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Original Research

Levels and Expression Patterns of Endogenous Hormones during Seed Germination of Dongxiang Wild Rice

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Abstract

Dongxiang wild rice (*Oryza rufipogon* Griff, hereafter DXWR), is very precious rice germplasm resource for maintaining genetic diversity of rice germplasm, but it has a strong dormancy property which will hinder the utilization of its germplasm resources. Here, the changes of endogenous hormone and the expression of endogenous hormone gene in DXWR and cultivated rice (9311) were analyzed using enzyme linked immunosorbent quantification and qRT-PCR. The result showed that only 30% of DXWR germination rate at 36-h and the highest germination rate displayed 83% at 92-h. The content of ABA and JA in DXWR mature seeds was higher than 9311, and the contents of GA and IAA for DXWR only slightly fluctuated during germination. The expression of ABA catabolic genes *NCED* and *OsABA8OH* in DXWR fast decreased before germination onset, however *GAMYB* and *DAD* (catabolic genes of GA and IAA, respectively) are low expression quantity during seed germination. These results provide important information about the seed dormancy release mechanism of DXWR and provide the theoretical basis for applying this wild rice germplasma.

Keywords: Dongxiang wild rice, endogenous hormones, germination rate, gene expression

Introduction

Seed dormancy is the incapacity of an intact viable seed to complete germination under favorable conditions [1]. The low level of seed dormancy assures quick and even germination of plant seeds, however, it can also result in unwanted early germination at the mother plant (pre-harvest sprouting) and lowered seed quality [2]. On the other hand, high dormancy is regarded as a negative trait owing to germination retardation and shorten the length of the growing season [1]. Thus, a good understanding of the regulatory mechanism of seed dormancy can provide a theoretical basis for ecological understanding and crop management.

In the past few years, many studies have focused on elucidating the regulation of seed germination and dormancy. Many hypotheses have been proposed and rejected, but the regulatory principle behind changes in dormancy and induction of germination is still a 'black' box [3, 4]. The majority of proposing mechanisms were related to certain plant hormones. There is considerable evidence that abscisic acid (ABA) and gibberellic acids

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(GA) are the primary phytohormones for seed dormancy establishment and maintenance [3, 5, 6]. Nishimura et al. showed that *AHG1*, a central negative regulator of ABA response in germination, and upstream components *DOG1* constitute an important regulatory system for seed dormancy and germination [7]. Frey et al. showed that the seed of ABA-deficient mutants germinated faster than those of the wild-type [8]. The transgenic rice seeds expressing the positive regulator factors of ABA biosynthesis gene was hypersensitive to ABA during early seedling growth and seed germination [9, 10]. Another important hormone, GA, has an antagonistic effect on the control of dormancy and germination [9, 11]. Changes in the ABA/GA balance were referred to the expression of dormancy in many species including rice and many cereal crops [4, 12]. Other plant hormones such as indole-3-acetic acid (IAA) and jasmonic acid (JA) also play an important role in inducing and maintaining seed dormancy. Exogenous IAA treatment can delay seed germination, and inhibited pre-harvest sprouting on mother plants [13, 14]. A recent study revealed that TOR-interacting protein (metabolic gene of IAA) positively regulates seed germination by maintaining the nutritional and hormonal balance [15].

Dongxiang wild rice (*Oryza rufipogon* Griff., hereafter DXWR), located in Dongxiang county (28°14′N latitude and 116°30′E longitude), Jiangxi Province, China, is considered to be the most northerly wild rice grown in the world. DXWR contains many excellent traits, such as the resistance to diseases and insect pests, various abiotic stresses, and improvement of biomass accumulation and nutrient quality, and is one of the very precious rice germplasm resource for maintaining genetic diversity of rice germplasm and exploring the excellent traits to modify cultivated rice [16]. It was noted that DXWR possess stronger dormancy than cultivated rice and that this dormancy could be released by after-ripening [17,18]. However, dormancy type and mechanism of DXWR seed are poorly understood. Here, we compared the changes of the endogenous hormones in cultivar and DXWR to investigate the changes in endogenous hormone contents in seed of DXWR during the release of dormancy. In addition, we also examined the expression levels of known genes functioning in biosynthesis and signaling pathway of ABA, IAA, GA and JA. These results provide important information about the seed dormancy release mechanism of DXWR and provide the theoretical basis for applying this wild rice germplasma.

