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Sequence variation and expression analysis of seed dormancy- and germination-associated ABA- and GA-related genes in rice cultivars

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Abscisic acid (ABA) and Gibberellic acid (GA) play key roles in regulating seed dormancy and germination. First, when examining germination of different rice cultivars, we found that their germination timing and dormancy status are rather distinct, coupled with different GA/ABA ratio. Second, we studied genomic sequences of ABA and GA dormancy- and germination-associated genes in rice and discovered single nucleotide polymorphisms and insertions/deletions (Indels) in both coding and regulatory sequences. We aligned all these variations to the genome assemblies of *9311* and *PA64s* and demonstrated their relevance to seed dormancy both quantitatively and qualitatively based on gene expression data. Third, we surveyed and compared differentially expressed genes in dry seeds between *9311* and *PA64s* to show that these differentially expressed genes may play roles in seed dormancy and germination.

Keywords: dormancy and germination, ABA, GA

INTRODUCTION

Seed dormancy has been defined as a temporary failure of seed germination under conditions that favor germination (Bewley, 1997). The initiation of dormancy can either occur in the dry state (after-ripening) or be triggered by imbibition under defined conditions in mature seeds (Gubler et al., 2005). Pre-harvest sprouting of cereal grain leads to reduced grain yield and poor quality products, resulting in economic losses of cultivars. Strong dormancy also prevents grain such as barley from being re-planted immediately after harvest, causing delayed or non-uniform germination. Both situations would result in poor crop establishment and grain performance.

Seed dormancy and germination are regulated by nearly all plant hormones. Several studies have shown that ethylene, auxin, and brassinosteroids promote the germination of dormant seeds, but it is now generally accepted that ABA and GA are the leading regulators (Koornneef et al., 2002; Gubler et al., 2005). ABA deficiency during seed development is associated with the absence of primary dormancy in mature seeds, whereas over-expression of ABA biosynthesis genes can increase seed ABA content and enhance seed dormancy or delay germination. ABA plays important roles in many cellular processes including seed development, dormancy, germination, vegetative growth, and environmental stress responses. ABA level can increase significantly during seed maturation and in response to environmental stresses. In addition to hormone content, the transition from the dormant to the non-dormant state of many seeds is characterized by a decrease in ABA sensitivity and an increase in GA sensitivity (Chiwocha et al., 2005).

Recently, the major enzymes involved in ABA and GA metabolism pathways have been identified. ABA metabolic genes have been identified such as ABA biosynthetic gene NCED that encodes a 9-*cis*-epoxycarotenoid dioxygenase (NCED) and ABA catabolic gene CYP707A that encodes a ABA 8′-hydroxylase (CYP707A; Seo and Koshiba, 2002; Lefebvre et al., 2006; Okamoto et al., 2006; Yang and Guo, 2007). Genes associated with GA metabolism have also been identified such as GA biosynthetic gene GA20ox that encodes a GA 20-oxidase (GA20ox), GA3ox that encodes a GA 3-hydroxylase (GA3ox), and GA catabolic gene GA2ox that encodes a GA 2-oxidase (GA2ox; Yamauchi et al., 2004; Finch-Savage et al., 2007). However, no potential receptors that could perceive this increase in abscisic acid had been identified until recent reports of four abscisic acid binding proteins: the chloroplast protein magnesium protoporphyrin-IX chelatase H subunit (Shen et al., 2006), the membrane-associated protein G protein coupled receptor 2 (Liu et al., 2007; Verslues and Zhu, 2007), two GPCR-type G proteins (GTG1 and GTG2) (Ma et al., 2009; Pandey et al., 2009), and the PYR/PYL/RCAR family proteins (Ma et al., 2009; Park et al., 2009). Since there have been controversies over structural and functional classification of ABA receptors, we carried out analyses on the recently available recent receptor candidates in this study.

The identification of GA receptors has also improved our understanding of GA function in relation to germination (Nakajima et al., 2006).

Rice is one of the most important cereal crops, providing food for billions of people. In addition, it is also a model plant with various cultivars and mutants for the study of heterosis and domestic process. *9311* is a cultivar of *Oryza sativa* L. ssp. *indica* – the major rice subspecies grown in Asia-Pacific regions. Pei-Ai 64s (*PA64s*) has a major background of *indica* and a minor background of *japonica* and *javanica*, and the first two are two other commonly cultivated subspecies in China. *9311* and *PA64s* as parental lines show significant difference in their phenotype of seed dormancy. Their F1 offspring, an elite super hybrid rice *LYP9*, also shows significant heterosis including weak dormancy like its paternal line *9311*. Compared to its wild ancestor *O. rufipogon*, cultivated rice typically exhibits reduced dormancy (Veasey et al., 2004; Sweeney and Mccouch, 2007). Therefore, to elucidate the molecular mechanisms regulating rice seed dormancy and germination is of importance for both plant biology and crop development. Toward this end, we identified genes encoding ABA and GA metabolic enzymes and found that there were many single nucleotide polymorphisms (SNPs) and Indels in both protein-coding and regulatory regions between different rice cultivars. We also found differentially expressed transcription units in the seed of our rice model *9311* and *PA64s*. We demonstrate that seed dormancy state in rice may be influenced by both gene sequence variation as well as expression patterns of ABA and GA metabolic genes.

MATERIALS AND METHODS

PLANT MATERIALS

Mature seeds from fresh harvest of *9311*, *PA64s, IR24, SHOEMED*, *AZUCENA*, *DOURADO AGULHA*, *MAKALIOKA34*, and *IR*36 were harvested at the maturing stage then dried and stored at room temperature. The seeds of *9311* and *PA64s* were provided by the Chinese National R&D Center on Hybrid Rice, Changsha, China. The seeds of *IR24, SHOEMED*, *AZUCENA*, *DOURADO AGULHA*, *MAKALIOKA34*, and *IR*36 were provided by the International Rice Research Institute (IRRI). Six months after harvesting the seeds were used for the experiments. Mature rice embryos were separated manually from their seeds using scalpels, and then stored in −80°C immediately before RNA extraction.

GERMINATION EXPERIMENTS

For the germination experiment, we started with 50 seeds in a Petri dish with multiple layers of wet filter papers (sterile double-distilled water) at 37°C. The germination rate was calculated daily based on radical emergence. Experiments were performed in triplicate for each cultivar examined.

DETERMINATION OF ABA AND GA LEVELS

Mature rice embryos were homogenized in 80% (v/v) acetone. After adding internal standards ^{13}C-abscisic acid (ABA) and ^{3}H-labled GA_3, the homogenate was shaken for 10 h on ice in darkness and then centrifuged at 2,000*g* for 30 min. The precipitate was then re-extracted, and the combined supernatant was evaporated to remove residual acetone. After a series of organic extractions, the extracts were purified through C18 column. ABA was methylated with diazomethane, whereas GA_3 were trimethylsilylated with BSTFA at 100°C for 60 min. Gas chromatography–electron impact ionization mass spectrometry was carried out to determine ABA and GA concentrations. The following mass-to-charge ratio peaks were used for quantification: for ABA, 192 (labeled) and 190 (endogenous); and for GA_3, 506 (labeled) and 504 (endogenous). ABA and GA concentrations of *9311* and *PA64s* from each individual assay of the triplicate are provided in **Data Sheet S1** in Supplementary Material.

