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Physiological analysis on pre-harvest sprouting in recombinant inbred rice lines

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Abstract Pre-harvest sprouting (PHS) in rice production is usually caused by high temperature and humidity or continuous rains. It frequently happens in F_1 in hybrid rice seed production. The PHS or “Physiologically germinated” seeds are of lower quality, by which the hybrid rice seed production is badly affected every three years at a loss of 20% or even 50% yield in seed production over the vast Yangtze River Valley and Southwest China. It is estimated that PHS causes an average decrease of seed activity by 10%. A recombinant inbred line population including 304 lines, derived from a cross between Indica rice (*Oryza sativa* L.) cultivars Zhong-156 and Gumei-2, was used to study the PHS physiology. Based on the data of sprouting rate in panicles and sprouting rate in grains, two kinds of lines, namely easy-to-sprout lines and hard-to-sprout lines, were selected to investigate their physiological differences when PHS happened. The experiment was conducted in a special field with a microclimate of higher temperature and humidity. The results indicated that it was easier to produce PHS from the female parent GM-2 than the male parent ZH-156, besides, the GA_1 content and amylase activity in GM-2 grains were higher than those in ZH-156. However, the abscisic acid (ABA) content in GM-2 grains was lower than that in ZH-156. Higher temperature and higher humidity facilitated the GA_1 increment from milk ripe stage to yellow ripe stage. GM-2 and the easily-sprouting lines showed an even higher increase in GA_1 than ZH-156 and the hardly-sprouting lines, which enhanced the amylase activity and induced pre-harvest sprouting. This may be the physiological basis for pre-harvest sprouting induced by higher temperature and higher humidity, and these special characteristics must be inherited from their parents.

Keywords pre-harvest sprouting, physiological analysis, hybrid rice seed F_1 , amylase activity, recombinant inbred lines

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1 Introduction

Pre-harvest sprouting (PHS) usually happens under the condition of continuous rains or high humidity and temperature before harvest in cereal plants, especially in hybrid rice seed production. The hybrid seed quality or grain quality will be severely degraded by PHS. This happens every three years and becomes a troublesome problem in hybrid rice seed production over the Yangtze River Valley and Southwest China, resulting in a yield reduction of 10%–50%. Yet, there is still no effective solution to it up to now (Huang et al., 1995; Duan et al., 1991).

Both sprouting rate in panicles (SRP) and sprouting rate in grains (SRG) were used as pre-sprouting indices; special rice lines were used as plant materials to identify the physiological traits during pre-sprouting. By data analysis, this paper tries to provide a theoretical basis for the pre-sprouting control through breeding.

2 Materials and methods

2.1 Materials

An Indica variety Zhong-156 was crossed with another Indica type variety Gumei-2 and self-crossing was continued to get recombinant inbred lines (RIL) in 1995. This system was composed of 304 plants, provided by the Gene Orientation Research Group of the China National Rice Research Institute (CNRRI).

The pre-experiment was started in the year 2002 to observe growth durations of those recombinant rice lines in the experiment station of CNRRI. The differences among those recombinant lines were identified and the differences of the earliest and the latest were up to 16 days in growth duration. Those rice lines were planted officially during May to October 2003 by stages in seeding to facilitate flowering synchronization. The seeds were treated with a fungicide (*Jinzhongling* in Chinese) for 36 h at 30°C, with seeds/fungicide solution being 1/2.5 (W/V) before sowing, and kept in a dark room for 48 h for germination. Those germinated seeds

were directly sowed at a trial plot of 2 m² and in the main field at 1.5 g/m² (dry weight), which was replicated three times.

Samples were taken from special materials identified from 304 lines, and were classified into two types: the easy-to-sprout group (ESG) and the hard-to-sprout group (HSG). No.34, No.104 and No.276 belonged to the former and No.36, No.162 and No.272 belonged to the latter. Individual plants with good uniformity were selected from the two groups for the sprout-inducing treatment in a “bath room”.

2.2 Methods

2.2.1 Sprout-inducing treatment

In order to create a good environment for sprouting, a special “bath room” was built for each plant 15 days after anthesis. The “bath room” was 1 m × 1 m × 2.5 m in size, covered with a special film with good light transparency and air penetration and little water penetration. Besides, it had high humidity (85%–95%) and suitable temperature (28°C–30°C) for germination. Fixed volume of water was supplied by spraying on foliages every 3 h from 7:00 to 18:00.