Experimental

Materials

The rice (*Oryza sativa* L.) cultivar 93-11 and the wild rice (DXWR) were grown in the field at Rice Research Institute-Jiangxi Academy of Agricultural Sciences, Jiangxi, China. The mature seed was harvested, dried to around 20% water content, then sealed and stored at -20ºC, keeping the seed dormancy until use in subsequent experiments.

Seed Germination

Approximately 100 mature and dormant seeds were placed in a filter paper lined Petri dish, soaked in 10 ml of double distilled water, and then cultured in a 28ºC lightproof chamber, and then Petri dishes for each rice sample were performed. The seed whose embryo breaks through the seed coat is recorded as germination. Germination rates were measured by randomly counting 100 seeds per dish and repeat three Petri dishes. During germination, 50 seeds were collected at 12 h interval and immediately frozen in liquid nitrogen, until 96 h.

Enzyme Linked Immunosorbent Quantification of Endogenous Hormones

The frozen rice seeds were ground into powder, and approximately 500 mg powder were homogenized in 5mL of ice-cold PBS (0.01M, pH = 7.4), and then the homogenates were centrifugated for 5 minutes at 5000×g to get the supernatant. ABA, GA3, IAA and JA were analyzed by enzyme linked immunosorbent assay employing monoclonal antibodies, respectively (Abcam, USA) according to the protocol. Standard curves with different ABA, GA3, IAA and JA dilutions were constructed to calculate ABA, GA3, IAA and JA concentrations in the sample, with each measurement repeated 3 times.

RNA Extraction from Rice Endosperm

Total RNAs from rice endosperm at different treatments were isolated using the SV Total RNA Isolation System (Promega, Peking, China) according to the manufacturer's instructions with some modification. To remove the abundant starch in rice seed, the RNAiso for Polysaccharide-rich Plant Tissue-Takara, Japan-buffer was added to the samples after grinding. Recombinant DNase I (RNase-free) (Takara, Japan) was used to digest contaminating genomic DNA. RNA concentrations were measured spectrophotometrically using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE), and only samples with A260/A280 of 1.8-2.0 were used for further analysis. The RNA quality was checked with an RNA gel for two clear bands of 18S and 28S rRNAs [19].

Quantitative Real-Time PCR (qRT-PCR)

From total RNA (5 ug), first-strand cDNA was synthesized with oligo(dT) using the Frist-Strand Synthesis of cDNA kit (Promega, Peking, China).

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Table 1. Sequences of qRT-PCR primers.

Gene name	Primer sequences 5' to 3'
Actin	forward: GAACTGGTATGGTCAAGGCTG reverse: ACACGGAGCTCGTTGTAGAAG
ABA8OH	forward: CTACTGCTGATGGTGGCTGA reverse: CCCATGGCCTTTGCTTTAT
GAMYB	forward: CTGGGGACACTTCACCTCAT reverse: ATGATACCGTCGCCAAGAAC
DAD	forward: ACCTCATCGACGGGTTCAC reverse: CGGTTCACGTACTCCTTCTTCA
LOX	forward: GCATCCCCAACAGCACATC reverse: AATAAAGATTTGGGAGTGACATATTGG
NCED	forward: CTCACCATGAAGTCCATGAGGCTT reverse: GTTCTCGTAGTCTTGGTCTTGGCT

Gene-specific primers of starch synthesis related genes used in the subsequent qRT-PCR were listed in Table 1. Additionally, the housekeeping gene *Actin* was used as internal control to normalize the concentration of cDNA present in each sample. The qRT-PCR was carried out using StepOne TM and StepOne Plus TM Real-Time PCR Systems (ABI-USA), according to the kit manuals accompanying the SYBR® Premix Ex Taq (Takara, Dalian, China). The relative expression levels were calculated by the method of $2^{-\Delta CT}$[20].

Results and Discussion

Germination Rates of Wild Rrice (DXWR) and 9311

According to germination test results (Fig. 1), although all seeds started to germinate after 24 h rehydration, we observed significant differences between cultivar 9311 and wild rice. 9311 showed the 83% germination rate within 36-h imbibition and the highest germination rate (91%) within 72-h imbibition, in contrast, only 30% of wild rice at 36-h and the highest germination rate displayed 83% at 92-h. According to the method described by Wan et al. [21], the germination percentage was used to evaluate the dormancy levels, deep dormant, medium dormant and non-dormant displayed <2%, 50%-80% and >80% germination after four days' imbibition, respectively. So, the DXWR was regarded as medium dormant. Hilhorst et al. described that some seeds with physiological dormancy may not germinate under favorable conditions, but the seeds with medium dormant can germinate slowly in a narrow light temperature range [22]. The germination rate of DXWR is uniform and slow during 24 h-96 h imbibition, indicating that the dormancy of DXWR seed was non-physical dormancy.

