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Abstract

Seed dormancy is an important agronomic trait in cereals. Using deep dormant (*N22*), medium dormant (*ZH11*), and non-dormant (*G46B*) rice cultivars, we correlated seed dormancy phenotypes with abscisic acid (ABA) and gibberellin (GA) metabolism gene expression profiles and phytohormone levels during seed development and imbibition. A time course analysis of ABA and GA content during seed development showed that *N22* had a high ABA level at early and middle seed developmental stages, while at late developmental stage it declined to the level of *ZH11*; however, its ABA/GA ratio maintained at a high level throughout seed development. By contrast, *G46B* had the lowest ABA content during seed development though at early developmental stage its ABA level was close to that of *ZH11*, and its ABA/GA ratio peaked at late developmental stage that was at the same level of *ZH11*. Compared with *N22* and *G46B*, *ZH11* had an even and medium ABA level during seed development and its ABA/GA ratio peaked at the middle developmental stage. Moreover, the seed development time-point having high ABA/GA ratio also had relatively high transcript levels for key genes in ABA and GA metabolism pathways across three cultivars. These indicated that the embryo-imposed dormancy has been induced before the late developmental stage and is determined by ABA/GA ratio. A similar analysis during seed imbibition showed that ABA was synthesized in different degrees for the three cultivars. In addition, water uptake assay for intact mature seeds suggested that water could permeate through husk barrier into seed embryo for all three cultivars; however, all three cultivars showed distinct colors by vanillin-staining indicative of the existence of flavans in their husks, which are dormancy inhibition compounds responsible for the husk-imposed dormancy.

Keywords

Seed development, Seed imbibition, Gene expression, Abscisic acid (ABA), Gibberellin (GA), ABA/GA ratio, Rice

Introduction

By definition, seed dormancy is the inability of a viable seed to germinate under favorable conditions (Finch-Savage and Leubner-Metzger, 2006) and determined by genetic factors with a substantial environmental influence (Bewley, 1997, Graeber et al., 2012). In spite of numerous factors affecting dormancy, the

phytohormones, abscisic acid (ABA) and gibberellins (GAs), remain of great interest (Kermode, 2005, Kucera et al., 2005, Gianinetti and Vernieri, 2007).

In *Arabidopsis* seed development, there exist two ABA accumulation phases. The first major peak occurred in the middle phase of seed development, which was mainly derived from maternal tissues while the second minor peak occurring later was the result of *de novo* ABA biosynthesis in the embryo (Karssen et al., 1983, Finkelstein et al., 2002, Xiong and Zhu, 2003). Kanno et al. (2010) demonstrated that ABA was synthesized in both maternal and zygotic tissues during seed development, and maternal ABA can be translocated to the embryos and induce seed dormancy. ABA content was appreciably high in developing cereal grains, such as wheat (*Triticum araraticum*) and barley (*Hordeum vulgare*), and substantially declined as the grains underwent maturation and desiccation (Benech-Arnold et al., 1999, Jacobsen et al., 2002). Furthermore, ABA deficient mutants in maize (*Zea mays*), *Arabidopsis* and tomato (*Solanum lycopersicum*) lost their dormancy and resulted in precocious seed germination (Groot and Karssen, 1992, Mccarty, 1995, Koornneef et al., 2002). On the contrary, overexpression of ABA biosynthesis genes increased seed ABA content, deepened seed dormancy and delayed germination (Finkelstein et al., 2002, Nambara and Marion-Poll, 2005, Holdsworth et al., 2008).

However, mounting evidence also revealed no direct correlation between ABA content and seed dormancy (Black, 1991). For example, dormancy intensity in tomato was inconsistent with its ABA content (Hilhorst, 1995, Bewley et al., 2012), *Arabidopsis* mutants, *rdo1* and *rdo2*, lacked dormancy but possessed normal level of ABA (Léon-Kloosterziel et al., 1996), and application of ABA could recover all traits of *Arabidopsis* ABA deficient mutant, *aba*, except seed dormancy (Koornneef et al., 1989). Generally, ABA effect is dependent on the seed sensitivity to ABA, which is related to the balance between ABA biosynthesis and catabolism (Ni and Bradford, 1992, LePage-Degivry et al., 1996, Schmitz et al., 2002, Feurtado et al., 2007). ABA content can be correlated with seed dormancy variation under the condition of similar seed sensitivity to ABA among different dormancy phenotypes (Hilhorst, 1995, De Castro and Hilhorst, 2000) and sensitivity thresholds are also crutial for such a correlation (Bradford and Trewavas, 1994). ABA biosynthesis, turnover, and sensitivity are therefore most likely modulated during seed dormancy (Kermode, 2005, Gianinetti and Vernieri, 2007).

Moreover, the absence of such a correlation can be explained by a proposed role of ABA in seed dormancy, that is, ABA indirectly acts in the physiological modulation/maintenance of seed dormancy (Gianinetti and Vernieri, 2007) and is in concert with other endogenous components (Kermode, 2005, Kucera et al., 2005, Hauser et al., 2011). Another important phytohormone, GA, has an antagonistic role in controlling dormancy and germination (Finch-Savage and Leubner-Metzger, 2006, Finkelstein et al., 2008, Nambara et al., 2010). Changes in the ABA/GA balance are involved in the expression of dormancy in many species including *Arabidopsis* and many cereal crops (Kucera et al., 2005, Finch-Savage and Leubner-Metzger, 2006, Fang et al., 2008, Finkelstein et al., 2008). In particular, a high ABA/GA ratio in early seed development programming is critical for the germination suppression and maturation induction (Koornneef et al., 1982, White et al., 2000).

Seed dormancy in rice (*Oryza sativa* L.) has been undertaken to improve breeding program in pursuit of a balance between adequate control of pre-harvest sprouting and ensuring high rate of germination for deep dormant cultivars (Gao et al., 2008, Xie et al., 2011). Currently, the main approach to understand the underlying mechanism of rice seed dormancy depends on quantitative trait loci (QTL) analysis, which genetically dissects embryo- (Takeuchi et al., 2003, Gu et al., 2010, Sugimoto et al., 2010) and seed coat-imposed dormancy (Gu et al., 2003, Gu et al., 2005). It is found that the seed coat-imposed dormancy, associated with pericarp color in the lower epidermal cells, is controlled by a pleiotropic gene that regulates ABA and flavonoid synthesis in early seed development (Gu et al., 2011). Besides organic compounds, the importance of seed coat impermeability to water and/or oxygen is underscored in the study of seed dormancy as well (Kelly et al., 1992, Debeaujon et al., 2000).