SEQUENCE SIMILARITY ANALYSIS AND ALIGNMENT OF AMINO ACID SEQUENCES

We acquired sequences of seed dormancy- and germination-associated genes from *Arabidopsis* and rice databases (Goff et al., 2002; Yu et al., 2002). We analyzed ABA metabolism-related genes from rice *Nipponbare* including *OsNCED*1 (AY838897), *OsNECD*2 (AY838898), *OsNCED*3 (AY838899), *OsNCED*4 (AY838900), *OsNCED*5 (AY838901), *OsCYP707A*5 (AB277270), *OsCYP707A*6 (NM_001068556), *OsCYP707A*7 (NM_001069901), and *OsGPCR* (CM000147), and homologs from *Arabidopsis* including *AtNCED*1 (AT3G63520), *AtNCED*2 (AT4G18350), *AtNCED*3 (AT3G14440), *AtNCED4* (AT4G19170), *AtNCED*5 (AT1G30100), *AtNCED*6 (AT3G24220), *AtNCED*9 (AT1G78390), *AtCYP707A*1 (AT4G19230), *AtCYP707A*2 (AT2G29090), *AtCYP707A*3 (AT5G45340), *AtCYP707A*4 (AT3G19270), and *AtGCR*2 (AT1G52920) (Note: not all NCED enzymes are involved in ABA biosynthesis in *Arabidopsis*). GA metabolism-related genes from rice are *OsGA20ox*1 (AC096690), *OsGA20ox*2 (NM_001051549), *OsGA20ox*3 (AP005840), *OsGA20ox*4 (NM_001062119), *OsGA3ox*1 (NM_001048721), *OsGA3ox*2 (AC144738), *OsGA2ox*1 (AC119288), *OsGA2ox*2 (NM_001048899), *OsGA2ox*3 (NM_001050827), *OsGA2ox*4 (AC132485), *OsGA2ox*5 (NM_001062846), and *OsGID1* (AB211399), and homologs from *Arabidopsis* are *AtGA20ox*1 (At4g25420), *AtGA20ox*2 (At5g51810), *AtGA20ox*3 (At5g07200), *AtGA3ox*1 (At1g15550), *AtGA3ox*2 (At1g80340), *AtGA3ox*3 (At4g21690), *AtGA2ox*1 (AT1G78440), *AtGA2ox*2 (AT1G30040), *AtGA2ox*3 (AT2G34555), and *AtGID*1 (AT3G05120). Alignment of sequences and phylogenetic analysis were carried out by using ClustalW with default parameters and MEGA4.1 with neighbor-joining method (Tamura et al., 2007). All sequences of *9311*, *PA64s*, and *Nipponbare* used in this study are provided in **Data Sheet S2** in Supplementary Material. For each gene pair, we calculated the number of non-synonymous substitutions per non-synonymous site (*Ka*) and the number of synonymous substitutions per synonymous site (*Ks*) using the maximum-likelihood method (Goldman and Yang, 1994). Ka/Ks = 1 indicates neutral evolution, where the number of non-synonymous changes at each possible non-synonymous site is the same as the number of synonymous changes per synonymous site. Ka/Ks < 1 suggests purifying selection, where selection generally eliminates deleterious mutations; Ka/Ks > 1 indicates positive selection, where selection bring more amino acid changes.

TEMPLATE PREPARATION AND REAL-TIME PCR VALIDATION

Total RNA was purified from each sample using Trizol (Invitrogen) according to the manufacturer's instructions. Eight microgram total RNA of each sample was used for first-strand cDNA synthesis in 25 μl reaction containing 5 μl 5 × RT buffer, 2.5 μl 10 mM dNTP, 50 ng random primer, 50 ng oligoDT(15), 2.5 μl RNase inhibitor

(20 U/μl), 4 μl reverse transcriptase (50 U/μl; Invitrogen), 2.5 μl DTT. Reverse transcription was performed at 42°C for 60 min with a final denaturation at 70°C for 15 min.

Specific primers for Real-time PCR in the experiments are listed as follows:

OsNCED1 (upper: 5′-TGGAGCACATGGAGCTAGT-3′, lower: 5′-CCGAAGTAGCCGTACCTG-3′); *OsNCED2* (upper: 5′-GCTTGACTTTGATCTCGTC-3′, lower: 5′-TCTCCGGTGTCCTCTTC-3′); *OsNCED3* (upper: 5′-CCACCATGATCCACGACT-3′, lower: 5′-AGAGGTGGAAGCAGAAGC-3′); *OsNCED4* (upper: 5′-GCTTGACTTTGATCTCGTC-3′, lower: 5′-AGAGGTGGAAGCAGAAGC-3′); *OsNCED5* (upper: 5′-CATCTTCAACGAGTCGGA-3′, lower: 5′-GTAGGCGTACCTCGTCTTC-3′); *OsCYP707A5* (upper: 5′-AGATTGCCAAGGAGAAAGA-3′, lower: 5′-TGAAGGTGAAGGAGAGGATG-3′); *OsCYP707A6* (upper: 5′-AGACGAGGAGCATGACACT-3′, lower: 5′-CGGGTTGTGATGGATGTT-3′); *OsCYP707A7* (upper: 5′-GCTCACCTTCTTCCTCAAC-3′, lower: 5′-GCCATTCCTTTGCTTGAT-3′); *OsGA20ox1* (upper: 5′-CATGCGCCTCAACTACTAC-3′, lower: 5′-GAGCGCCATGAAGGTGT-3′); *OsGA20ox2* (upper: 5′-TCATGCGGTGCAACTACTA-3′, lower: 5′-GTGTCGCCGATGTTGAT-3′); *OsGA20ox3* (upper: 5′-GCTCACCTTCTTCCTCAAC-3′, lower: 5′-GCCATTCCTTTGCTTGA-3′); *OsGA20ox4* (upper: 5′-CGTCGCCGATTACTTCTC-3′, lower: 5′-TAGTAGTTGCACCGCATGA-3′); *OsGA3ox1* (upper: 5′-CGACGAGTTGCTGAGGTT-3′, lower: 5′-AGGTGAAGAAGCCCGAGT-3′); *OsGA3ox2* (upper: 5′-ATGCCCTACTTCCTCGGT-3′, lower: 5′-CTTGTCCTCTTCCTTCGCTA-3′); *OsGA2ox1* (upper: 5′-TCAATGTTGGTGATGTCCTC-3′, lower: 5′-GCTGGCTGTGATTGTCTCT-3′); *OsGA2ox2* (upper: 5′-AGATCATCTCCGTGCTCC-3′, lower: 5′-CTCGACTTCTCGCTGTTCA-3′); *OsGA2ox3* (upper: 5′-CGACTCCTTCTTCGTCAAC-3′, lower: 5′-TGCAATCCTCTGTGCTAAC-3′); *OsGA2xo4* (upper: 5′-GCCAACAAGCTCAAGTC-3′, lower: 5′-GAGAGGTAGGCAGCCTTC-3′); *OsGA2ox5* (upper: 5′-CATTTCTGGTAACTAAAGGC-3′, lower: 5′-GAGCAGCACATAATCACAT-3′).

Real-time PCR was performed with Quant SYBR Green PCR kit (BIO-RAD). The data were quantitated by referencing to a PCR product of house-keeping gene of GAPDH under the same reaction condition (94°C for 4 min, then 40 cycles of 94°C for 15 s, 60°C for 20 s, and 68°C for 30 s). Melting curves for each PCR reaction were carefully monitored to avoid non-specific amplifications. Gene expression quantification was performed with the comparative Ct formula normalized with the expression of GADPH.

STATISTIC ANALYSIS

We used the unpaired Student's *t*-test to analyze expression levels of ABA and GA, transcripts of ABA- and GA- related genes in dry seeds when comparing the two groups of *9311* and *PA64s*, and Ka/Ks ratios. Statistical significance ($p < 0.01$) was determined based on the *t*-test in GraphPad Prism 5. Wilcoxon rank sum test was performed to compare SNPs and Indels between coding regions and regulatory regions, also between paralogs and orthologs.

RESULTS

GERMINATION TEST AND ABA/GA CONCENTRATION MEASUREMENT OF RICE SEEDS FROM VARIOUS CULTIVARS

We initiated our study by carrying out a germination experiment using seeds from various cultivars (*9311, PA64s, IR24, SHOEMED, AZUCENA, DOURADO AGULHA, MAKALIOKA34,* and *IR36*) with diverse genetic backgrounds (**Figure 1**). Although all seeds started to germinate after 1 day rehydration, we observed significant differences among the cultivars. For instance, *IR24* showed the highest germination rate (82%) and about half of the seeds from *9311*, *SHOEMED, IR36*, and *AZUCENA* germinated within 36-h imbibition. In contrast, only 29% of *MAKALIOKA34*, 14% of *DOURADO*, and less than 10% of *PA64s* seeds germinated at this time period.

Since rice germination rate is highly associated with the ABA/GA ratio (Nonogaki et al., 2010), we quantified ABA and GA in *9311* and *PA64s* dry seeds (see Materials and Methods). The endogenous ABA level of *9311* was obviously lower than those of *PA64s* and the GA/ABA ratio was much higher in *9311* than in *PA64s*, which is consistent with our experimental results that *PA64s* exhibited stronger dormancy than *9311* (**Figure 2**).