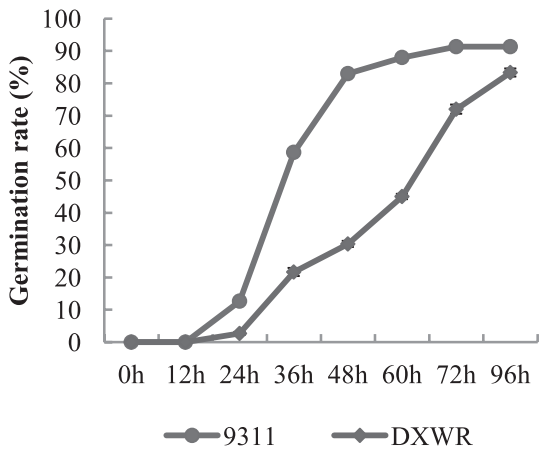

Fig. 1. Germination rates of wild rice (DXWR) and cultivated rice (9311).

The Levels of Endogenous Hormones in Wild Rice (DXWR) and Cultivated Rice (9311)

Since the germination rate of rice is closely related to the levels of endogenous hormones, we quantified ABA, GA, IAA and JA in 9311 and DXWR at different seed germination time points. Researches suggested that seed dormancy is partly depended on the level of ABA contained in mature seeds [6, 23]. The content of ABA in DXWR mature seeds was higher than 9311 (Table 2). During the germination of 9311 seeds, the lowest ABA content was 24 h, and the seeds began to germinate at this time point, consistent with previous studies which have revealed the loss of dormancy and germination of grain seeds were related to the decrease of seed ABA level during imbibition [4, 12]. Interestingly, during 9311 and DXWR seed germination, the change trend of JA content is similar to ABA content, suggested that JA has an inhibitory effect on the germination process which can support by the exogenous JA application delays seed germination [23].

In contrast, a significant peak of GA and IAA occurred after the germination onset at 36 h for 9311, and the contents of GA and IAA for DXWR only slightly fluctuated (Table 2), which coincided with germination

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Table 2. The content of endogenous hormones in wild rice (DXWR) and cultivated rice (9311).

Content / Time	ABA (ng/mL)		IAA (μmol/L)		GA (pmol/L)		JA (pmol/L)	
	9311	DXWR	9311	DXWR	9311	DXWR	9311	DXWR
0 h	13.9±0.9	17±0.4	3.7±0.1	4.4±0.2	23.1±1.1	29.6±0.9	478±22.6	495±25.6
12 h	13.9±0.3	17.5±0.3	3.8±0.1	4.5±0.2	18.1±1.0	22.0±0.9	386.9±23.3	547.7±6.8
24 h	12.5±0.7	14.4±0.7	2.9±0.1	4.4±0.1	24.7±0	30.3±1.5	476.3±0.6	467.3±28.6
36 h	14.9±0.5	18.5±1.3	4.9±0.2	4.8±0.3	36.3±1.2	35.6±1.2	555±15.2	602.4±7.5
48 h	15.6±0.8	19.8±1.3	3.8±0.1	4.9±0.1	31.9±1.7	33.4±0.4	512.7±16.6	640.2±29.4
60 h	19.6±1.2	18.4±1.3	4.4±0.1	4.7±0.1	24.7±0.3	35.9±1.3	487.0±26.3	606.0±10.1
72 h	16.1±0.4	17.4±1.2	4.2±0.2	4.3±0.1	27.8±0.9	33.2±1.0	517.9±12.8	545.5±5.3
96 h	13.4±0.2	18.4±0.9	3.9±0.1	4.9±0.1	27.8±1.6	30.6±0.9	507.2±21.8	541.8±16.6

phenotypes of these two rice varieties (Fig. 1). Xiao et al. found that the content of IAA in the embryo gradually increased during seed germination, and then reach a maximum at 12 h, but then decreased at 48h [24]. Jia et al. found that GA biosynthesis and signaling transduction specifically act on seed dormancy release [25]. Several physiological and genetic analyses have demonstrated the requirement of GA for germination; GA can promote seed germination through enhancing the growth potential of the embryo and overcoming the mechanical barriers imposed by covering layers surrounding embryo [4, 12].