2.2.2 Pre-sprouting observation

The observation was conducted every 2 days from the day after “raining”, and based on 100 panicles; the pre-sprouting rate was counted according to the following methods (Wang et al., 2000).

Sprouting rate in panicle (SRP) is the number of panicles that had 3 sprouted grains in 100 panicles.

Sprouting rate in grains (SRG) is the number of sprouted grains in 100 panicles, divided by the total number of fertile grains in 100 panicles.

2.2.3 Amylase activity measurement

Based on a technique (Zhu et al., 1990), thirty water-saturated grains (before germination) were selected for enzyme extraction at 0°C, and the enzyme was activated at 40°C. This extraction was divided into three samples, one of which was treated in a 100°C water-bath for enzyme passivation (CK), another was treated in a 70°C water-bath for β-amylase passivation (for α-amylase measurement), and the rest was used for amylase activity measurement (α-amylase + β-amylase). The activity was expressed by mg (glucose) · (100grains)⁻¹ · h⁻¹, with parallel errors of three replicates less than 5%.

2.2.4 Endogenous hormones measurement

Water-saturated grains (before germination) were ground in an ice-bath with 3 mL 80% cold methanol, then the slurry was diluted with another 3 mL cold methanol, and centrifuged in the centrifuge tube at 8 500 × g for 20 min after 1 h sedimentation, and finally purified with Sep-PAKC₁₈ (Waters). This purified extraction was used for Enzyme-linked Immunosorbent Assay (ELISA) measurement.

GA₁ and ABA were determined by ELISA (Zhen and Zhou, 1995; Wu et al., 1988). Hormone-protein complex was coated to a solid carrier (Polystyrene microtitration panel). Then the purified extraction sample and rabbit anti-hormones antibodies were dripped into the hole of a microtitration panel for reaction, with the supernatant discarded and the hole cleaned before adding enzyme-labeled double-antibody for taking reaction. Finally, the enzyme quantity combined with the solid phase (indexed by optical density (OD) volume) was determined. Hormone level was negatively correlated with the enzyme, and was showed by logarithm volume of OD.

3 Results

3.1 Differences between recombinant lines and their parents on pre-sprouting status

There were significant differences concerning pre-sprouting (Fig. 1). Firstly, there existed a difference of the starting time for pre-sprouting between rice lines, as Gumei-2 (GM-2, male parent of the recombinant lines) sprouted at 2 days after “raining” treatment while Zhong-156 (ZH-156, female parent of the recombinant lines) sprouted at 6 days after “raining”. Secondly, the SRP and SRG were up to 21.5% and 15.6%, respectively for GM-2 at the time of 2 days after “raining”, but the SRP and SRG were only 0.8% and 1.1% for ZH-156. At the same time, there were significant differences between the 3 lines from the ESG and the 3 lines from HSG. For example, the SRP and the SRG were 0% and 0.8% respectively for No.162 from HSG at the time of 10 days after “raining”. At the same time however, the SRP and the SRG were 31.5% and 19.8%, respectively for No.34 from ESG.

3.2 Differences in GA₁ and ABA level among parents and some RIL

The dynamic changes of endogenous GA₁ and ABA for ZH-156 and GM-2 during maturation are shown in Fig. 2. Firstly, GA₁ level was high during the early filling stage (0–5 days after anthesis) for parents, 800 pmol/gFW and 1 100 pmol/gFW for ZH-156 and GM-2, respectively, then fell down sharply. GA₁ was beyond the minimal level of ELISA at the time of 25 days after anthesis for ZH-156; only 50 pmol/gFW was detected in GM-2 at the same time. It was also undetectable for GA₁ at 30 days after anthesis in MG-2 grains. Secondly, the dynamic changes of ABA showed a two-apex curve during maturation. Peak values were located on the 5th and the 20th days after anthesis for ZH-156, but on the 5th and the 15th days for GM-2. There was no significant difference between ZH-156 and MG-2 as the first peak value occurred, but the second peak value of ZH-156 was significantly higher than that of GM-2. Thirdly, the ABA content in GM-2 was 340 pmol/gFW 30 days after anthesis (maturity stage) and the ABA content in ZH-156 was 510 pmol/gFW, 50% higher than that of GM-2.