IDENTIFICATION OF RICE ABA AND GA METABOLIC GENES

Because ABA and GA metabolic genes and their receptors are not well-annotated in the rice genome assemblies, we extracted well-annotated *Arabidopsis* genes from the public databases and searched for orthologs and paralogs from the rice databases[1]. To confirm the predicted protein

[1]www.ncbi.nlm.nih.gov/blast/

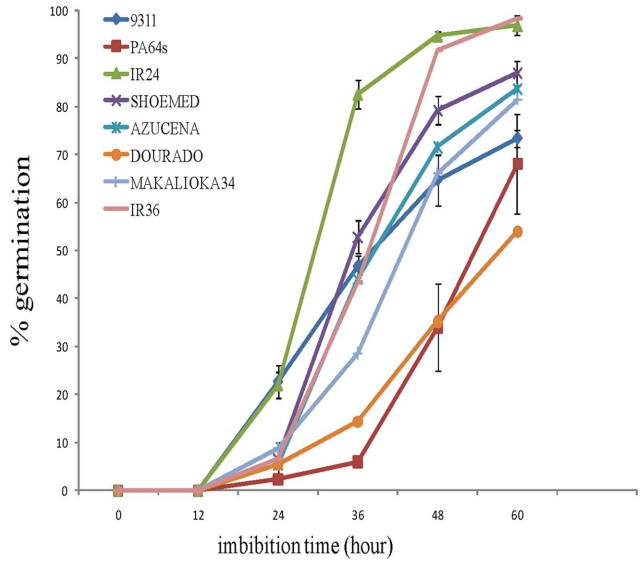

FIGURE 1 | Germination test for rice cultivars *9311, PA64s, IR24, SHOEMED, AZUCENA, DOURADO AGULHA, MAKALIOKA34,* and *IR36* (after imbibition). Measurements are averaged over three replicates with error bars representing SE.

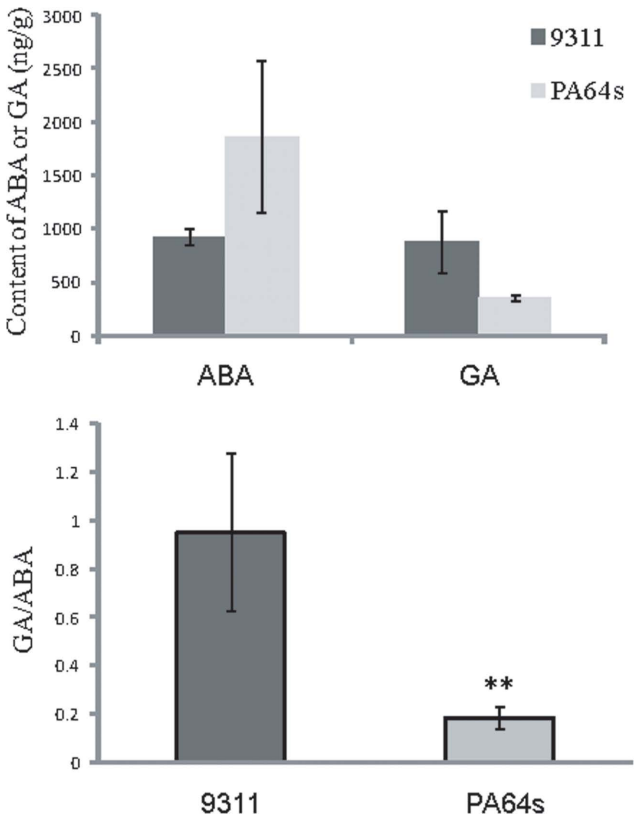

FIGURE 2 | Quantification of endogenous ABA and GA levels in dry seeds of *9311* and *PA64s*. Measurements are averaged over three replicates with error bars representing SE. The GA/ABA ratio is significantly different in *9311* and *PA64s* (*t*-test: $^{**}p < 0.01$).

sequences, we used PFAM database[2] to identify conserved domains and found that rice has five NCED, three CYP707A, four GA20ox, two GA3ox, and five GA2ox gene family members. They are distributed on chromosome 1 (*OsGA20ox*2, *OsGA3ox*2, *OsGA2ox*2, and *OsGA2ox*3), chromosome 2 (*OsNCED*1 and *OsCYP707A*5), chromosome 3 (*OsNCED*3 and *OsGA20ox*1), chromosome 5 (*OsGA20ox*4, *OsGA3ox*1, *OsGA2ox*1, *OsGA2ox*4, and *OsGA2ox*5), chromosome 7 (*OsNCED*4), chromosome 8 (*OsCYP707A*6), chromosome 9 (*OsCYP707A*7), and chromosome 12 (*OsNCED*2, *OsNCED*5, and *OsGA20ox*3). Our annotations were further confirmed in GBROWSE[3] (**Table 1**).

PHYLOGENIC ANALYSIS OF ABA AND GA METABOLIC GENES

We identified five NCED genes in rice and nine in *Arabidopsis* (Schwartz et al., 2003; Tan et al., 2003; Kushiro et al., 2004). *OsNCED*2 and *OsNCED*5 are both located on chromosome 12 and *OsNCED*1, *OsNCED*3, and *OsNCED*4 are located on chromosomes 2, 3, and 7, respectively. The amino acid sequence of *Arabidopsis AtNCED*4 showed higher similarities with *OsNCED*1 and *OsNCED*2 (51–57% identities) but relatively lower identities with *OsNCED*3 to *OsNCED*5 (ranging between 33 and 36%). Phylogenetic analysis (**Figure 3A**) revealed that *OsNCED*3 and *OsNCED*5, which were more similar to each other (80% identity) than any other reported NCED proteins from *Arabidopsis*, were also closely related to *AtNCED*2, *AtNCED*3, *AtNCED*5, *AtNCED*6, and *AtNCED*9 (59–63% identities).

Since *Arabidopsis* CYP707A has four paralogs (*AtCYP707A*1, *AtCYP707A*2, *AtCYP707A*3, and *AtCYP707A*4), we tried really hard to find each of their orthologs in rice. However, we only identified three rice CYP707A genes; one of them is identical to what was reported previously (Saika et al., 2007). *OsCYP707A*5, *OsCYP707A*6, and *OsCYP707A*7 reside on different chromosomes: 2, 8, and 9, respectively. We observed independent gene duplication events in both rice and *Arabidopsis* (**Figure 3A**).

As to GA3ox in rice, we identified only two previously reported genes (Itoh et al., 2001), matching to four GA3ox orthologs in *Arabidopsis* (Hedden et al., 2001). *OsGA3ox*1 and *OsGA3ox*2 are located on chromosomes 5 and 1, respectively. The amino acid sequence of *OsGA3ox*1 and *OsGA3ox*2 displayed about 37% similarities to *Arabidopsis* GA3ox proteins. Phylogenetic analysis (**Figure 3B**) revealed that GA3ox genes duplicated separately after the monocot and dicot divide.

We identified four *OsGA20ox* genes; two were previously reported in rice (Sasaki et al., 2002) and five were reported in *Arabidopsis* (Hedden et al., 2001). In rice, *OsGA20ox*1, *OsGA20ox*2, *OsGA20ox*3, and *OsGA20ox*4 are located on chromosomes 3, 1, 12, and 5, respectively. The amino acid sequence of *OsGA20ox*1 has the highest homology with *Arabidopsis* GA20ox proteins (52–57% identities). In comparison, *OsGA20ox*2 and *OsGA20ox*4 have 46–51% identities, respectively. Phylogenetic analysis (**Figure 3B**) revealed that GA20ox proteins were subdivided into two groups. *OsGA20ox*2 and *OsGA20ox*4 were more closely related (64% identity) than the other *OsGA20ox* proteins, and they formed one separate branch, but then *OsGA20ox*1 and *OsGA20ox*3 were grouped with *AtGA20ox*1, *AtGA20ox*2, and *AtGA20ox*3 to form another.

