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The Expanded and Diversified Calmodulin-Binding Protein 60 (CBP60) Family in Rice (*Oryza sativa* L.) Is Conserved in Defense Responses against Pathogens

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Abstract: Plant disease management is key to sustainable production of staple food crops. Calcium (Ca^{2+}) signal and phytohormones play critical roles in regulating plant defense responses against pathogens. The Ca^{2+} signals are sensed, decoded and transduced by calmodulin and other Ca^{2+}-binding proteins, followed by interaction with and modulation of activities of target proteins such as calmodulin-binding proteins (CBPs). Members of the Arabidopsis *CBP60* gene family, *AtCBP60g* and *AtSARD1*, have emerged as major regulators of immune responses. In this study, we identified a 15 member *CBP60* gene family in rice (*Oryza sativa*) of which *OsCBP60g-3*, *OsCBP60g-4*, *OsCBP60a* and *OsSARD-like1* genes were consistently upregulated in rice seedlings in response to infection with both fungal (*Magnaporthe oryzae*) and bacterial (*Xanthomonas oryzae*) pathogens as well as by salicylic acid (SA). *OsCBP60g-4* and *OsCBP60g-3* were induced maximally by SA and brassinosteroid (BR), respectively, and *OsCBP60g-4* was expressed at 3-fold higher levels in the *M. oryzae* resistant rice genotype (IC-346004) as compared to the susceptible rice genotype (Rajendra Kasturi). The considerable expansion of the immunity clade and the up-regulation of several *OsCBP60* genes in response to pathogens and defense hormones supports the importance of further investigating *OsCBP60* genes as targets for increasing disease resistance in rice.

Keywords: calmodulin-binding protein 60 (CBP60); *AtCBP60*; *OsCBP60*; *Xanthomonas oryzae* pv. *oryzae*; *Magnaporthe oryzae*; brassinosteroid; salicylic acid; jasmonic acid

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1. Introduction

Rice, as a staple food of more than half of the world's population, is key to food security in most Asian countries. The global demand for rice will continue to increase due to the predicted population growth to 9 billion by 2050 [1]. As with other crops, both biotic and abiotic stresses constrain the production of rice, and with the emergence of new diseases and pests associated with global warming, the situation is likely to be further exacerbated. Rice yield losses due to pests and pathogens at a global scale range from 20 to 40% [2]. Of the nearly 70 diseases that can occur in rice, losses due to bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae*, and blast caused by *Magnaporthe oryzae*, are paramount [3]. The study of the molecular interactions between rice and *X. oryzae* and *M. oryzae* has made rice a model monocotyledonous plant in understanding pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) [4,5].

Phytohormones and a variety of secondary messengers play key roles in mediating cellular responses to various stress stimuli [6,7]. Of the known phytohormones, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have been implicated in disease resistance in rice plants [8]. The plant steroid hormone, brassinosteroid (BR), plays vital roles in plant growth and development, and also modulates abiotic and biotic stress responses [9,10]. BR has also been implicated in rice immune mechanisms [11], but due to its cross-talk with other defense hormones [12,13], the role of BR in rice immunity requires further investigation.

Calcium ion (Ca^{2+}) is an ubiquitous and a highly versatile secondary messenger involved in regulating plant growth and stress responses [14,15]. Calcium signatures are formed by changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in response to developmental and environmental stimuli, including pathogen signals [16]. The calcium signatures are decoded by Ca^{2+} sensors such as calmodulin (CaM) and CaM-like (CML) proteins, calcium-dependent protein kinases (CDPKs) and calcineurin B-like (CBL) proteins that translate the signal to molecular, physiological and metabolic responses [17]. CaM, the primary receptor of intracellular Ca^{2+}, functions by interacting with and modulating the activities of various target proteins, such as transcription factors, kinases, phosphatases and metabolic enzymes [18,19]. Although there is no well-defined consensus sequence for a CaM-binding domain (CBD), several characteristic features such as higher propensity for helical conformation, net positive charge within the binding region, and hydrophobic anchor residues separated by certain number of amino acids, have allowed for prediction of CBD within a protein [20,21].