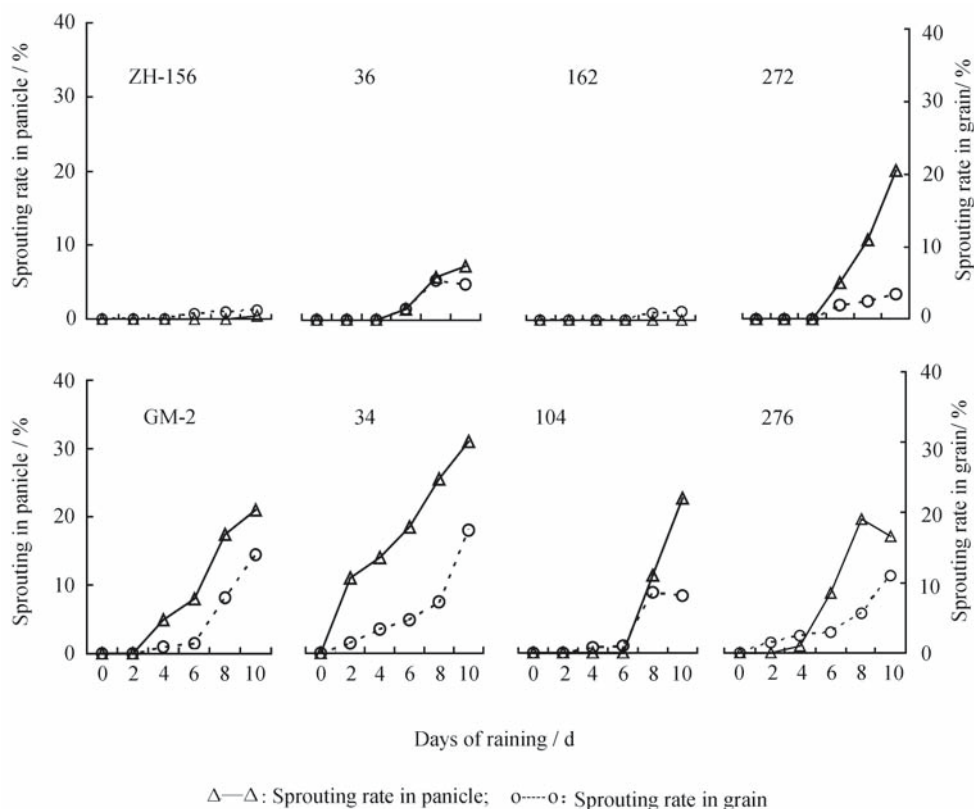


Fig. 1 Pre-sprouting rate in parents and in some RIL

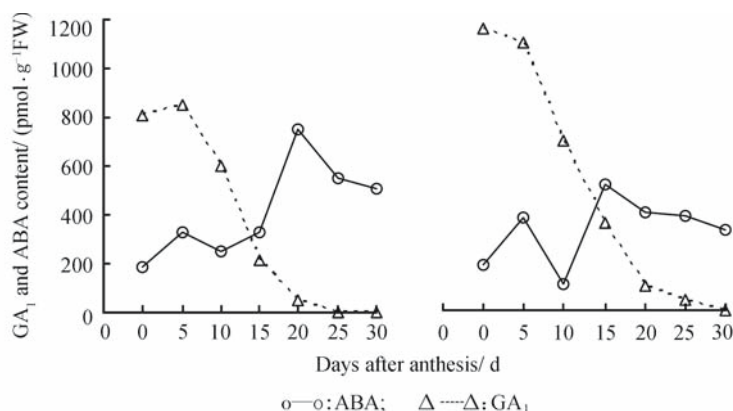


Fig. 2 Endogenous GA_1 and ABA contents in grains of ZH-156 and GM-2 during filling stage

The dynamic changes of ABA and GA_1 for six recombinant lines are shown in Fig. 3. Those data were sampled from a “mini-environment” within 10 d continuous “Raining” starting at the time of 15 days after anthesis. Firstly, GA_1 level in filling grains was increased with the “Raining” for several days. Specifically, the GA_1 contents of ZH-156 and No.36, No.162, No.272 (from hardly-sprouting group) were increased from 100 pmol/gFW to 550 pmol/gFW within 10 days of “Raining” while the GA_1 contents of MG-2 and No.34, No.104, No.276 (from easy-sprouting group) were increased from 50 pmol/gFW to 1 980 pmol/gFW. Secondly, during 10 days of “Raining” starting from the 15th day after anthesis, the endogenous ABA contents in filling grains were raised.

The ABA contents of ZH-156 and No.36, No.162, No.272 were increased from 200 pmol/gFW to 1 000 pmol/gFW within 10 days, about 4 times more than those at the beginning of “Raining” day. At the same time, the ABA contents of GM-2 and No.34, No.104, No.276 were increased by 2.15, 1.5, 2.5 and 1.75 times, respectively when compared with those at the beginning of “Raining” day.