To better understand the underlying mechanism of rice seed dormancy variation, we carried out a large-scale screening using rice germplasms and three rice cultivars representing three different degrees of seed dormancy were selected. We performed a comprehensive analysis of ABA and GA metabolism gene expression profiles and corresponding hormone amounts during seed development and imbibition processes and proposed that ABA/GA ratio before late seed development (i.e., during early and middle seed development) and the flavan compounds in the husk are two major aspects responsible for the dormancy variation in rice.

Results

Selection of rice cultivars representing different levels of seed dormancy

To select rice cultivars representing different levels of dormancy under normal field conditions, approximately 300 rice cultivars were repetitively screened in Beijing, Lingshui (Hainan Province), and Hangzhou (Zhejiang Province), China. Dormancy levels were evaluated by germination percentage following the method described by Wan et al. (1997). Using germination test results (Fig. S1), we selected three representative cultivars for further investigation: non-dormant *G46B* and deep dormant *N22*, which displayed >80% and <2% germination after seven days' imbibition, respectively, and the cultivar *ZH11*, whose dormancy was regarded as medium (50%–80% germination after seven days' imbibition).

Sensitivity to exogenous ABA and GA of the three cultivars is well correlated with seed dormancy

To determine the sensitivity of three rice cultivars to exogenous ABA, dehusked seeds of non-dormant *G46B*, medium dormant *ZH11*, and deep dormant *N22* were incubated in water, 5 and 10 μmol/L ABA, and the germination percentage and plumule length were respectively measured on moistened filter paper on the 7th day after imbibition (Fig. 1A). The three cultivars had the same germination percentage without addition of ABA (Fig. 1B). By contrast, the germination rate was significantly lower for *N22* (55.21%) than for *G46B* (91.23%) and *ZH11* (84.76%) at the presence of 5 μmol/L ABA. In incubation with 10 μmol/L ABA, the germination rate of *G46B* and *ZH11* was 87.41% and 65.74%, respectively, which was significantly higher than 33.11% for *N22* (Fig. 1B). Moreover, the plumule length measurement after seven days of imbibition in ddH_2O showed that the average plumule length of *G46B* (4.31 ± 0.12 cm) was significantly longer than those of the other two cultivars (3.42 ± 0.32 cm for *ZH11* and 3.12 ± 0.41 cm for *N22*; $P < 0.01$) (Fig. 1C). The average plumule length exhibited significant difference after incubation with 5 μmol/L ABA (1.32 ± 0.12 cm for *G46B*, 0.27 ± 0.07 cm for *ZH11*, and 0.06 ± 0.05 cm for *N22*; $P < 0.01$), while 10 μmol/L ABA considerably inhibited seed germination of the three cultivars and resulted in the lowest overall average plumule length in all three treatments (0.32 ± 0.03 cm for *G46B*, 0.03 ± 0.04 cm for *ZH11*, and 0.00 ± 0.09 cm for *N22*; $P < 0.01$) (Fig. 1C). These results indicated that the sensitivity of *G46B* to exogenous ABA was weaker than that of *ZH11* and *N22*, and the rice seed dormancy were correlated with ABA sensitivity.

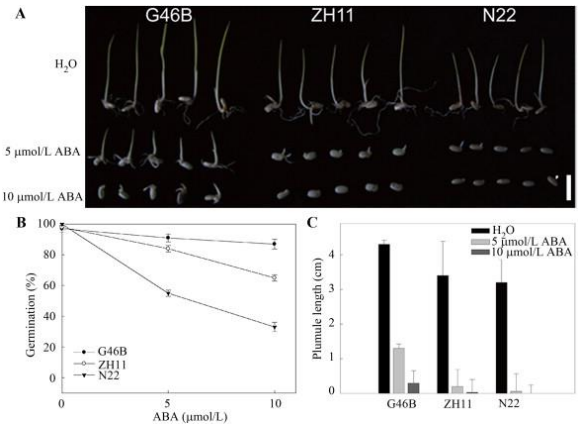

Fig. 1. ABA sensitivity of rice cultivars *G46B*, *ZH11*, and *N22* (non-thermally treated).

A: Germination of dehusked rice seeds in response to exogenous ABA after seven days of imbibition (compared to H_2O control). Scale bar, 1 cm. **B:** Germination rate for seeds incubated at 0, 5, and 10 μmol/L ABA after seven days of imbibition. **C:** Measurement of plumule length seven days after the imbibition. Error bars represent standard errors.

The α-amylase activity reflects the ability of endosperm starch degradation and thus indicates the state of seed dormancy (Yamaguchi, 1998). It was found that the difference of α-amylase activity between cultivars were proportional to their difference in embryo response to ABA (Benech-Arnold et al., 1999). The relative α-amylase activity in 10^{-7} mol/L GA_3 for *G46B* was nearly 9 times higher than that in water (Fig. 2). For *ZH11*, there was also a significant difference with or without 10^{-7} mol/L GA_3. However, α-amylase activity for *N22* did not have appreciable change with or without exogenous GA_3. These results suggested that aleuronic layer of *N22* was less sensitive to GA compared to that of the other two cultivars. Taken together, the rice dormancy variation was associated with their sensitivity to exogenous ABA and GA.

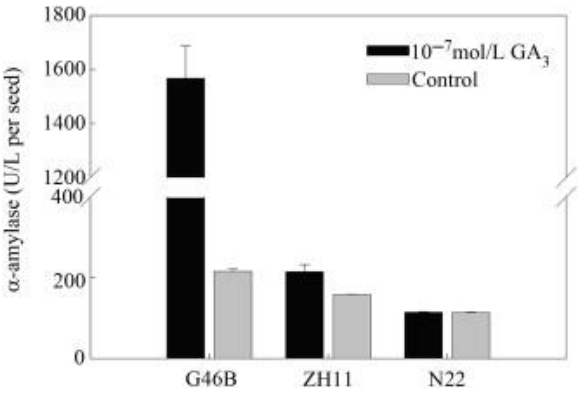

Fig. 2. GA_3 induction of α-amylase activity.

