁ synthesized in the Tan-ginbozu mutant (Kobayashi et al., 1989) is enough to induce a limited amount of GA-modulated *Amy* genes late during germination.

The results (Figures 1 and 2) obtained by treating intact germinating grains with exogenous GA₃ are not fully explained by our understanding about α -amylase gene modulation by hormones and sugars. Exogenous GA₃ had a positive effect on all *Amy* genes expressed in the aerobic aleurone (Figure 1A), even though GA₃ treatment does not induce as much *Amy1A* as observed in non-mutant cultivars (Hwang et al., 1999). Experiments with embryoless half-grains (Figure 3) under 'physiological' conditions, i.e., with exogenous glucose added to mimic the metabolic environment of germinating grains, show that GA₃ had a positive effect on *Amy3D* expression. The regulation of *Amy3D* gene expression by GA₃ is not expected as *Amy3D* does not have a GARE in its promoter sequence (see Introduction). Indeed expression of *Amy3D* does not require exogenous GA₃ in the absence of glucose (Figure 3), but GA₃ exerts a positive effect on the expression of *Amy3D* if the embryoless half-grains are incubated in the presence of glucose, i.e., GA₃ appears to de-repress *Amy3D* from sugar repression (Figure 3). This observation suggests that under nor-

mal conditions the level of endogenous GAs is unable to de-repress *Amy3D* from glucose inhibition since this gene is transiently expressed, and its expression negatively correlates with soluble sugars levels in the tissue (Karrer and Rodriguez, 1992). Additional experiments are needed to clarify the role of gibberellins in the induction of genes other than *Amy1A*, but the results reported in this paper suggest that, besides direct effects of gibberellins on *Amy* genes, exogenous GA₃ may affect the expression of some genes indirectly (e.g., by de-repressing them from glucose repression).

Addition of exogenous GA₃ under aerobic conditions allows the expression of *Amy1A* and *Amy3E* in aleurones (Figure 1A) but not in embryos (Figure 2A) of Tan-ginbozu. Expression of *Amy3D* is higher than that of *Amy1A* and *Amy3E*, showing a peculiar bi-phasic pattern of accumulation (Figure 1A). A transient expression of *Amy3D* in embryos is well known in wild-type rice grains (Hwang et al., 1999) and is observed also in Tan-ginbozu aleurones with the transcript level increasing at day 1 and falling to a low level at day 2 (Figure 1A). α -Amylase isoenzymes encoded by *Amy1A* and *Amy3D* have distinct functional roles in starch breakdown during grain germination (Terashima et al., 1995). The *Amy1A* enzyme has a much higher activity to starch granules, while *Amy3D* degrades preferentially oligosaccharides (Terashima et al., 1995). It is tempting to speculate that the relatively low expression of *Amy1A* in Tan-ginbozu leads to a slow rate of starch degradation, thus not allowing glucose to accumulate to levels repressing *Amy3D* expression. This proposal is compatible with the transient expression of *Amy3D* observed between day 0 and 2, since *Amy3D* degrades rapidly oligosaccharides stored in the grains, this resulting in transient glucose accumulation that may repress *Amy3D* expression (Terashima et al., 1995). The rise of *Amy3D* expression in Tan-ginbozu at day 7 (Figure 1A, aerobic conditions) is however not observed with wild-type M202 grains, since starch degradation by *Amy1A* is vigorous in this cultivar at this germination stage (Hwang et al., 1999; our unpublished observation).

Under anoxia expression of *Amy1A* and *Amy3E* is negligible in Tan-ginbozu grains (Figures 1B and 2B). Addition of exogenous GA₃ stimulated the expression of these two genes in the aerobic aleurone, but not under anoxia, in agreement with the limited GA-responsiveness of wild-type aleurones under anoxia (Loreti et al., 2002b; Perata et al., 1993).

The overall conclusion is that gibberellins are not required for rice germination under anoxia. This is supported by the lack of positive effects of exogenous GA₃ on the germination of Tan-ginbozu grains. This conclusion does not challenge the role of α -amylases under anoxia because the vigorous expression of *Amy3D* (and to a minor extent *Amy3B/C*) compensates for the absence of the GA-modulated *Amy1A*-encoded enzyme.

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