3.3 Amylase contents among recombinant lines and their parents

Amylase contents in grains changed during ripening (Fig. 4). Firstly, peak values for ZH-156 and GM-2 appeared 10 days

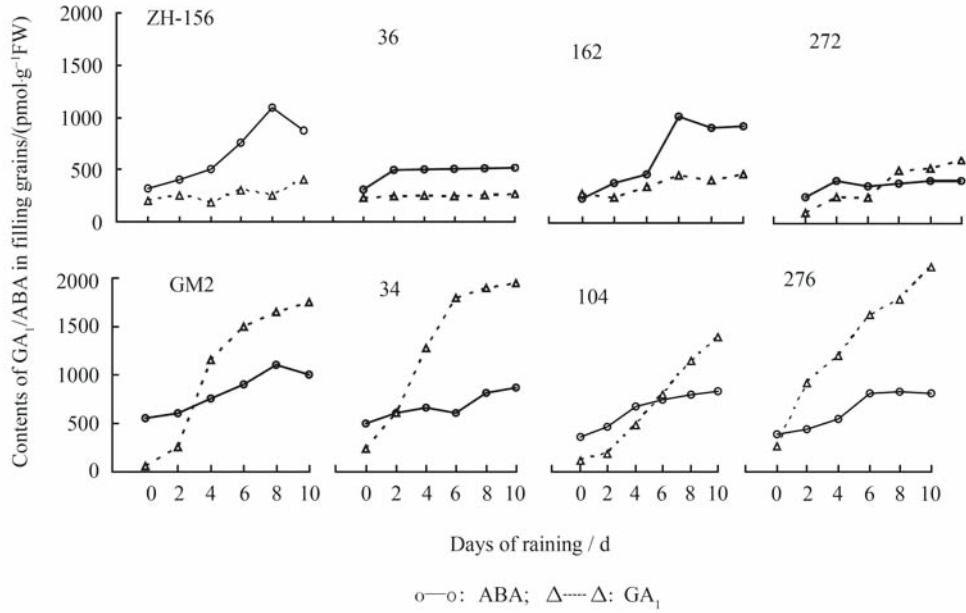


Fig. 3 Effect of raining on endogenous GA_1 & ABA content in parents and RIL grains during maturity

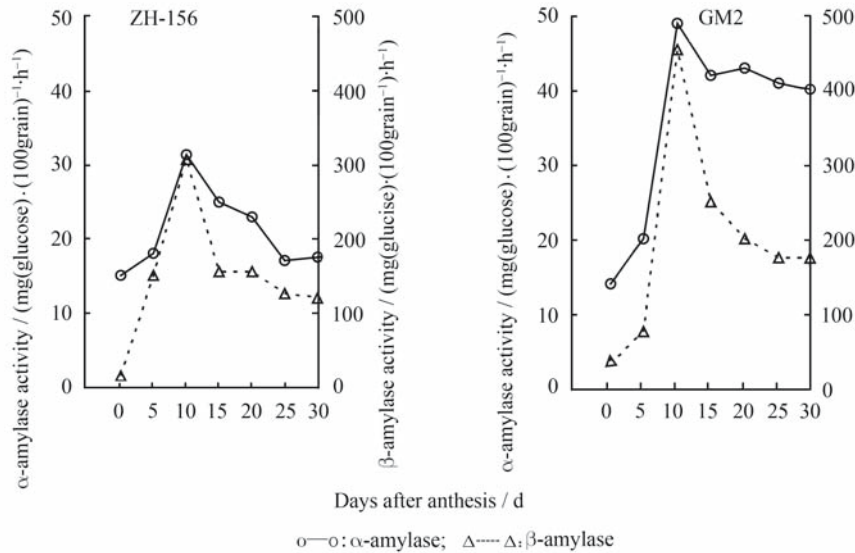


Fig. 4 Amylase activity of grains in cultivars ZH-156 and GM-2

after anthesis, which was just in the duration of embryo development. This result was similar to that of the former researchers (Wu et al., 1988). Secondly, the amylase content in grains of GM-2 was significantly higher than that of ZH-156. The α -amylase contents in GM-2 grains and ZH-156 grains were $49 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ and $33 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$, respectively. At the same time, the β -amylase contents in GM-2 grains and ZH-156 grains were $480 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ and $300 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$, respectively. Thirdly, the slow decrease in amylase contents took place from the 10th day on after anthesis. Thus, the α -amylase and β -amylase activity of ZH-156 were $17 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ and $110 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$, respectively at ripening

(about 30 days after anthesis). Meanwhile, the α -amylase and β -amylase contents of GM-2 were $40 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ and $185 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$, respectively, which were higher than those of ZH-156.