Embryoless half seeds (non-thermally treated) were incubated for 60 h at 25 °C in culture medium with and without 10^{-7} mol/L GA_3. Values are the means ± SE (n = 5, biological replicates) and error bars represent standard errors. For significance an ANOVA test was performed. Asterisks indicate means that are significantly different ($P < 0.01$).

Expression of ABA and GA metabolism genes during seed development

Differences in seed dormancy can be attributable to differential expression of key hormone metabolism genes. We therefore assayed the expression of genes related to ABA and
GA biosynthesis and catabolism using qPCR during seed development (Fig. 3A), including before pollination (BP)

to 10 days after pollination (DAP, early developmental stage), 10 to 20 DAP (middle developmental stage), and 20 to 30 DAP (late developmental stage).

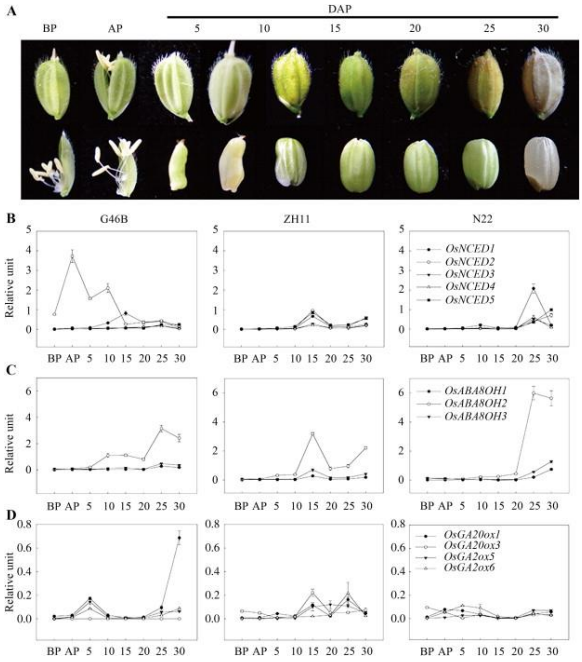

Fig. 3. Gene expression profiling during seed development.

A: Seed morphological changes (*ZH11*) during the seed development from "before pollination" (BP) to "at pollination" (AP) and 5 to 35 "days after pollination" (DAP); **B–D:** qPCR analysis of metabolic gene expression profile of ABA and GA during seed development in rice cultivars *G46B*, *ZH11*, and *N22*. Relative expression levels of ABA and GA metabolic genes before pollination" (BP), at pollination (AP), and from 5 to 30 days after pollination (DAP) were shown. **B:** Expression levels of five members of the ABA biosynthesis genes *OsNCED*. **C:** Expression levels of three members of the ABA catabolic genes *OsABA8OH*. **D:** Expression levels of two members of the GA biosynthesis genes *OsGA20ox* and two members of the GA catabolic genes *OsGA2ox*. Values are the means ± SE (*n* = 3, biological replicates), and error bars represent standard errors.

The expression levels of genes involved in ABA synthesis and turnover were examined during seed development. The 9-*cis*-epoxycarotenoid dioxygenase (*OsNCED*) and ABA 8'-hydroxylase (*OsABA8OH*) genes are rate-limiting steps of ABA biosynthesis and catabolism, respectively (Fang et al., 2008), and five *OsNCED* paralogs and three *OsABA8OH* paralogs were found in rice, among which *OsNCED2* and *OsABA8OH2* were predominantly expressed during seed development while the expression levels of other paralogs were quite low (Fig. 3B and C). Interestingly, the peak of *OsNCED* transcripts occurred at different stages of seed development for the three rice cultivars: early stage for the non-dormant *G46B*, middle stage for the medium dormant *ZH11*, and late stage for the deep dormant *N22* (Fig. 3B). In contrast, the peak of *ABA8OH2* transcripts occurred at late, middle, and late stage for *G46B*, *ZH11*, and *N22*, respectively (Fig. 3C). This indicated that the *G46B* seeds had low ABA content throughout seed development, whereas *N22* transcripts in ABA synthesis and turnover coincidently peaked at late developmental stage, suggest that the mature *N22* seeds accumulated high ABA content during early and middle seed development, which could activate ABA catabolism gene expression at late stage.

The GA20 oxidase (*OsGA20ox*) and GA2 oxidase (*OsGA2ox*) are genes in the committed steps of GA biosynthesis and catabolism, respectively (Sakamoto et al., 2004). Expression analysis revealed that the transcripts of biosynthesis gene *OsGA20ox1* had two peaks occurring at the early and late stage of seed development, while the transcripts of the GA catabolism genes *OsGA2ox5* and *OsGA2ox6* peaked at early developing stage

overlapping with the first *OsGA20ox1* peak in terms of the peak time for *G46B* (Fig. 3D). This indicated that *G46B* accumulated active GAs at the late stage of seed development. For *ZH11*, the transcript levels of GA biosynthesis and catabolism genes peaked in the middle and late developmental stages simultaneously, suggesting that the balance expression of GA metabolism genes led to a lower level of active GAs for *ZH11* than *G46B* (Fig. 3D). *N22* had minor changes in GA metabolism gene transcripts, suggesting that *N22* accumulated few amount of active GAs during seed development (Fig. 3D).