Several CaM-binding protein (CBP) families with distinct roles in stress responses have been identified in plants [22,23]. An eight-member plant-specific gene family in Arabidopsis, *AtCBP60*, comprises of members that are induced by pathogen infection and play critical roles in SA-mediated immunity [24,25]. Two closely related members of the *AtCBP60* family, *AtCBP60g* and *SAR DEFICIENT1* (*SARD1*), promote SA production in response to recognition of microbe associated molecular patterns (MAMPs) and impact both SA-dependent and SA-independent gene expression [26,27]. AtCBP60g binds CaM via its CBD located at the N-terminus, while AtSARD1 appears to lack a CBD and does not bind CaM [26,28]. Studies in Arabidopsis suggest that *AtCBP60g* responds to the initial activation of Ca^{2+} flux in response to plant-pathogen interaction, which leads to SA production and subsequent immunity-related gene expression, while *AtSARD1* functions similarly but in a Ca^{2+}-independent manner such that immune responses continue to persist even after Ca^{2+} levels have returned to the normal range [25]. Recently it was demonstrated that *AtCBP60b* positively regulates immunity by activating the expression of *AtSARD1* and *AtCBP60g* and other immune response genes [29]. In contrast to the positive regulation of immune responses by *AtCBP60g*, *AtSARD1* and *AtCBP60b*, knockdown of the *CBP60a* ortholog in cotton increased SA levels and resistance against pathogen, indicating that *CBP60a* is a negative regulator of immunity [30]. Phylogenetic analysis of CBP60 protein sequences of diverse plant species has revealed that CBP60a, CBP60g and SARD1 form an immune-related clade and that these subfamilies are evolving at a fast rate, likely due to strong selection pressure from pathogen effectors [31]. Interestingly, *AtCBP60g* and *AtSARD1* have also been linked with cold and drought stress responses [24,32], and to be induced by BR (P. Krishna, personal communication).

While the roles of a subset of *CBP60* genes in immune regulation are well documented in dicotyledonous plants [25,33], there is little information on the *CBP60* genes and their functions in monocotyledonous plants [33]. Considering the growing importance of the roles of *CBP60* genes in biotic and abiotic stress responses, we undertook a study of the *CBP60* gene family in rice, a model monocotyledonous plant. Fifteen *OsCBP60* genes were identified in rice. Of these, 12 gene products fell in the immunity clade, indicating a significant expansion of the *CBP60g* and *SARD1* subfamilies in rice. Five *OsCBP60* genes within the immunity clade were upregulated by both pathogens, SA and BR. Single nucleotide polymorphisms (SNPs) were identified within the putative promoter region of *OsCBP60g-4*, which was expressed at a higher level in *M. oryzae* resistant genotype as

compared to a susceptible genotype. These results provide strong preliminary evidence for an expanded role of the *OsCBP60* gene family in defense against pathogens in rice.

2. Materials and Methods

2.1. Sequence Analysis

Similarity searches of nucleotide and amino acid sequences were carried out using BLASTP at the National Center for Biotechnology Information (NCBI) GenBank database and the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) (accessed on 14 June 2016) database. A phylogenetic tree was constructed using the neighbour-joining (NJ) method based on the genetic distance of the protein sequences using the MEGA 7 (http://www.megasoftware.net/) (accessed on 14 June 2016) tool. The chromosomal positions of *OsCBP60s* were studied using Phytozome v12.1 database (https://phytozome.jgi.doe.gov/pz/portal.html) (accessed on 16 August 2017). In order to identify the conserved motif structures encoded by the OsCBP60s, gene structure analysis was carried out using the Gene structure display server GSDS 2.0.

Using Boxshade (https://embnet.vital-it.ch/software/BOX_form.html) (accessed on 12 September 2021) and CLUSTAL Omega (accessible through https://www.ebi.ac.uk) (accessed on 12 September 2021), the sequences for the conserved CaM and DNA-binding domains were analysed. The Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html) (accessed on 15 September 2021) and CalModulin intEraction Learning System (CaMELS; https://camels.pythonanywhere.com/) (accessed on 15 September 2021) were used to further examine OsCBP60 amino acid sequences for the presence of potential CBD. In addition, amino acid sequences were submitted to https://heliquest.ipmc.cnrs.fr/ for the detection of amphipathic helices.

Subcellular localization of proteins was analysed using Balanced Subcellular Localization Predictor tool (BaCelLo) (http://gpcr.biocomp.unibo.it/bacello/info.htm) (accessed on 25 October 2021).

Upstream sequences of genes were obtained from the Rice Annotation Project Database (http://rapdb.dna.affrc.go.jp/tools/dump) (accessed on 13 April 2018). The putative promoters regions (1000 bp and 2000 bp upstream of transcription start site) of Arabidopsis and rice *CBP60* genes were searched for the presence of TGACG motif as binding site for TGA1/TGA4, W-box [(T)TGAC(C/T)] for WRKY70, and CAMTA motif [(A/C/G)CGCG(C/G/T)] for CAMTA, using PlantPAN 3.0 (http://PlantPAN.itps.ncku.edu.tw/) (accessed on 20 September 2021) platform. BES1 (CANNTG, CACGTG and CACTTG) and BZR1 (CGTGT/CG) binding sites were manually searched within the putative promoter sequences of the genes [10,34,35].