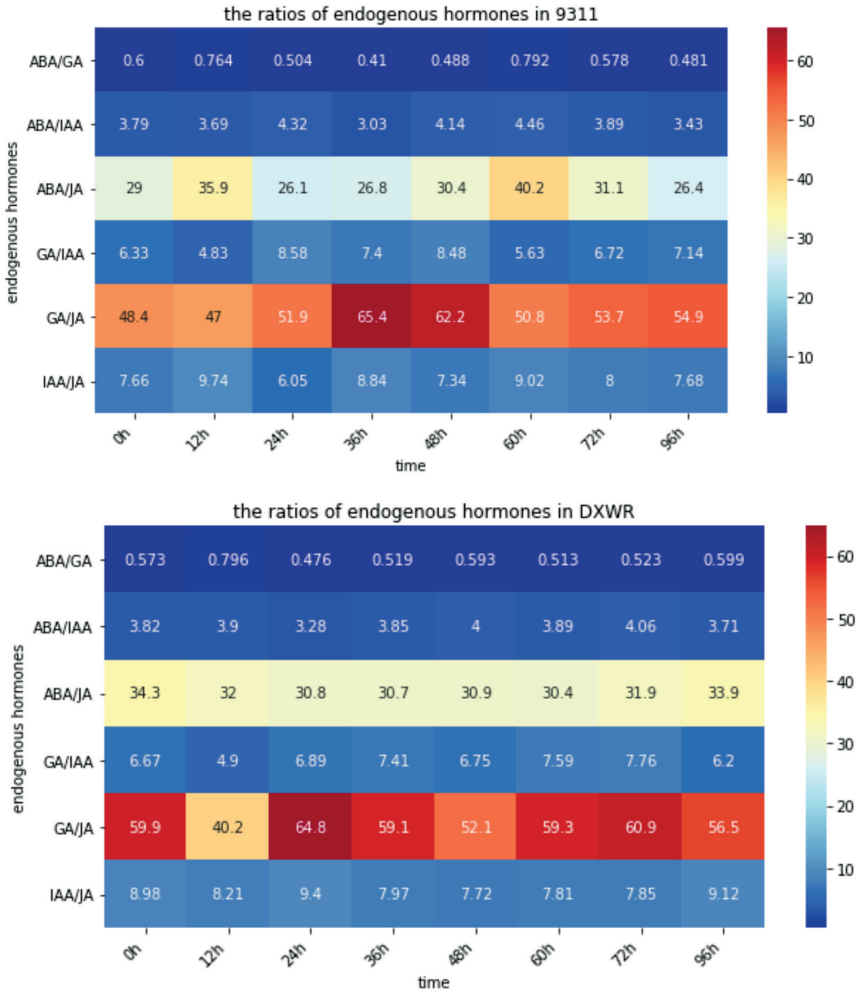

Fig. 2. The ratios of endogenous hormones (ABA, IAA, GA and JA) in wild rice (DXWR) and 9311.

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The Ratios of ABA/GA, ABA/IAA and ABA/JA in Wild Rice (DXWR) and 9311

Dynamic variations of the ABA/GA ratio during germination showed that DXWR had a similar ratio with 9311 during 24 h imbibition, but significantly higher ratio than 9311 during 24 h-48 h imbibition (Fig. 2), consistent with previous studies showed that a high ABA/GA ratio is critical for the induction of maturation and germination suppression in early seed development programming [25]. In our results, the ABA/IAA ratio for DXWR only slightly fluctuated, but one ratio slack at 36 h and two ratio peaks at early and late stages of seed germination for 9311 were observed (Fig. 2), suggesting IAA can promote seed germination. Shuai et al. reported that exogenous IAA treatment represses seed germination by enhancing ABA biosynthesis, while impairing GA biogenesis [26]. In addition, the ratio of ABA/JA for DXWR almost no change, on the contrary, after an initial increased ABA/JA for 9311 and then decreased again before the germination onset (12 h) (Fig. 2). At the later stage of germination, ABA/JA ratio reached its peak value, and then decreased rapidly to the lowest value, similarly with Wilen et al. reported the ABA in combination with JA resulted in a higher degree of seed germination inhibition at 23ºC in maize, wheat and cornflower than either growth regulator applied separately [27].