ABA and GA levels during seed development

To determine whether ABA and GA levels correlated with the expression profiles of their metabolism genes during seed development, we measured ABA and GA_3 amounts over the time course of seed development by indirect competitive enzyme-linked immunosorbent assay (icELISA) and quantified ABA at early, middle and late developing stages by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Fig. 4). ABA accumulation occurred at the early stage of grain filling, and the ABA content of *N22* peaked higher than that of the other two cultivars (Fig. 4A). The ABA icELISA results are well fitted with ABA quantification by LC/MS/MS (Fig. 4A and C). The ABA level of deep dormant cultivar *N22* was high at early (258.67 pg/mg) and middle (275.85 pg/mg) stages, then abruptly declined (54.12 pg/mg) and remained low at late stage. In contrast, ABA amounts in *G46B* were low during seed development (<50 pg/mg). For *ZH11*, average ABA amounts remained around 85 pg/mg during seed development (Fig. 4C). Meanwhile, GA_3 levels were high in early seed development for all three cultivars and the non-dormant, medium dormant and deep dormant cultivars exhibited high, medium, and low peak levels, respectively (Fig. 4B). Taken together, though high ABA or GA accumulation during the early and middle stages of seed development were not correlated with the expression of their corresponding hormone metabolism genes (Fig. 3), the levels of ABA and GA_3 at early and middle development stages are well correlated with the dormancy phenotypes of the three cultivars.

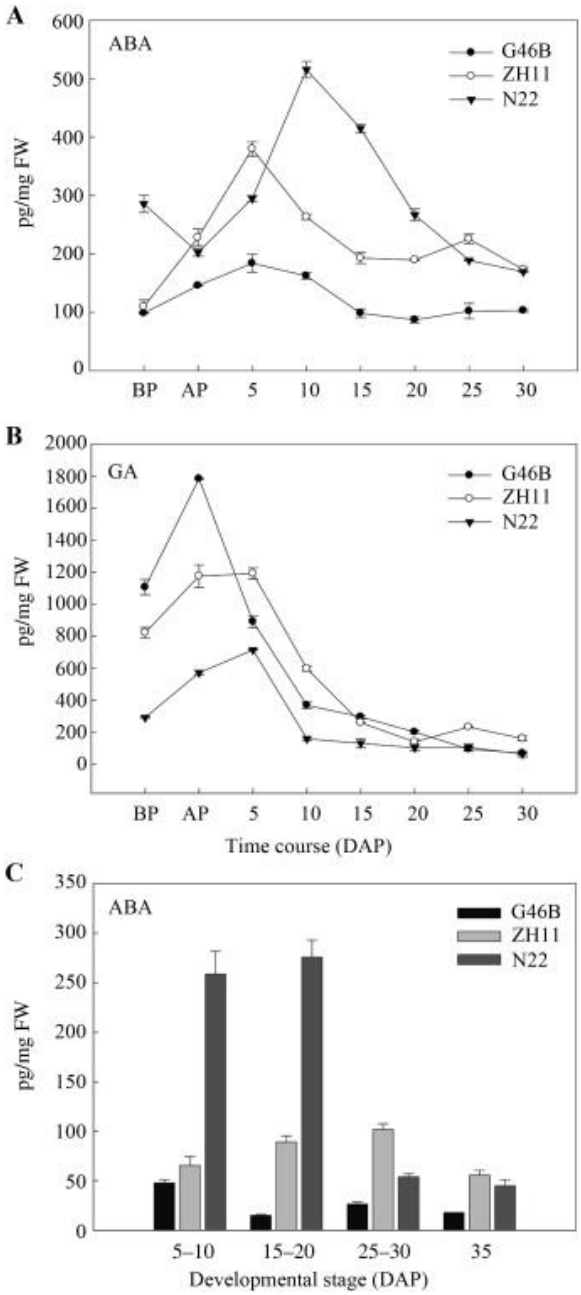

Fig. 4. ABA and GA levels during seed development in rice cultivars *G46B*, *ZH11*, and *N22*.

A and **B:** Indirect competitive enzyme-linked immunosorbent quantification of phytohormone levels during seed development. BP, before pollination; AP, after pollination; DAP, days after pollination. **C:** Liquid chromatography-tandem mass spectrometry quantification of ABA. Data presented are mean values of nine biological repeats with SD.

Expression of ABA and GA metabolism genes during seed imbibition

We further analyzed the expression profiles of ABA and GA metabolism genes during seed imbibition (Fig. S2). The transcript levels of ABA metabolism genes in *G46B* did not significantly change during seed imbibition, while the levels of its GA biosynthesis gene *OsGA20ox1* peaked at 36 h, indicating that active GAs were synthesized (Fig. 5A-a, d, g). For the deep dormant cultivar *N22* treated thermally, expression of ABA catabolism genes kept a relatively high level before 36 h while *OsNCED2* were highly expressed prior to 12 h and peaked again at its germination onset (48 h), however, levels of *OsGA20ox1* peaked at 48 h and 24 h (Fig. 5A-c, f, i). This suggested

that a complicated antagonism or regulation between ABA and GA existed during the imbibition of *N22*.
For *ZH11*, expression levels of the ABA and GA metabolism genes changed little during seed imbibition, but had small peaks around the time of coleoptile emergence, suggesting that a milder antagonism between ABA and GA during the imbibition of *ZH11* (Fig. 5A-b, e, h and Fig. S2).

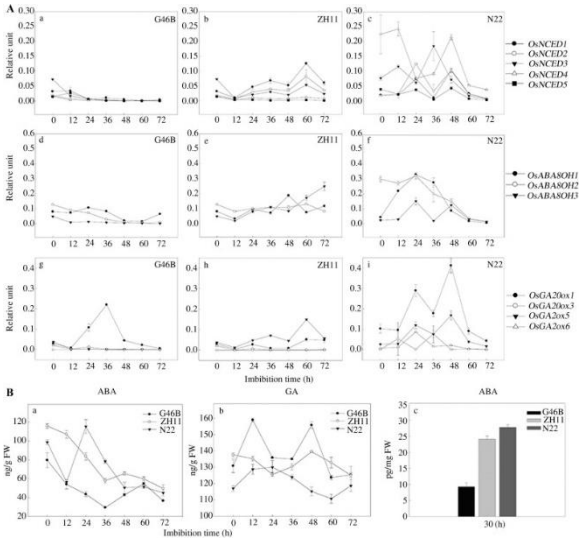

Fig. 5. Gene expression profiling and ABA and GA levels during seed imbibition.