2.2. Plant Materials and Treatments

Rice cv. Rajendra Kasturi (*Oryza sativa* L. sp. indica cv. Rajendra Kasturi) was used in this study. Seeds of Rajendra Kasturi were grown in earthen pots containing 2:1 soil:cocopit in a greenhouse maintained at 28 °C. *M. oryzae* (isolate B157, corresponding to international race IC 9) was obtained from Dr. Bharat Chattoo Genome Research Centre, M.S. University, Vadodara, Gujarat. *M. oryzae* was grown on Potato Dextrose Agar (PDA) medium at 28–30 °C. For infection with the fungus, 21-day-old rice seedlings were inoculated with conidial suspensions (1×10^5 spores/mL) of *M. oryzae* as described previously [36–38]. For mock treatment, rice seedlings were treated with an equal volume of distilled water.

X. oryzae pv. *oryzae* was isolated from blight infected rice field at Bihar Agricultural College, Sabour (NCBI GenBank: MH986180) [39]. *X. oryzae* was grown on a nutrient agar medium at 28 °C [39,40]. Leaf infection with *X. oryzae* was performed using the leaf clipping method [38,41]. Rice leaves were clipped with scissors dipped in bacterial suspension ($1 \times 10^{8-9}$ cfu/mL) in saline (0.9%) containing 0.05% Triton-X-100. Sterile water containing 0.05% Triton-X-100 was used for mock treatment. Leaf samples were collected at 12 h, 24 h and 48 h after pathogen and mock inoculation, quick frozen in liquid nitrogen and stored at −80 °C till further use.

Rice genotypes IC-346004, IRBB55, Pusa Basmati-1 and Tetap were also used for pathogens treatment as described for Rajendra Kasturi.

For SA treatments, 21-day-old rice seedlings were sprayed with 3 mM sodium salicylate containing 0.05% Triton-X-100 [38]. Seedlings sprayed with distilled water containing 0.05% Triton-X-100 served as a mock treatment. For JA treatment, seedlings were grown for 21 days in black portrays (9 cm diameter and 9 cm height) containing a hole at the bottom for water absorption from a tray (20X14X7 cm) containing 1 litre of water. Seedlings were then placed in another tray containing either 100 µM JA [42] or water for mock treatment. Leaf samples were collected at 12 h, 24 h and 48 h after treatment.

For EBR treatment, surfaced sterilized rice seeds were placed on ½ Murashige and Skoog (MS) medium containing either 1 µM EBR or 0.02% ethanol (solvent of EBR) in test tubes [10,43]. The test tubes were closed with sterilized cotton plugs. Seedlings were grown for 15 days in the presence of EBR before leaf tissue was collected for gene expression analysis.

SNP analysis was carried out using DNA isolated from rice varieties IC-346004 (resistant to *M. oryzae*) and Rajendra Kasturi (susceptible to *M. oryzae*).

2.3. qRT-PCR Analysis

Total RNA was isolated using the SV Total RNA isolation kit (Promega). Random hexamer primers (Promega) were used for the synthesis of cDNA from total RNA by reverse transcription. cDNA was diluted in nuclease free water (1:5) and used for Quantitative real-time RT-PCR (qRT-PCR) analysis. qRT-PCR was carried out using SYBR Green dye in Light Cycler system (Applied Biosystem). Each qRT-PCR quantification was carried out in triplicate using gene specific primers (Supplementary Table S1). *ACTIN* was used as reference gene to normalize the gene expression data. The fold-change in expression level was calculated using the $2^{-\Delta\Delta Ct}$ method of relative quantification compared with control [44].

2.4. Promoter Mining

Genomic DNA was extracted from young seedlings of IC-346004 and Rajendra Kasturi using the DNA extraction kit (Qiagen, Germantown, MD, USA). The 1000 bp upstream region of *OsCBP60g-4* was amplified using specific primers (Fp: GCTGTGGACACTTCCTAGCC and Rp: GCAACTCACGCGGTGACACG) and the amplified product was sequenced. The sequences of IC-346004 and Rajendra Kasturi were compared with the reference sequence (Nipponbare; AP014967.1) available at NCBI by generating sequence alignment using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) (accessed on 12 July 2020) to identify Single Nucleotide Polymorphisms (SNPs).

2.5. Statistical Analysis

Gene expression data was statistically analysed using the computer software SPSS. Significance of differences were analysed by one-way analysis of variance (ANOVA). Comparison among treatment means was performed using the Least Significant Difference (LSD) multiple-comparison test.