The DXWR has the similar GA/IAA ratio with 9311 during 24 h imbibition, but the GA/IAA ratio for 9311 was significantly higher than DXWR during 24 h-48 h imbibition (Fig. 2). In addition, the GA/IAA ratio for DXWR decreased to the lowest value at 12 h, during 24h-96h imbibition, the GA/IAA ratio only slightly fluctuated and was closed to the GA/IAA ratio before germination (Fig. 2). GA can promote the synthesis of IAA by tryptophan, meanwhile, it can inhibit the

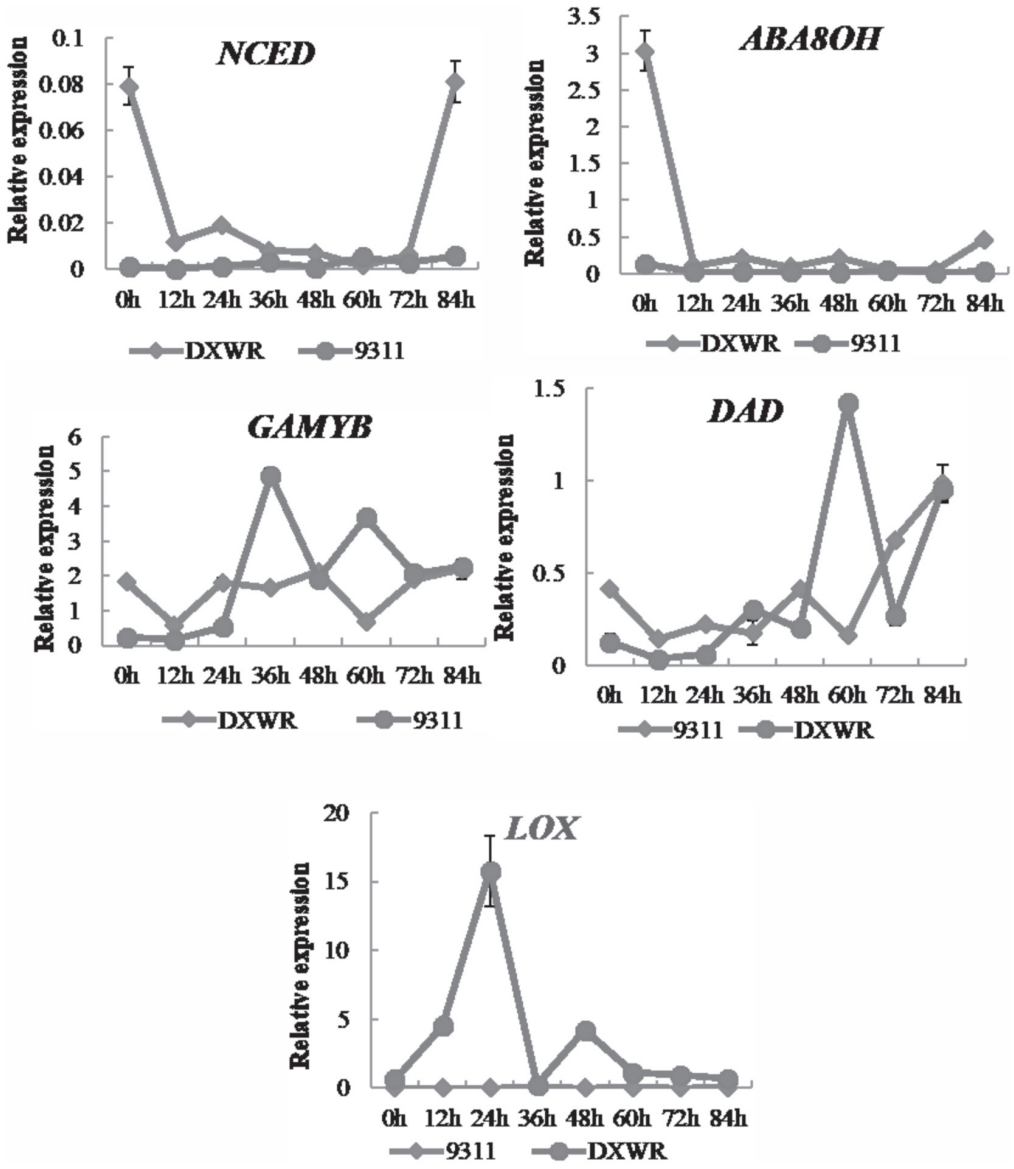

Fig. 3. qRT-PCR analysis expression of endogenous hormones metabolism genes in wild rice (DXWR) and 9311.