A: qPCR analysis of metabolic gene expression profiles of ABA and GA during imbibition in rice seeds of cultivars *G46B*, *ZH11*, and *N22*. Relative expression levels of ABA and GA metabolic genes from 0 to 72 h of imbibition were shown. **a–c:** Expression levels of five members of the ABA biosynthesis genes *OsNCED*. **d–f:** Expression levels of three members of the ABA catabolic genes *OsABA8OH*. **g–i:** Expression levels of two members of the GA biosynthesis genes *OsGA20ox* and two members of the GA catabolic genes *OsGA2ox*. Values are the means ± SE (*n* = 3, biological replicates), and error bars represent standard errors. **B:** ABA and GA levels during imbibition in rice cultivars *G46B*, *ZH11*, and *N22*. Indirect competitive enzyme-linked immunosorbent quantification of phytohormone levels during 72 h of seed imbibition. Liquid chromatography-tandem mass spectrometry quantification of ABA during 30 h of seed imbibition. Data presented are mean values of nine biological repeats with SD.

ABA and GA contents during seed imbibition

To determine ABA and GA levels after the antagonism effect between ABA and GA during seed imbibition, we measured ABA and GA_3 amounts at different time-points in a certain interval during 72 h imbibition. In general, ABA levels were lower during seed imbibition compared to seed development and declined as imbibition onward (Fig. 5B-a). A significant small peak of ABA biosynthesis was observed only for thermally treated *N22* right before radicle protrusion (Fig. 5B-a and Fig. S2). From the late stage of seed development (35 DAP) to 30 h of imbibition, ABA levels for *ZH11* were almost as high as *N22*, and were the lowest for *G46B* (Fig. 5B-c), indicating that seed dormancy is not determined by the absolute ABA content of mature seeds. In contrast, two peaks of GA_3 occurred before and after the germination onset at 36 h for *G46B*, and GA_3 contents for *ZH11* and *N22* only slightly fluctuated (Fig. 5B-b), which coincided with germination phenotypes of these three rice cultivars (Fig. S2). In addition, the GA_3 content for *N22* was low and further decreased after its germination onset at 48 h, which correlated with our observation of its slow radicle elongation.

Husk-imposed dormancy and *de novo* ABA biosynthesis during seed imbibition

To assess the husk permeability during imbibition, the percent of water uptake during the first 36 h of imbibition was measured (Fig. S3). The results demonstrated that seeds of three cultivars underwent similarly sustainable

water uptake in the first 8 h, suggesting that the husks of three rice cultivars had similar water uptake ability and thus husk permeability did not affect seed dormancy. However, after 8 h, water uptake quickly increased for *G46B*, *ZH11* and thermally treated *N22*, while untreated *N22* did not (Fig. S3). This indicated that after 8 h imbibition, most cultivars began to initiate a series of germination events, such as RNA and protein (enzyme) repair and synthesis, which needed to absorb more free water. *N22* seeds with husk (untreated thermally) were incapable of germinating; however, when they were dehusked, germination percent could reach up to 90% after 60 h of imbibition (Fig. 6C). It was likely that some inhibitory effects of flavonoids released and leached out from the husks, and the vanillin-staining assay agreed with the existence of the flavan compounds (Fig. S4) and these compounds were detected in wheat (Kato et al., 2003). In all, this correlation suggested that the existence of germination inhibitory compounds in the husk could also affect the dormancy variation.

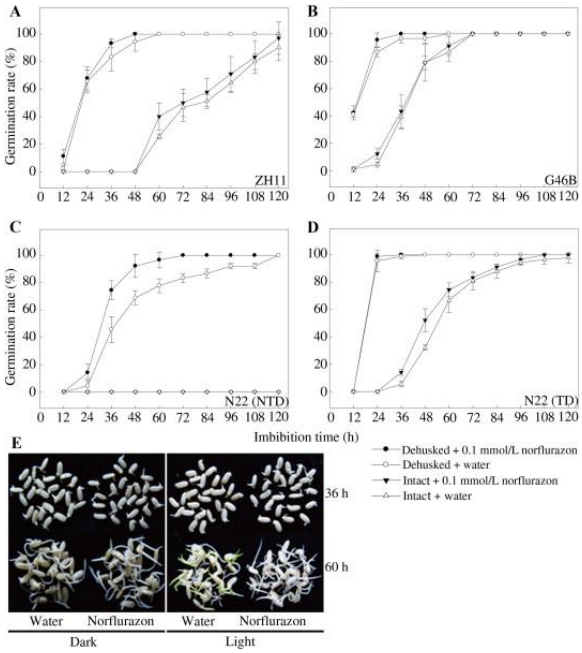

Fig. 6. Effect of the ABA biosynthesis inhibitor norflurazon on seed germination among *G46B*, *ZH11*, and *N22*.

A–C: Norflurazon effect on dormant in intact/dehusked seeds. NTD, non-thermally treated. **D:** Norflurazon effect on thermally treated (TD), intact/dehusked *N22* seeds. **E:** Validation of norflurazon treatment for *ZH11* by comparing the effect under dark and light conditions.

To evaluate the contribution of *de novo* ABA biosynthesis to seed dormancy during seed imbibition, seeds germinated in the presence of the bleaching herbicide norflurazon, which interferes with ABA biosynthesis due to a block in the pathway of carotenoid biosynthesis (Chamovitz et al., 1991). Norflurazon stimulated germination of intact and dehusked seeds of *G46B*, *ZH11*, and *N22* without or with thermal treatment (Fig. 6A–D). These results suggested that *de novo* ABA biosynthesis during seed imbibition accounted for germination retardation to a certain extent. However, norflurazon didn't stimulate the germination of intact *N22* seeds without thermal treatment, but can stimulate the germination when seeds were dehusked (Fig. 6C), suggesting that not only ABA but also some compounds in *N22* husks can attenuate or even prohibit seed emergence. In addition, seedlings exposed to light appeared yellowish, which was in agreement with the function of norflurazon, namely, capable of blocking carotenoid biosynthesis and thus affecting chlorophyll biosynthesis and photosynthetic activity (Fig. 6E).