We identified five GA2ox genes in rice as compared to seven previously reported *Arabidopsis* counterparts (Schomburg et al., 2003); four (*OsGA2ox*1, *OsGA2ox*2, *OsGA2ox*3, and *OsGA2ox*4) of them were reported previously (Sakamoto et al., 2001; Sakai et al., 2003). *OsGA2ox*1, *OsGA2ox*4, and *OsGA2ox*5 are located on chromosome 5, and *OsGA2ox*2 and *OsGA2ox*3 are on chromosome 1. Although some *OsGA2ox* genes are localized on the same chromosomes, they are well separated with large phylogenetic distance that suggests early duplication events. *OsGA2ox*3 showed highest similarity to *Arabidopsis* GA2ox (49–51% identities), whereas the other *OsGA2ox* showed identities between 35 and 48% with the *Arabidopsis* counterparts. Phylogenetic analysis (**Figure 3B**) revealed that *OsGA2ox*1 has lower identities with those of *Arabidopsis* counterparts, constituting a separate clade.

COMPARATIVE ANALYSIS OF ABA AND GA METABOLIC GENES AMONG RICE CULTIVARS

Using a threshold of nucleotide identity over 95% and coverage over 80%, we defined orthologous gene pairs in *9311*, *PA64s*, and *Nipponbare* (also including a 3-kb upstream sequence containing promoters and UTRs) for comparative analysis (**Table 2**). Although *9311*, *PA64s*, and *Nipponbare* share a common ancestor of *O. sativa*, there are many sequence variations between orthologs that may be a result of artificial selection. In general, there are significantly more SNPs and Indels in coding regions between paralogs than between orthologs (Wilcoxon rank sum test: $p < 0.01$). Compared to coding regions, regulatory regions have significantly higher numbers of

[2]http://www.sanger.ac.uk/resources/databases/pfam.html

[3]http://gbrowse.ncpgr.cn/cgi-bin/gbrowse/japonica/

Table 1 | ABA and GA metabolism-related genes in *Nipponbare*.

Number	Gene name	Entry name	DNA sequence length (bp)	Annotation	Chromosome
ABA BIOSYNTHESIS					
1	*OsNCED1*	AY838897	1917	9-*cis*-epoxycarotenoid dioxygenase 1	II
2	*OsNCED2*	AY838898	1731	9-*cis*-epoxycarotenoid dioxygenase 2	XII
3	*OsNCED3*	AY838899	1827	9-*cis*-epoxycarotenoid dioxygenase 3	III
4	*OsNCED4*	AY838900	1749	9-*cis*-epoxycarotenoid dioxygenase 4	VII
5	*OsNCED5*	AY838901	1842	9-*cis*-epoxycarotenoid dioxygenase 5	XII
ABA CATABOLISM					
6	*OsCYP707A5*	AB277270	1416	ABA 8′-hydroxylase 1	II
7	*OsCYP707A6*	NM_001068556	1521	Cytochrome P450 family protein	VIII
8	*OsCYP707A7*	NM_001069901	1503	Cytochrome P450 family protein	IX
GA BIOSYNTHESIS					
9	*OsGA20ox1*	AC096690	1113	Putative gibberellin 20-oxidase	III
10	*OsGA20ox2*	NM_001051549	1170	Gibberellin 20-oxidase	I
11	*OsGA20ox3*	AP005840	1104	Putative gibberellin 20-oxidase	XII
12	*OsGA20ox4*	NM_001062119	1332	Gibberellin 20-oxidase	V
13	*OsGA3ox1*	NM_001048721	1113	GA 3 beta-hydroxylase	V
14	*OsGA3ox2*	AC144738	1155	Putative gibberellin 3 beta-hydroxylase	I
GA CATABOLISM					
15	*OsGA2ox1*	AC119288	1149	Gibberellin 2-oxidase	V
16	*OsGA2ox2*	NM_001048899.1	1008	Gibberellin 2-oxidase	I
17	*OsGA2ox3*	NM_001050827	984	Gibberellin 2-oxidase	I
18	*OsGA2ox4*	AC132485	729	Putative gibberellin 2-oxidase	V
19	*OsGA2ox5*	NM_001062846	1062	Gibberellin 2-oxidase	V
ABA AND GA PERCEPTION					
20	*OsGPCR*	CM000147	1386	Lanthionine synthetase C-like	X
21	*OsGID1*	AB211399	1065	Gibberellin insensitive dwarf 1	V

substitutions and Indels (Wilcoxon rank sum test: $p < 0.01$), which may also collectively contribute to the differential expression of these genes.

Based on the degree of sequence variations, we can readily identify the gene family members and their relationship with regard to obvious inheritances from their ancestor subspecies, *indica* or *japonica*. For instance, in NCED family, three genes (*OsNCED1*, *OsNCED3*, and *OsNCED5*) of *PA64s* and *9311* were inherited from the same *indica* ancestor as shown in **Figure 4**, which is consistent with lower variation between *9311* and *PA64s* (**Table 2**). In another gene family, *OsCYP707A6* and *OsCYP707A7* of *PA64s* were from *indica* (**Figure 4B**). In GA20ox family, *PA64s* inherited *OsGA20ox2* from *indica*, whereas other genes were not obviously separated into *indica or japonica* (**Figure 4D**). In GA3ox family, both *OsGA3ox1* and *OsGA3ox2* were from *indica* background, and in GA2ox family, *PA64s* inherited *OsGA2ox4* from its *indica* background (**Figures 4E and 4F**). ABA receptor (*OsGPCR*) of *PA64s* was most likely from *japonica or javanica* rather than from *indica* background. Both ABA and GA receptors were found with large amount of variations between *9311* and *PA64s* due to frequent hybridization with complex genetic background during rice domestication and breeding. We concluded that the dormancy trait of *PA64s* was mainly inherited from *indica*, though it also maintained a partial background of *japonica* and *javanica*. The mixed background may contribute to its significant difference in germination when compared with *9311*.

SEQUENCE AND FUNCTIONAL ANALYSIS OF ABA AND GA METABOLIC GENES OF *9311*, *PA64s*, AND *NIPPONBARE*

We essentially identified two classes of differences among the three cultivars: SNPs and Indels (**Table 2**). Some genes are obviously more conserved (by and large due to selection) than others, such as *OsGA3ox2* and *OsGA2ox3*, who have no Indel or SNP discovered in either *9311* or *PA64s* (**Table 2**). One possible reason is that these genes play major functional roles and therefore are strongly selected. Another explanation is that these genes were recently duplicated and therefore less mutation was accumulated. Other genes in *9311*, *PA64s*, and *Nipponbare* appear high variable, such as *OsGA20ox4* (41 SNPs and 2 Indels with a total length of 540 bp) and *OsGA2ox5* (65 SNPs and 9 Indels with a total length of 154 bp) (**Table 2**). One possible reason for the variability is that they are relative older or less conserved due to weaker selection.

We have further scrutinized some SNPs and Indels of protein domains of the related genes to look for potential changes in protein structure and function. Ninety-seven candidate SNPs and 618 candidate Indels involving amino acid changes were found to lie in domains of RPE membrane protein, cytochrome P450, 2OG-Fe(II) oxygenase superfamily, lanthionine synthetase C-like protein, and alpha/beta hydrolase fold, as well as other residues associated with specific functions predicted by pfam (**Table 3**). We found that 2OG-Fe(II) oxygenase of OsGA3ox family is highly conserved but alpha/beta hydrolase fold of *OsGID1* has 24 SNPs and 172 amino acid Indels (**Figure 5**). Compared to the sequences of *PA64s* and