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activity of IAA-oxidase and peroxidase, promote the synthesis of IAA, inhibit its degradation and increase the content of IAA [12].

Expression of ABA, GA, IAA and JA Metabolism Genes in DXWR and 9311

Differences in expression of key endogenous hormone metabolism genes may lead to differences in seed dormancy. Thus, we analyzed the expression of genes connected with ABA, GA, IAA and JA biosynthesis and catabolism using qRT-PCR during seed germination. The main catabolic pathway of ABA is the reaction catalyzed by OsABA8OH (ABA 80-hydroxylase), and the key enzyme that regulations ABA biosynthesis is NCED (9-cis-epoxycarotenoid dioxygenase) [28, 29]. The *OsABA8OH* genes is a rate-limiting step in the process of ABA biosynthesis [28]. *OsABA8OH* has 3 homologous in rice, among which *OsABA8OH2* is mainly expressed during seed development [30]. The expression of *NCED* and *OsABA8OH* genes was also affected by after-ripening. According to the results obtained by qRT-PCR, there is low transcription level of *NCED* and *OsABA8OH* in 9311 and it did not significantly change during seed germination. In contrast, the expression of ABA catabolic genes *NCED* and *OsABA8OH* in DXWR fast decreased before germination onset and the latter peaked again at 96h (Fig. 3). Many studies showed that the transcript levels of *OsABA8OH* in imbibed grains were strongly increased as a result of post harvest storage [31, 32], and the expression of the *NCED* and *OsABA8OH* homologues in imbibed grains was observed at very low, constant level [31, 33].

The gene product of *GAMYB* was critical for the induction of α-amylase in aleurone, and *GAMYB* is the first identified GA signaling protein that regulates other transcription factors, and is a positive regulators of seed dormancy [34]. Liu et al. found that the expression of *GAMYB* peaked at early, middle, and late stages of seed development for deep dormant, medium dormant, and non-dormant, respectively, *GAMYB* has a positive correlation with the gene expression time [35]. Expression analysis revealed the transcript of GA and IAA metabolism gene *GAMYB* and *DAD* in 9311 had two peaks occurring at 48h and 72h respectively. In contrast, for DXWR both the *GAMYB* and *DAD* are low expression quantity during seed germination (Fig. 3), indicating that ABA and GA crosstalk during seed dormancy.

JA is synthesized in a series of steps, beginning with the release of linolenic acid (LA) and then converted to 13-hydrogen peroxide by lipoxygenase (LOX) [36]. It is low expression of *LOX* in 9311 and it almost no change during seed germination. By contrast, the expression of *LOX* in DXWR was increasing prior to 24h and firstly peaked 24h, then quickly drop to the lowest level at 48h and the latter peaked again at 60h (Fig. 3). At present, research on JA function is mainly about its resistance to diseases, insect pests and various abiotic stresses, but the research on seed dormancy is less. In this research, we found JA, as a new plant growth regulator, has an inhibitory effect on the germination process, and it had some similar physiological effects to ABA.

Conclusions

Seed dormancy is a quantitative trait. Primary dormancy was induced at seed maturity and reached a higher level in freshly harvested seed. We found only 30% of DXWR germination rates at 36-h and the highest germination rate displayed 83% at 92-h, regarded as medium dormant. The content of ABA and JA in DXWR mature seeds was higher than 9311. Interestingly, during 9311 and DXWR seed germination, the change trend of JA content is similar to ABA content, suggested that JA has an inhibitory effect on the germination process. The ABA/GA ratio showed that DXWR had a significantly higher ratio than 9311 during 24 h-48 h imbibition, revealed that a high ABA/GA ratio is critical for the induction of maturation and germination suppression. The expression of ABA catabolic genes *NCED* and *OsABA8OH* in DXWR fast decreased before germination onset, however *GAMYB* and *DAD* are low expression quantity during seed germination, showed that seed germination is related to the regulation of endogenous hormone catabolic genes.

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Conflict of Interest

The authors declare no conflict of interest.

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