ABA/GA ratio during seed development and imbibition

The dynamic changes of the ABA/GA ratio during seed development and imbibition showed that *N22* had significantly higher ABA/GA ratio than *G46B* during the seed development while the ratio of *ZH11* was in

intermediate level (Fig. 7). Moreover, two ratio peaks occurred at middle and late stages of seed development for *N22*. For *G46B*, ABA/GA ratio remained low until 20 DAP and had an increase at late development stage. At harvest stage (30 DAP), *N22* had the highest ratio, and surprisingly *ZH11* had a lower ratio than *G46B* (Fig. 7). In addition, the ABA/GA ratio for both *G46B* and *ZH11* during the imbibition was low and experienced a slight decrease while for thermally treated *N22*, after an initial decrease the ABA/GA ratio increased again before the germination onset (48 h) (Fig. 7). These results were consistent with the observation of *de novo* ABA biosynthesis of *N22* during imbibition. In conclusion, *N22* kept higher ABA/GA ratio during seed development and imbibition, which can be accountable for its deep dormancy phenotype.

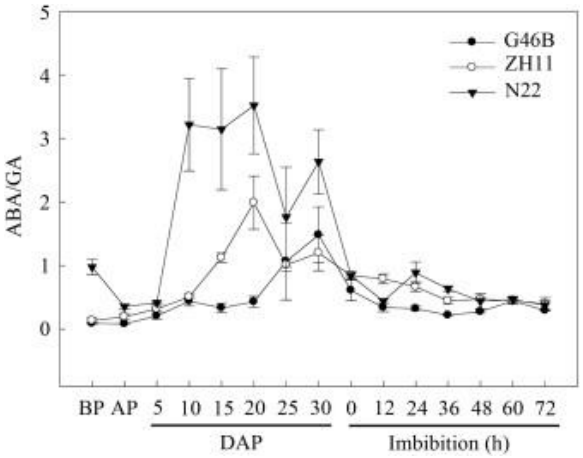

Fig. 7. ABA to GA ratios during seed development and imbibition in rice cultivars *G46B*, *ZH11*, and *N22*.

Discussion

By screening approximately 300 rice cultivars, three rice cultivars representing three different seed dormancy levels were selected. Using the selected rice cultivars, we investigated the underlying mechanism of seed dormancy by comparing different dormancy features: (1) differences in sensitivity of mature embryos to ABA are observed in rice cultivars (Fig. 1); (2) different dormancy intensity exhibits different capacity to produce α-amylase activity during imbibition (Fig. 2); (3) ABA/GA ratio before late seed development (i.e., early and middle seed development phases) determines embryo-imposed dormancy (Fig. 3, Fig. 4, Fig. 5, Fig. 7); (5) flavan compounds in husk determine husk-imposed dormancy (Fig. S4); and (6) *de novo* ABA biosynthesis during seed imbibition also accounts for seed dormancy variation (Fig. 6).

In response to exogenous ABA and GA, three rice cultivars were in line with the rule regarding the relationship between seed dormancy and phytohormone responsiveness, that is, the seed dormancy level is positively correlated with its sensitivity to ABA and negatively correlated with its capacity to produce α-amylase with exogenous application of GA (Yamaguchi, 1998, Gianinetti and Vernieri, 2007). Thus, it is justifiable to correlate embryo-imposed dormancy with ABA and GAs in rice cultivars.

To address how seed dormancy works with ABA and GA during seed development, we monitored all five rice *OsNCEDs* which are key regulators of ABA biosynthesis in developing seeds, and all three rice *OsABA8OH* which are rate-limiting factors for ABA inactivation (Fang et al., 2008). With aid of bio-chip data (http://ricexpro.dna.affrc.go.jp/GGEP/index.html) and semi-quantitative PCR results (data not shown), expression levels for two of the four rice key GA biosynthesis genes (*OsGA20ox1* and *OsGA20ox3*) and two of the six rice key GA catabolism genes (*OsGA2ox5* and *OsGA2ox6*) were used. Meanwhile, active phytohormones of ABA and GA were measured in the same time-points. Interestingly, high level of ABA (and GAs) content at early developmental stage seemed to be independent of the synthesis of their metabolism genes because high level of ABA occurring at the early stage of seed development (Fig. 4A and C) was not concomitant with transcript

accumulation of ABA metabolism genes (Fig. 3B and C), which was inconsistent with some of previous studies (Huh et al., 2013). A similar inconsistency occurred for GA counterparts (Figs. 3D and 4B). These inconsistencies were presumably due to maternal effects (Finkelstein et al., 2002, Xiong and Zhu, 2003, Donohue, 2009). High levels of maternal ABA biosynthesis capacity passed down for seeds of *N22* and *ZH11*, which became active thus leading to the high ABA amount during early seed development, and alternatively, the ABA is directly transported from maternally vegetative tissues to the embryos. It was also possible that active ABA molecules have three possible fates: remained functional, inactivated through hydroxylation, or degraded (Schroeder and Nambara, 2006). Therefore, seeds with high levels of ABA biosynthesis gene transcripts do not necessarily generate high levels of active ABA instantly, but release the stored ABA at a later stage. With regard to GA, *G46B* had highest GA content at early seed development while *N22* had the least (Fig. 4B), which did not match with the pattern changes of GA metabolism gene expression (Fig. 3D). A similar mechanism of maternal effects can also account for such an inconsistency. Notwithstanding inconsistent results between gene transcripts and hormone levels, in the three rice cultivars, *N22* having highest ABA content and lowest GA content during seed development and *G46B* having highest GA content and lowest ABA content are consistent with a general principle – ABA is positively correlated with seed dormancy while GA is negatively correlated. Moreover, our results agree with the classic model, that is, the state of seed dormancy correlated with ABA/GA ratio (Fig. 7) (White et al., 2000), which was further clarified by Penfield and King (2009). The core feature of this model is that a heterodimetric complex that promotes germination exists and the conglomerate of one monomer is affected by ABA and the other affected by GA. It is noteworthy that at 30 DAP, the ABA/GA ratio of *G46B* was not lower than that of *ZH11* (Fig. 7), intimating that a fine-tuning regulation or other mechanisms, for example interaction with other phytohormone, was involved in seed dormancy.