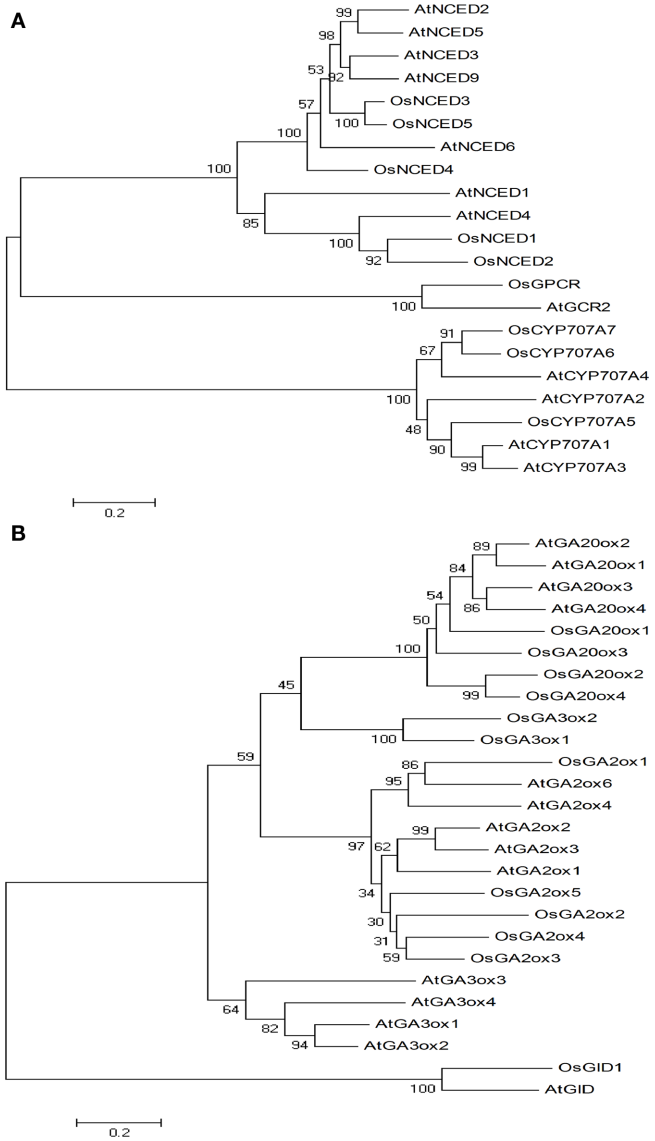

FIGURE 3 | Neighbor-joining trees for ABA (A) and GA (B) metabolic genes were constructed by using MEGA4.1 and generated based on full-length amino acid sequences from rice and *Arabidopsis*.

Nipponbare, 9311 has a large insertion in the alpha/beta hydrolase fold (Ollis et al., 1992), and this insertion may lead to enhanced sensitivity to GA. Sequence polymorphisms in the functional domains that differ between the three subspecies may be one of the direct causes for functional variation, although further functional tests are required to confirm their precise roles.

DIFFERENT FUNCTIONAL CONSTRAINTS ON ABA AND GA METABOLIC GENES BETWEEN *9311*, *PA64s*, AND *NIPPONBARE*

Following a gene duplication event, the two duplicates may be subjected to different selective constraints and even new functions. To investigate whether or not different rice cultivars have undergone different types of artificial selection, we identified reciprocal best matches – orthologs – among ABA and GA metabolic genes from *Arabidopsis* and rice. We calculated non-synonymous and synonymous substitution rate (*Ka* and *Ks*) for each pair (**Figure 6**). As indicated by Ka/Ks < 1, all of pairwise comparisons appear to undergo purifying selection. Interestingly, the *Ka*/*Ks* ratio of *OsGA20ox2* is significantly different from other members of OsGA20ox family (*t*-test: $p < 0.01$), indicating that it may evolve faster than others. This difference may be either a result of positive selection or relaxed purifying selection. Our results suggest that genes with multiple copies tend to evolve in different patterns, consistent with the view that one of the duplicates may undergo purifying selection after gene duplication while the other may enjoy more relaxed selective pressure. Furthermore, we also found that some of these genes (such as *OsNCED1* and *OsGA3ox2*) had different *Ka*/*Ks* values between different rice cultivars.

EXPRESSION ANALYSIS OF NCED, CYP707A, GA20ox, GA3ox, AND GA2ox GENES IN *9311* AND *PA64s*

In imbibing seeds, ABA and GA contents are regulated by ABA biosynthetic enzyme NCED, ABA catabolic enzyme CYP707A, GA biosynthetic enzymes GA20ox and GA3ox, and GA catabolic enzyme GA2ox. We detected expression levels of these genes which are important for the regulation of ABA and GA contents (Xiong and Zhu, 2003; Welsch et al., 2008). We also performed real-time PCR to confirm the expression levels of 19 genes in *9311* and *PA64s*, including 6 genes (*OsNCED*3, *OsCYP707A*5, *OsGA20ox*2, *OsGA3ox*2, *OsGA2ox*1, and *OsGA2ox*5) high-abundance and 13 (*OsNCED*1, *OsNCED*2, *OsNCED*4, *OsNCED*5, *OsCYP707A*6, *OsCYP707A*7, *OsGA20ox*1, *OsGA20ox*4, *OsGA20ox*3, *OsGA3ox*1, *OsGA2ox*2, *OsGA2ox*3, *OsGA2ox*4) low-abundance transcripts. Our results showed that the expression of ABA and GA metabolic genes was relative low and that there were significant differences between *9311* and *PA64s* (**Figure 7**). For instance, the expression of ABA synthetic gene *OsNCED*3 was 3.9-folds higher (*t*-test: $p < 0.01$) and catabolic gene *OsCYP707A*5 was 3.5-folds lower (*t*-test: $p < 0.01$) in *PA64s* than in *9311*; this result implied a lower ABA level in *9311* (Barrero et al., 2006). The GA synthetic gene *OsGA3ox*2 was expressed at a much higher level (2.8-folds) in *9311* than in *PA64s*. In contrast, both of GA catabolic genes *OsGA2ox*1 and *OsGA2ox*5 were expressed at a much higher level in *PA64s* than in *9311*; this result suggested higher GA level or less dormant seeds in *9311*. Indeed, a correlation between the expression level of GA metabolism genes and the content of active GA has been reported (Busov et al., 2003; Oh et al., 2006). The differential expression among gene family members also confirmed the complexity of the regulatory network, also footnoted by higher variations in regulatory regions.

DISCUSSION

Studying the plasticity of dormancy and germination is of necessity in plant biology and crop breeding (Roberts, 1961). We engaged our study on seed germination and discovered that dormancy states are different among rice cultivars, consistent with previous publications (Seshu and Dadlani, 1991; Gu et al., 2004, 2006; Veasey et al., 2004). Aligning sequences in coding and regulatory regions, we observed large amount of Indels and SNPs in genes that are related to regulate the ABA and GA metabolic pathways. For instance, two Indels of 306 bp in the coding sequence and two Indels of 260 bp in the upstream regulatory region of *OsCYP707A5* between *9311*

Table 2 | Single nucleotide polymorphisms and Indels among *9311, PA64s,* and *Nipponbare.*

Gene name	Genes on chromosome[a]			Regulation region		
	9311	PA64s	SNP no.[b]	SNP rate[c]	Indel no.[d]	Indel length[e]
OsNCED1	Chr02_3173	Chr02_4135	43|33|10	0.0141|0.0108|0.0033	12|7|6	116|96|20
OsNCED2	Chr12_1047	Chr12_0855	31|139|158	0.0102|0.0425|0.0478	8|8|16	96|540|614
OsNCED3	Chr03_3073	Chr03_2198	NA|NA|NA	NA|NA|NA	NA|NA|NA	NA|NA|NA
OsNCED4	Chr07_0366	Chr07_0350	100|174|228	0.033|0.0577|0.0757	3|16|9	60|30|22
OsNCED5	Chr12_2021	Chr12_1628	53|125|125	0.0173|0.0399|0.0406	9|10|12	116|264|164
OsCYP707A5	Chr02_3170	Chr02_4132	7|17|11	0.0023|0.0054|0.0035	3|3|2	2|262|260
OsCYP707A6	Chr08_2087	Chr08_1729	37|38|3	0.012|0.012|0.001	16|13|7	32|58|56
OsCYP707A7	Chr09_1329	Chr09_1219	262|262|2	0.084|0.0845|0.0007	25|25|0	204|204|0
OsGA20ox1	Chr03_4546	Chr03_3632	9|10|1	0.003|0.0033|0.0003	6|4|3	80|36|44
OsGA20ox2	Chr01_4645	Chr01_4410	34|NA|NA	0.011|NA|NA	7|NA|NA	188|NA|NA
OsGA20ox3	Chr12_1260	Chr12_1026	0|0|0	0|0|0	0|0|2	0|0|2
OsGA20ox4	Chr05_1936	Chr05_1801	45|45|0	0.0147|0.0147|0	9|9|0	102|102|0
OsGA3ox1	Chr05_0563	Chr05_0539	28|28|0	0.009|0.009|0	18|18|0	242|242|0
OsGA3ox2	Chr01_0611	Chr01_0665	40|40|0	0.0133|0.0133|0	6|6|0	12|12|0
OsGA2ox1	Chr05_0449	Chr05_0429	5|NA|NA	0.0016|NA|NA	6|NA|NA	10|NA|NA
OsGA2ox2	Chr01_0837	Chr01_0926	19|14|7	0.0063|0.0047|0.0023	11|6|8	28|18|12
OsGA2ox3	Chr01_3778	Chr01_3512	36|39|3	0.0119|0.0129|0.001	7|7|0	42|42|0
OsGA2ox4	Chr05_2528	Chr05_2378	370|18|348	0.117|0.0059|0.1103	69|10|67	306|76|306
OsGA2ox5	Chr05_2857	Chr05_2689	36|33|7	0.012|0.011|0.0023	6|5|4	10|6|8
OsGPCR	Chr10_1648	Chr10_1340	NA|25|NA	0.5375|0.0079|0.5209	80|11|95	184|340|228
OsGID1	Chr05_1857	Chr05_1721	NA|NA|NA	NA|NA|NA	NA|NA|NA	NA|NA|NA