Figure 5 shows the dynamic changes of amylase activity in both RILs and their parents under the condition of raining, high humidity and high temperature during ripening. Firstly, the α -amylase activity in GM-2 grains increased from $69 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ to $120 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ during 10 days of "Raining"; and the α -amylase activity in ZH-156 grains increased from $40 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$ to $49 \text{ mg (glucose)} \cdot (100\text{grains})^{-1} \cdot \text{h}^{-1}$. Secondly, the α -amylase activity in No.34, No.104 and No.276 from the ESG of RILs increased sharply during 10 "Raining"

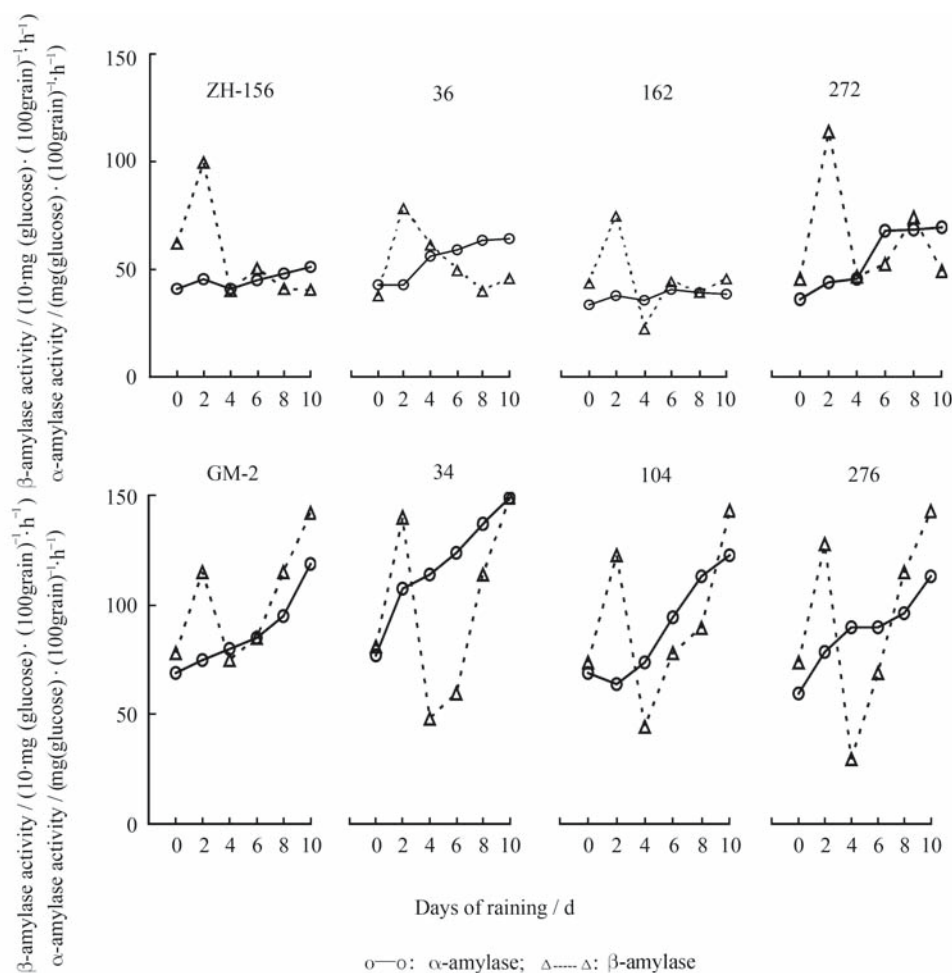


Fig. 5 Effects of raining on amylase activity in grains of recombinant lines during ripening

days. This result was similar to that of their parent—GM-2. However, the α -amylase activity in No.36, No.162 and No.272 from the HSG of RILs increased slowly during 10 “Raining” days. It was also similar to that of their parent—ZH-156. Thirdly, the peak values of β -amylase appeared 2 days after “Raining” treatment for all RILs and their parents, and the peak values from GM-2 and from the ESG of RILs were higher than that from ZH-156 and the HSG of RILs. Fourthly, the β -amylase activity in grains of No.34, No.104, No.276 and GM-2 began to rise from the 4th day after “Raining”, and it was sharply increased to 1 200–1 450 mg (glucose) \cdot (100grains) $^{-1} \cdot h^{-1}$ by the time of the 10th day after “Raining”. At the same time, no significant changes on the β -amylase activity were observed from ZH-156 and from the HSG of RILs.