In addition, the profiling of *OsGAMYB* expression was established, whose gene product is critical for floral organ development as well as the induction of α-amylase in aleurone (Kaneko et al., 2004). The expression levels of *OsGAMYB* peaked at early, middle, and late stages of seed development for *N22*, *ZH11*, and *G46B*, respectively (Fig. S5A). This indicated that the formation of seed dormancy has a positive correlation with the gene expression time.

The covering tissues have been hypothesized as a physical barrier to the germination of dormant seeds, and/or contain germination inhibitors (Bewley et al., 2012). Husk-imposed dormancy of *N22* was reported (Seshu and Sorrells, 1986) but its mechanism was not clarified. In rice, ABA and flavonoid synthesis in the lower epidermal cells of the pericarp tissue have been associated with husk-imposed dormancy (Gu et al., 2011). Our results demonstrated that the compounds rather than the husk permeability yielded husk-imposed dormancy in our rice cultivars using water uptake (Fig. S3) and vanillin-staining assay (Fig. S4). During seed imbibition, an obvious ABA increase was observed in *N22* and the expression of ABA and GA metabolism genes appreciably changed (Fig. 5A-c, f, i and 5B-a), suggesting the occurrence of ABA *de novo* biosynthesis and the existence of fine-tuning regulation on ABA and GA during seed imbibition. Such a detectable change was probably the attribute of deep dormant cultivar, *N22*, with some influence of thermal treatment. Nevertheless, no ABA accumulation had been observed for *G46B* and *ZH11* in imbibition. Moreover, there were no significant transcript changes of ABA and GA metabolism genes (Fig. 5A and Fig. S5B), which might be due to that the 12 h intervals for collecting materials were too long to capture swift changes of low dormant cultivars or the three cultivars have evolved different dormancy mechanisms. Additionally, the thermally treated *N22* had the highest *OsGAMYB* expression (Fig. S5B), indicating that *N22* required more GA to initiate germination which is consistent with the result of α-amylase assay (Fig. 2). Furthermore, expression levels of GA biosynthesis genes showed an abrupt increase at the late stage of seed development in *G46B* (Fig. 3D), which could account for the first peak of active GA at the onset of seed imbibition (Fig. 5B-b). Transcripts for *G46B* GA biosynthesis genes did not increase until 36 h after imbibition but there was an active GA accumulation at 12 h after imbibition (Fig. 5A-g). A possible explanation is that *G46B* seeds activated GA biosynthesis enzymes that had been synthesized in inactivated state during late

seed development. In contrast, the second GA peak right after the germination onset at 36 h was consistent with the up-regulation of the GA biosynthesis gene *GA20ox1* at this point (Fig. 5A-g). Thus, biosynthesis of active GA before and after the germination onset breaks dormancy and promotes germination, respectively, for *G46B*. Overall, the characteristics of GA biosynthesis gene transcripts in imbibition are consistent with different dormancy levels of three rice cultivars.

ABA and GA contents were measured on a tissue fresh weight basis during seed maturation and imbibition, as such, the significant difference in water content of the seeds throughout seed development and imbibition (Fig. S3) might be the reason that ABA content in *N22* declined at late developmental stage compared to previous stages. Nonetheless, it is still safe to conclude that ABA content in imbibition stages is lower than that in development stages.

Taken together, we conclude that seed dormancy imposed by embryo is determined by ABA/GA ratio during early and middle seed development phases, and the flavan compounds in husks determine husk-imposed dormancy and *de novo* ABA biosynthesis during seed imbibition affects seed dormancy variation as well.

Materials and methods

Plant materials

The rice (*Oryza sativa* L.) cultivars *G46B*, *ZH11*, and *N22* were used throughout the experiments. Approximately 20 spikelets per panicle in the center of florets at pollination were tagged with a marker to guarantee the same developmental stage for all samples. The spikelets were harvested from the field every five days after pollination (DAP) at six sequential time-points and then immediately frozen in liquid nitrogen. Thirty days after pollination, seeds attained harvest maturity with around 20% water content were harvested, sealed, and stored at −20°C to maintain dormancy. These grains were used in subsequent experiments.

Seed germination tests

Approximately 200 mature and dormant seeds were placed on three filter papers moistened with 8 mL of double-distilled water in 55 mm diameter Petri dishes and incubated at 28°C in a light-proof chamber. Seeds were first surface-sterilized with a solution containing 1% (w/v) sodium hypochlorite (NaClO) and 0.05% (v/v) Tween-20 and then rinsed with ddH_2O. Fungicide (Carbendazim, 0.5 g/L) was applied on the fourth day. Germination was scored at the first germination stage (S1) when radicle or coleoptile was visually ≥1 mm (Counce et al., 2000). Due to the extraordinarily deep dormancy of *N22*, which is unable to germinate right after harvest, some *N22* seeds were dried at 50°C for 72 h to break its dormancy prior to plating (Lu et al., 2011b). During imbibition, 30 seeds were collected every 12 h for 72 h, and then immediately frozen in liquid nitrogen. Moreover, to test if ABA biosynthesis occurs during imbibition, norflurazon (dissolved in pure dimethyl sulfoxide (DMSO) and then diluted to a final concentration of 0.1 mmol/L with ddH_2O) was applied. To assess ABA sensitivity, 50 dehusked seeds were imbibed in water (control) or in 5 or 10 μmol/L ABA (Lu et al., 2011a). The percentage of germinating seeds was scored and the plumule length was measured at the 7th day after incubation. For the water uptake assay, fifty gauze wrapped seeds were immersed in ddH_2O and the seed weight was measured at a 12 h interval until 72 h. The surface water was removed with paper tissue prior to the measurement. The assays were carried out in two independent seed batches with three replicates for each. Significance analysis was conducted by SAS® ver. 9.1.3 (SAS Institute, 1999).