Gene name	Genes on chromosome[a]			Coding region		
	9311	PA64s	SNP no.[b]	SNP rate[c]	Indel no.[d]	Indel length[e]
OsNCED1	Chr02_3173	Chr02_4135	5|5|0	0.0026|0.0026|0	1|4|1	48|239|189
OsNCED2	Chr12_1047	Chr12_0855	10|0|9	0.0057|0|0.0051	0|0|0	0|0|0
OsNCED3	Chr03_3073	Chr03_2198	8|16|8	0.0044|0.0088|0.004	0|2|2	0|129|129
OsNCED4	Chr07_0366	Chr07_0350	1|9|8	0.0006|0.0051|0.004	1|0|1	177|0|177
OsNCED5	Chr12_2021	Chr12_1628	1|4|1	0.0007|0.0022|0.001	0|0|0	0|0|0
OsCYP707A5	Chr02_3170	Chr02_4132	1|2|0	0.0007|0.0014|0	0|2|2	0|306|306
OsCYP707A6	Chr08_2087	Chr08_1729	1|6|0	0.0007|0.0036|0	0|2|0	0|159|0
OsCYP707A7	Chr09_1329	Chr09_1219	1|4|0	0.0007|0.0027|0	0|0|0	0|0|0
OsGA20ox1	Chr03_4546	Chr03_3632	2|2|0	0.0018|0.0018|0	0|0|0	0|0|0
OsGA20ox2	Chr01_4645	Chr01_4410	2|2|0	0.0017|0.0017|0	2|2|2	126|381|411
OsGA20ox3	Chr12_1260	Chr12_1026	0|0|0	0|0|0	0|0|0	0|0|0
OsGA20ox4	Chr05_1936	Chr05_1801	41|41|0	0.031|0.031|0	2|1|1	540|369|171
OsGA3ox1	Chr05_0563	Chr05_0539	3|5|2	0.0026|0.0043|0.017	2|6|4	18|84|66
OsGA3ox2	Chr01_0611	Chr01_0665	3|3|0	0.0027|0.0027|0	1|1|0	9|9|0
OsGA2ox1	Chr05_0449	Chr05_0429	3|2|2	0.00247|0.0017|0.002	1|1|1	63|471|534
OsGA2ox2	Chr01_0837	Chr01_0926	81|5|57	0.08|0.0049|0.057	4|1|5	276|3|279
OsGA2ox3	Chr01_3778	Chr01_3512	1|1|0	0.001|0.001|0	0|0|0	0|0|0
OsGA2ox4	Chr05_2528	Chr05_2378	3|32|8	0.0028|0.0318|0.011	1|10|2	336|276|62
OsGA2ox5	Chr05_2857	Chr05_2689	59|6|65	0.0527|0.0056|0.061	7|2|9	60|95|154
OsGPCR	Chr10_1648	Chr10_1340	10|40|38	0.0072|0.0288|0.027	2|5|4	597|388|553
OsGID1	Chr05_1857	Chr05_1721	20|20|18	0.0119|0.0019|0.017	5|0|5	708|0|708

[a] *Matched genes of 9311 and PA64s with chromosome and gene ID information.*
[b] *Number of SNPs between the rice cultivars. | Number of SNPs between PA64s and Nipponbare | Number of SNPs between 9311 and PA64s. NA strands for poor similarity.*
[c] *Rate of SNPs between 9311 and Nipponbare | Rate of SNPs between PA64s and Nipponbare | Rate of SNPs between 9311 and PA64s.*
[d] *Number of Indels between 9311 and Nipponbare | Number of Indels between PA64s and Nipponbare | Number of Indels between 9311 and PA64s.*
[e] *Total length (bp) of Indels between 9311 and Nipponbare | Total length (bp) of Indels between PA64s and Nipponbare | Total length (bp) of Indels between 9311 and PA64s.*

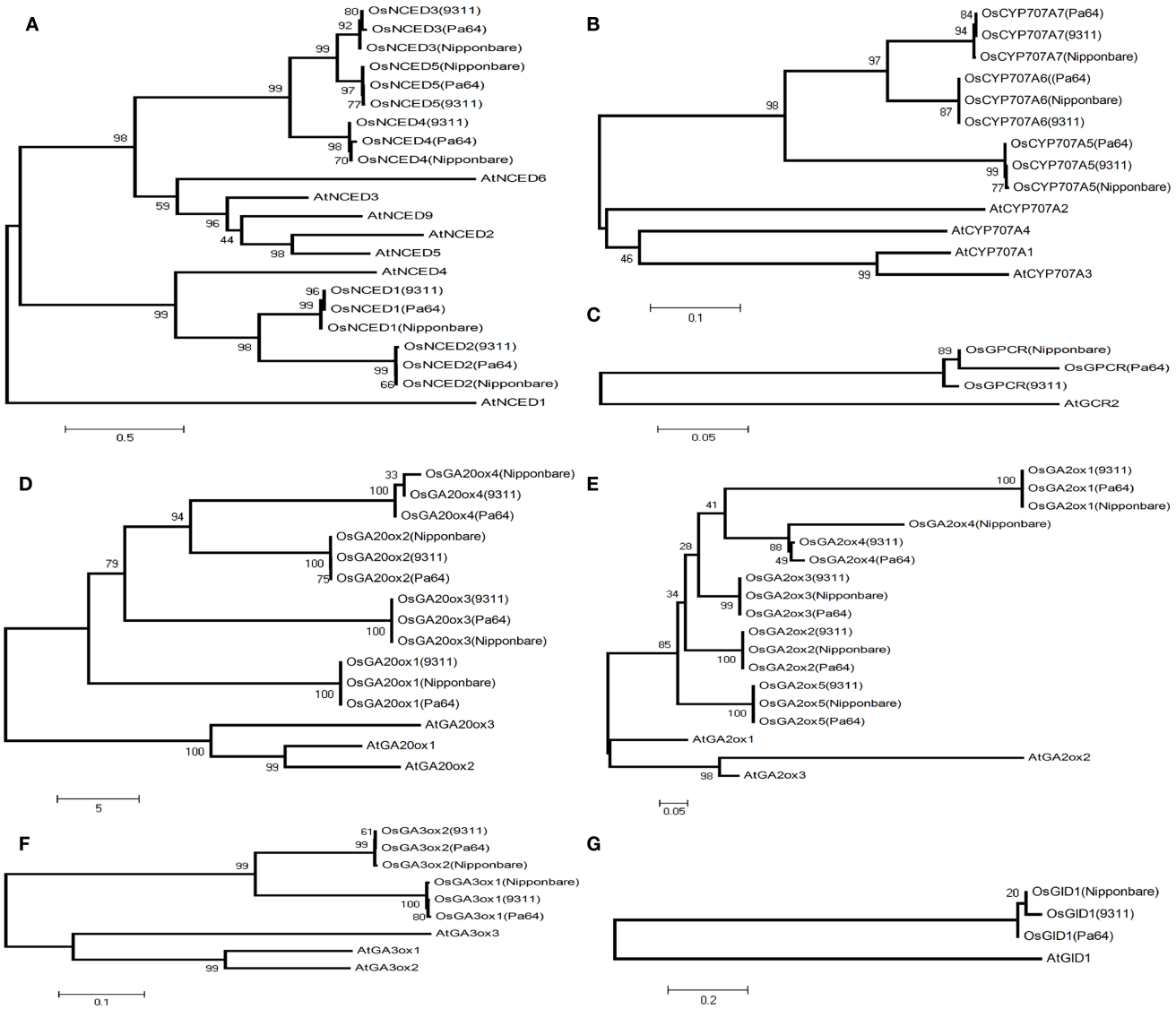

FIGURE 4 | Neighbor-joining phylogenetic analysis of (A) NCED, (B) CYP707A, (C) ABA receptor, (D) GA20ox, (E) GA2ox, (F) GA3ox, and (G) GA receptor among *9311*, *PA64s*, and *Nipponbare*.

and *PA64s* may affect the function of *OsCYP707A5* and the binding of its regulatory transcriptional factors. In addition, we compared genes differentially expressed in dry seed between *9311* and *PA64s*, and hypothesized that different cellular concentrations of ABA and GA may reflect the genetic variations among these genes, especially those related to the ABA and GA pathways (Xiong and Zhu, 2003; Welsch et al., 2008).