4 Discussion

Hybrid rice seed quality may be badly degraded by pre-sprouting, and has interfered with the benefits of hybrid rice seed production and the seed cost (Huang et al., 1995). Scientists have spent decades trying to find a way to control

pre-sprouting, mostly focusing on searching for a chemical regulator to temporarily inhibit the early germination (Huang et al., 1995; Duan et al., 1991; Wang et al., 2000). A technology to produce ABA by fermentation was developed at the end of the 20th century. This made it possible to stop pre-sprouting by application of ABA in plant (Tan et al., 1998). The authors of this paper conducted a comparative study on the effects of ABA and maleic hydrazide (MH) on pre-sprouting, and suggested that those two regulators had good inhibitory effects on pre-sprouting (Wang et al., 2000). However, there were some differences between ABA and MH concerning the effects such as the different effective concentration and the different seed vitality after seeding. It was observed that the germination rate was decreased and the seedling grew abnormally if treated with MH during the grain filling stage. The ABA treatment during the grain filling stage also had a negative influence on seedling quality, but showed better effects on inhibiting pre-sprouting (Wang et al., 2000). Thus, there is still a long way to go on the pre-sprouting research. Conducting physiological or genetic studies on pre-sprouting will bring success to the chemical control, and would also provide useful data to breeding for pre-sprouting resistance rice.

Grain ripening after anthesis is a biochemical process in which small-molecule substances like glucose are made into large-molecule starch for storage. However, germination is just a reverse process against maturation, in which starch is decomposed to glucose to supply energy for seedlings (Song and Fu, 1993; Okamoto and Akazawa, 1979; Walker-Simmons, 1989). The pre-sprouting in hybrid rice F_1 discussed in this paper is indeed a complex balance of the above-mentioned two processes. GA_1 and ABA contents decreased during the F_1 seed filling and development in a normal status, so did amylase activity (Figures 2 and 4). At the same time, if the factors like high temperature, high humidity or artificial “Raining” were built for the grain filling during 15 days after anthesis, both GA_1 /ABA contents and α -/ β -amylase activities increased in all tested materials. This increment was bigger in the easy-sprouting-group than in the hard-sprouting-group (Figures 3 and 5).

It was shown in this experiment that grain maturation was still on-going while in a “Raining” environment 15 days after anthesis, and β -amylase was still in accumulation (Fig. 4, Fig. 5). Besides, the decomposing of starch became mainstream if the condition access to germination and GA_1 content and amylase activity began to increase (Figures 3 and 5). Therefore, the composing process was reversed to decomposing process in maturing grains when high temperature and high humidity were encountered, then the GA_1 increasing intensity was stronger than that of the ABA, which activated amylase activity, especially for β -amylase activity, leading to pre-sprouting before harvest. This may be one physiological pre-sprouting hypothesis. This pre-sprouting trait was mostly inherited from their parents.

It was generally considered that GA played a trigger effect to activate synthesis of α -amylase during germination (Song and Fu, 1993; Choi et al., 1996; Murakami, 1996). By comparison study using two types of plant materials, which were easy-sprouting and hard-sprouting RILs, this paper suggests that GA_1 and amylase are two important physiological traits positively associated with pre-sprouting. Starch accumulation and aleurone development are still in process during grain maturation, the increasing GA_1 must excrete from grain (Murakami, 1996; Tanaka et al., 1970), and α -amylase may be transferred from epithelia to the developing endosperm (Okamoto and Akazawa, 1979; Okamoto and Akazawa, 1980). The existing ABA in the endosperm may be too weak to inhibit increment for GA_1 content and α -amylase activity (Okamoto and Akazawa, 1979; Okamoto and Akazawa, 1980; Tsuneo and Kazuyoshi, 1993).

The main function of β -amylase, like α -amylase, is also used to decompose starch, and plays an important role in seed

germination (Tsuneo and Kazuyoshi, 1993). It was observed that β -amylase activity was ten times higher in sprouting grains than that in normal developing grains (Tao et al., in press) in our recent experiments. Further studies are needed to identify the important function of β -amylase in the process of pre-sprouting during grain maturation.

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