RNA extraction from rice seed

Rice seeds at different developmental stages and the different treatments were powdered in a mortar and pestle with liquid nitrogen and then transferred to Eppendorf tubes with 800 μL ice-cold extraction buffer [200 mmol/L Tris-HCl (pH 9.0), 200 mmol/L LiCl, 5 mmol/L EDTA, 1% SDS, 1/1000 (v/v) β-mercaptoethanol]. After

vortexing, the sample was centrifuged at 13,000 r/min for 5 min, and the supernatant was collected. An identical volume of water/phenol was added to the supernatant before vortexing and centrifugation at 13,000 r/min for 10 min at 4°C. The water phase was transferred into new tubes and an identical volume of chloroform/isoamyl alcohol (24:1) was added. The mixture was centrifuged at 13,000 r/min for 5 min, the new water phase was collected into a new tube and a 1/300 volume of acetic acid and an identical volume of isopropanol was added. After storage at −20°C for 30 min, tubes were centrifuged at 13,000 r/min for 5 min, and the supernatant was discarded. The precipitate was dissolved in 30 µL TE buffer and a 1/5 volume of 10 mol/L LiCl was added. After storage overnight at 4°C, samples were centrifuged at 13,000 r/min for 10 min, and the precipitate was dissolved in 50 µL TE buffer with 125 µL ethanol added. The centrifuged precipitate was rinsed with 70% ethanol and then treated with DNase using the TURBO DNA-free kit (Ambion, Applied Biosystems, USA) to eliminate genomic DNA contamination. Finally, RNA concentrations were measured spectrophotometrically and the RNA quality and the accuracy of the concentration were checked with an RNA gel.

Quantitative real-time PCR

Two µg of RNA was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). First-strand cDNA synthesis products were diluted 5-fold, and 1 µL of cDNA was first used for semi-quantitative PCR. The quantitative PCR was performed using the Bio-Rad CFX96 Real-time System following established laboratory protocols (Tong et al., 2009). Efficiency calculation and normalization were performed using real-time PCR Miner (www.miner.ewindup.info/) (Zhao and Fernald, 2005) and data quality was confirmed through internal controls and no-template-controls (NTCs) and by comparing replicates' repeatability. An average expression value for each gene at each time-point was generalized from the normalized data. Actin1 and ubiquitin were used for the internal standards. Experiments were repeated three times.

Liquid chromatography-tandem mass spectrometry quantification of ABA

Approximately 150 mg of powdered rice seeds, as described for seed RNA extraction, were used for ABA quantification following a published method (Kojima et al., 2009) with minor modifications. Briefly, plant tissues were homogenized and extracted for 24 h in methanol containing 2H-ABA (CDN Isotopes) as an internal standard. Oasis Max solid phase extraction cartridges (150 mg/6 mL; Waters, Milford, USA) were used for purification after centrifugation. The entire sample was injected into a liquid chromatography-tandem mass spectrometry system consisting of an Acquity Ultra Performance Liquid Chromatograph (Acquity UPLC; Waters) and a triple quadruple tandem mass spectrometer (Quattro Premier XE; Waters).

Indirect competitive enzyme-linked immunosorbent quantification of ABA and GA_3

Approximately 200 mg powdered rice seeds was homogenized in 5 mL of ice-cold extraction medium (80% methanol, 1 mmol/L dibutyl hydroxy toluene) and extracted overnight at 4°C for indirect competitive enzyme-linked immunosorbent assay (icELISA). After centrifugation at 1000 *g* for 10 min, the supernatant was removed and passed through a C18-SepPak classic cartridge (Waters). The extract was evaporated and was diluted 8- and 16-fold in diluent [PBS; 0.1% (v/v) Tween-20; 0.1% (w/v) glutin] for GA_3 and ABA measurements, respectively. ABA and GA_3 were analyzed by competitive-binding ELISA employing commercially available monoclonal antibodies specific for (+)-ABA and GA_3, respectively (Sigma, St Louis, MO, USA) according to the Phytodetek protocol and essentially as previously described (Zhao et al., 2006). A standard curve of different ABA and GA_3 dilutions was constructed to calculate the sample ABA and GA_3 concentrations. Each measurement was repeated three times.

α-amylase activity assay

Alpha-amylase activity analysis using embryoless half seed were performed as described previously with some modifications (Yamaguchi, 1998). Rice seeds were surface-sterilized with 1% NaClO for 15 min, and then rinsed

three times with ddH_2O. Grains were then de-embryonated and transferred aseptically to 5 mL of aqueous buffer (0.02 mol/L calcium chloride, 0.05 mol/L sodium citrate, 10 mg/L streptomycin sulfate, pH 6.2) with or without 10^{-7} mol/L GA_3. This concentration is reportedly sufficient to induce the α-amylase saturation (Ueguchi-Tanaka et al., 2000). Samples were then incubated in dark at 25°C on an orbital shaker at 100 r/min for 60 h. Soaked half seeds were ground in their buffer and centrifuged at 3000 r/min for 15 min. Subsequently, the aqueous phase was divided into two portions with 2 mL to each tube and then kept at 37°C for 10 min. The α-amylase activity was determined by the Phadebas® Amylase kit according to the manufacturer's instructions (Magle, Sweden). Significance analysis was conducted by SAS® ver. 9.1.3 (SAS Institute, 1999).

Vanillin-staining assay

Intact mature seeds were incubated in a 1% (w/v) vanillin solution (SCRC, Beijing, China) at room temperature for 3 h as previously described (Aastrup et al., 1984). Vanillin turns red upon binding to flavan-3,4-diols (leucoanthocyanidins) and flavan-4-ols (catechins), which are present either as monomers or as terminal subunits of proanthocyanidins (Deshpande et al., 1986, Berridge et al., 1996).

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Supplementary data

The following is the supplementary data related to this article:

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Fig. S1. Seed germination tests and three cultivars' germination percent.

Fig. S2. Germination phenotype of three rice cultivars *G46B*, *ZH11*, and *N22*.

Fig. S3. Water uptake assay for harvested *G46B*, *ZH11*, and *N22* rice seeds over 36 h.

Fig. S4. The presence of catechins and proanthocyanidins in mature rice seeds determined by the vanillin assay compared with the original color of untreated rice seeds.

Fig. S5. Expression pattern of the GA marker *OsGAMYB* during seed development and imbibition for rice cultivars *G46B*, *ZH11*, and *N22* (thermally treated).

Table S1. qPCR primers used in this study.

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