As rice cultivars have been under strong artificial selection during domestication and the loss of seed dormancy is one of the most important traits in the domestication syndrome, dormancy- and germination-associated genes are absolutely the targets of selection. Thus, we further calculated *Ka/Ks* values for the ABA and GA metabolism-related genes and found that all were lower than 0.5; the result suggests that they are most likely undergoing purifying selection despite the fact that most of them belong to multi-gene families. Compared to other *OsNCED* family members, *OsNCED3*, due to their high expression levels, may play a major role in controlling dormancy and germination (Lefebvre et al., 2006; Hwang et al., 2010), which is consistent with that the ectopic expression of *OsNCED3* in *Arabidopsis* leads to a delay in seed germination (Hwang et al., 2010). Expression analysis of *CYP707A* genes suggests that *OsCYP707A5* may play a key role in seed dormancy and germination (Kushiro et al., 2004; Millar et al., 2006; Okamoto et al., 2006). One SNP and several Indels with a total length of 59 amino acids were identified in functional regions of *OsCYP707A5*, indicating its potential involvement in regulating ABA concentration.

Table 3 | Candidate SNPs and Indels identified in conserved regions as indicated by Pfam.

Gene	Change[a]	Position[b]	Indel length (aa)	Domain/family	Domain/family position
OsNCED1	E/K	448	63	RPE (retinal pigment epithelial) membrane protein	131–630
	G/R	465			
OsNCED2	I/L	188	0	RPE membrane protein	68–569
	V/A	199			
	R/Q	359			
OsNCED3	R/A	238	43	RPE membrane protein	108–601
	A/G	240			
	C/W	241			
	G/R	242			
	D/E	245			
	N/Y	254			
	L/F	257			
	V/I	258			
	D/G	282			
	C/R	492			
OsNCED4	P/S	87	59	RPE membrane protein	76–575
	V/L	109			
	G/A	116			
	V/L	125			
	A/T	131			
	G/S	549			
OsNCED5	A/V	260	0	RPE membrane protein	110–606
OsCYP707A5	A/T	237	59	Cytochrome P450	40–432
OsCYP707A6	NA	NA	4	Cytochrome P450	52–489
OsCYP707A7	N/H	69	0	Cytochrome P450	45–474
OsGA20ox1	NA	NA	0	2OG-Fe(II) oxygenase superfamily	208–307
OsGA20ox2	NA	NA	0	2OG-Fe(II) oxygenase superfamily	225–324
OsGA20ox3	NA	NA	9	2OG-Fe(II) oxygenase superfamily	199–304
	S/V	304			
	C/S	312			
	S/A	313			
	I/T	315			
OsGA20ox4	R/L	316	49	2OG-Fe(II) oxygenase superfamily	234–369
	L/V	318			
	L/F	320			
	T/R	321			
	T/Q	331			
	Q/S	347			
OsGA3ox1	NA	NA	0	2OG-Fe(II) oxygenase superfamily	226–327
OsGA3ox2	NA	NA	0	2OG-Fe(II) oxygenase superfamily	212–304
OsGA2ox1	NA	NA	0	2OG-Fe(II) oxygenase superfamily	190–321
OsGA2ox2	NA	NA	0	2OG-Fe(II) oxygenase superfamily	179–287
OsGA2ox3	NA	NA	0	2OG-Fe(II) oxygenase superfamily	174–278
OsGA2ox4	25	/	113	2OG-Fe(II) oxygenase superfamily	113–177
OsGA2ox5	F/P	206	0	2OG-Fe(II) oxygenase superfamily	179–292
	G/R	207			
OsGPCR	18	/	47	Lanthionine synthetase C-like protein	120–461
OsGID1	24	/	172	alpha/beta hydrolase fold	116–330

[a]*Amino acid changes in functional regions are indicated as well as Indels numbers.*
[b]*The position of amino acid changes in functional regions. NA stands for no change. Positions of three proteins, OsGA2ox4, OsGPCR, and OsGID1 were not listed due to a high number of changes.*
/ represents that there are too many SNPs to list in the Table.

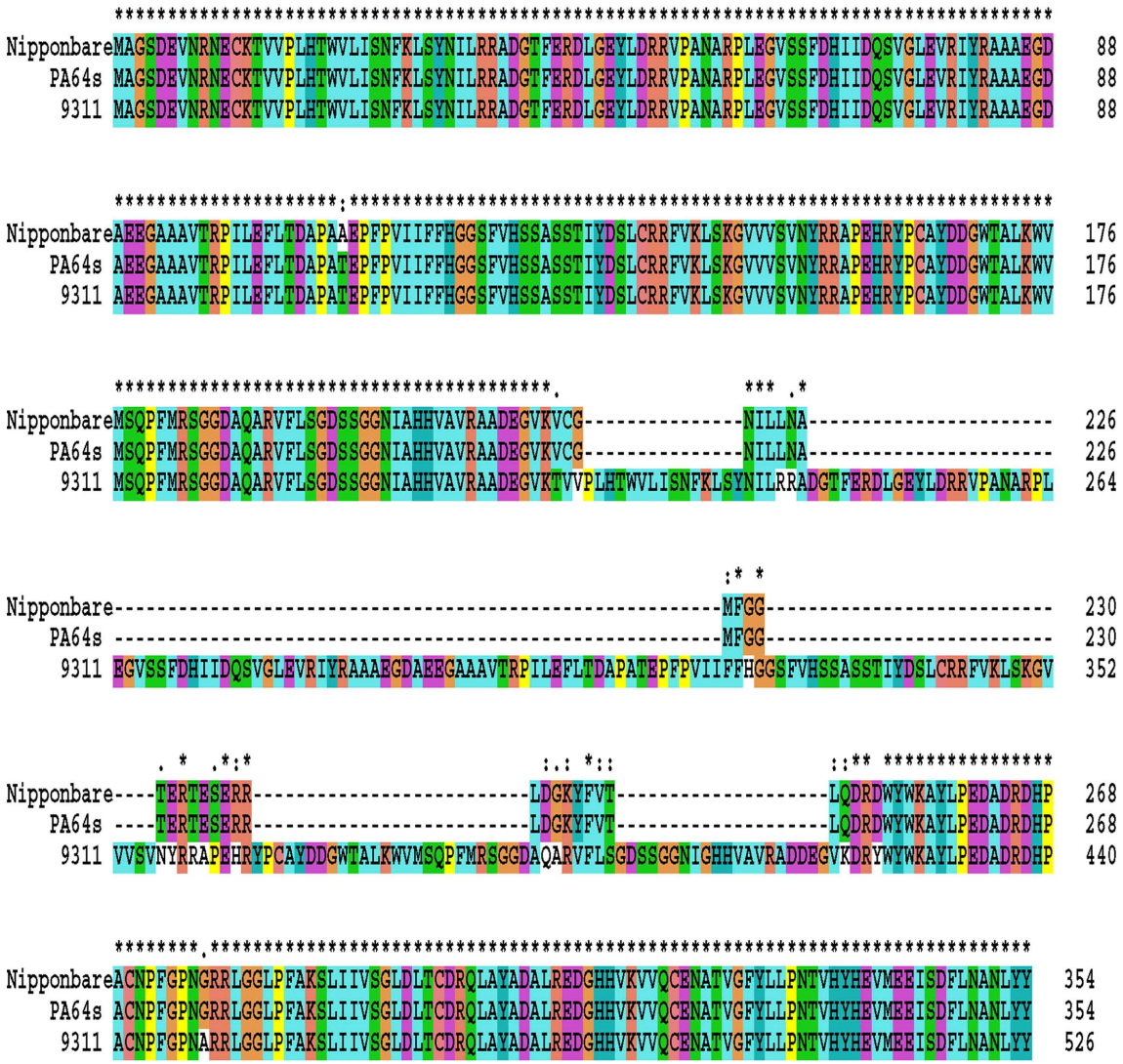

FIGURE 5 | Amino acid sequence alignment of GA receptors among *9311, PA64s,* and *Nipponbare*. Positions in the sequence where all three proteins are the same or physiochemically similar are marked with an asterisk above.

In *OsGA20ox* and *OsGA3ox* families, *OsGA20ox*2 and *OsGA3ox*2 may play major roles in the regulation of GA synthesis (Calvo et al., 2004); both are highly conserved as no SNP/Indel was found in their functional regions. Therefore, their differential expression may control the cellular concentration of GA (Mitchum et al., 2006; Rieu et al., 2008). By the same token, *OsGA2ox*1 and *OsGA2ox*5 of *OsGA2ox* family may play major roles in regulating GA catabolism (Mitchum et al., 2006) so do *OsGPCR* and *OsGID1* (Aleman et al., 2008; Hirano et al., 2008; Murase et al., 2008).

A balanced ABA/GA ratio governed by dynamics of hormone synthesis and catabolism regulates dormancy and germination through changing seed sensitivity to the external environment (Cadman et al., 2006; Seo et al., 2006). We propose a model extending the ABA/GA balance theory to address changes in gene function and expression (**Figure 8**). According to this model, different rice cultivars exhibit different dormancy states due to differential expression of ABA and GA metabolic genes. Environmental conditions, such as high temperature and light (Toh et al., 2008), can enhance expression of specific ABA biosynthesis genes (*OsNCED* family) and GA catabolic genes (*OsGA2ox* family), resulting in seed dormancy through induction of ABA dominance. Low temperature (Finch-Savage et al., 2007) can also enhance expression of specific ABA catabolic genes (*OsCYP707A* family) and GA biosynthesis genes (*OsGA20ox* and *OsGA3ox* families). In order to further illustrate molecular mechanisms of embryonic dormancy in rice, additional studies should be carried out, focusing on comparative sequence analysis and expression profiling of dormancy-associated genes among large samples of rice cultivars to construct gene regulation networks covering all the stages of seed dormancy and germination.

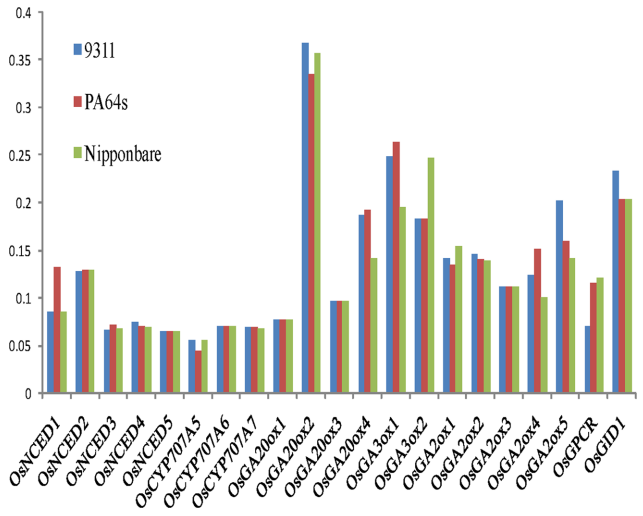

FIGURE 6 | *Ka/Ks* ratios of ABA and GA metabolic genes for different rice cultivars.

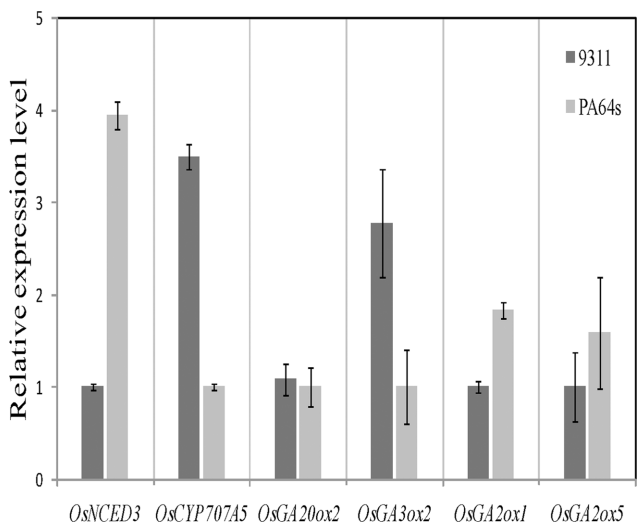

FIGURE 7 | Real-time PCR based analyses of *OsNCED3, OsCYP707A5, OsGA20ox2, OsGA3ox2, OsGA2ox1,* and *OsGA2ox5* discovered in *9311* and *PA64s* seeds. Transcripts were normalized with GAPDH as an internal control. Measurements were averaged over three replicates with error bars representing SE.

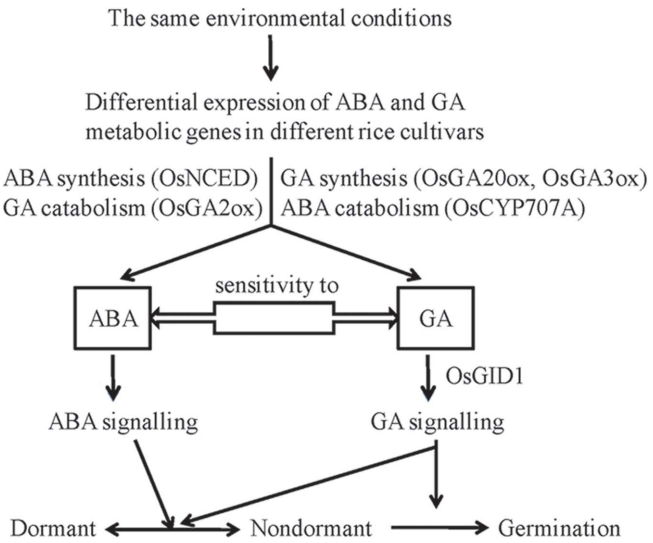

FIGURE 8 | A schematic model for the regulation of dormancy and germination by ABA and GA in response to the environment in rice seeds. Under the same environmental conditions, different rice cultivars exhibit different dormancy states due to their differential expression of ABA and GA metabolic genes. Environmental conditions such as higher temperature enhance expression of specific ABA biosynthesis genes (OsNCED family) and GA catabolic genes (OsGA2ox family), which result in dormancy by inducing dominance of ABA. Moreover, whether a seed remains dormancy or germinates may depend on a balanced ABA and GA metabolism influenced by genetic and epigenetic mechanisms, a collective outcome determined by sequence and expression variations of ABA and GA metabolic genes and the function of ABA/GA receptors.

CONCLUSION

In this study, we identified rice ABA and GA metabolism-related genes based on comparative and phylogenetic analyses among rice cultivars. We found that there are many SNPs and Indels in the coding and regulatory sequences of these genes due to their redundancy and diverse genetic background. We further traced the variations on the genome sequences of *9311* and *PA64s* and showed that sequence variations may lead to functional variations and variable seed dormancy states regulated by ABA and GA. In addition, we also surveyed and compared differentially expressed genes between *9311* and *PA64s* and demonstrated that differential expression of related genes may also play roles in the variable dormancy and germination states in rice. Although precise correlation between sequence variations and dormancy states need further experimental verification, sequence and expression analyses of ABA and GA metabolism-related genes among rice cultivars pave a way to study molecular mechanism of seed dormancy and germination.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Plant_Genetics_and_Genomics/10.3389/fpls.2011.00017/abstract

Data Sheet S1 | ABA and GA concentrations of *9311* and *PA64s* from each individual assay of the triplicate.
Data Sheet S2 | DNA sequences of *9311, PA64s,* and *Nipponbare* used in this study.

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