3. Results

3.1. Phylogenetic Analysis of CBP60 Families in Arabidopsis and Rice

CBP60 proteins in rice were identified by performing BLASTP searches at Phytozome11 using complete protein sequences of the eight Arabidopsis members as query against the rice genome (*Oryza sativa* v7_JGI). After removing the incomplete sequences, a total of 15 *OsCBP60/SARD1-like* genes were identified. The protein sequences of the 15 OsCBP60s and eight AtCBP60s were used to create a phylogenetic tree using neighbor-joining method with bootstrapping (500 replicates) in MEGA7 software and rooted with a moss CBP60 sequence (Figure 1). We have named OsCBP60s based on sequence homology with the Arabidopsis proteins (Table 1). The *OsCBP60s* were numbered according to their location

on consecutive chromosomes but the gene numbers do not correlate to the chromosome numbers. If more than one gene was on the same chromosome, the numbering of gene members of a subgroup was continued in a consecutive manner.

Table 1. Full list of *OsCBP60* gene loci with proposed systematic names, predicted subcellular localization, and location of CBD in OsCBP60 proteins.

S.No.	Gene ID	Proposed Name	Predicted Localisation [a]	Predicted CBD (Consensus *)
1	*LOC_Os02g08120*	OsCBP60bcd-1	Nucleus	C-terminus
2	*LOC_Os02g35470*	OsCBP60bcd-2	Nucleus	C-terminus
3	*LOC_Os04g36660*	OsCBP60bcd-3	Nucleus	C-terminus
4	*LOC_Os03g32160*	OsCBP60a	Nucleus	C-terminus
5	*LOC_Os01g04280*	OsSARDL-1	Nucleus	none
6	*LOC_Os08g27170*	OsSARDL-2	Nucleus	none
7	*LOC_Os09g13890*	OsSARDL-3	Nucleus	none
8	*LOC_Os03g18960*	OsCBP60g-1/OsSARDL	Nucleus	none
9	*LOC_Os03g56660*	OsCBP60g-2	Nucleus	none
10	*LOC_Os11g44600*	OsCBP60g-3	Nucleus	none
11	*LOC_Os11g44680*	OsCBP60g-4	Chloroplast	none
12	*LOC_Os12g36110*	OsCBP60g-5	Nucleus	none
13	*LOC_Os12g36910*	OsCBP60g-6	Nucleus	none
14	*LOC_Os12g36920*	OsCBP60g-7	Nucleus	none
15	*LOC_Os12g36940*	OsCBP60g-8	Nucleus	none

[a] Subcellular localization predictions by BaCelLo tool. * Full details of CBD prediction results in Supplementary Table S1.

The phylogenetic tree analysis identified two main clades with high reliability, which were further divided into sub-clades (Figure 1). Three rice proteins that grouped in clade I were designated as orthologs of AtCBP60b, c and d and named OsCBP60bcd-1, OsCBP60bcd-2 and OsCBP60bcd-3 (Figure 1). Proteins closely related to AtCBP60e and AtCBP60f were absent in rice. A total of 12 OsCBP60/SARD1-like proteins grouped in the immunity-related clade II with AtCBP60a, AtCBP60g and AtSARD1. One of these, designated as OsCBP60a, appears to be orthologous to AtCBP60a. The three proteins forming a branch with AtSARD1 have been named as SARD-like (SARDL): OsSARDL-1, OsSARDL-2 and OsSARDL-3. The remaining eight proteins form a diverse group with AtCBP60g, although the position of OsCBP60g-1/SARDL remained unclear based on bootstrap values. To gain a better understanding of the position of OsCBPO60g-1/SARDL, a phylogenetic tree of CBP60 homologs from Arabidopsis, rice and cotton was constructed using NJ and ME algorithms (data not shown). While the phylogenetic tree analysis grouped OsCBP60g-1/SARDL with CBP60gs from Arabidopsis and cotton, exon analysis of all OsCBP60 proteins showed that similar to OsSARDL proteins, OsCBP60g-1/SARDL has a shortened C-terminus (Figure 2). Pairwise sequence alignment indicated that OsCBP60g-1/SARDL shares greater similarity with OsSARDL as compared to OsCBP60g proteins. Due to this discrepancy, we have named this protein as OsCBP60g-1/SARDL. Figure 2 indicates that in contrast to OsCBP60g-1/SARDL, the rest of OsCBP60gs (OsCBP60g-2-8) gained new C-terminal exons (Figure 2).

OsCBP60g-1/OsSARDL and *OsCBP60g-2* are located far apart on chromosome 3, while *OsCBP60g-3* and *OsCBp60g-4* are located close together on the chromosome 11 (Table 1). Four *OsCBP60g* genes (*OsCBP60g-5* to *OsCBP60g-8*) are encoded on chromosome 12. Also located on chromosome 3 is *OSCBP60a*. The *OsSARDL1-3* are located on chromosomes 1, 8

and 9, respectively. *OsCBP60bcd-1* and *OsCBP60bcd-2* are located on chromosome 2 and *OsCBP60bcd-3* on chromosome 4.

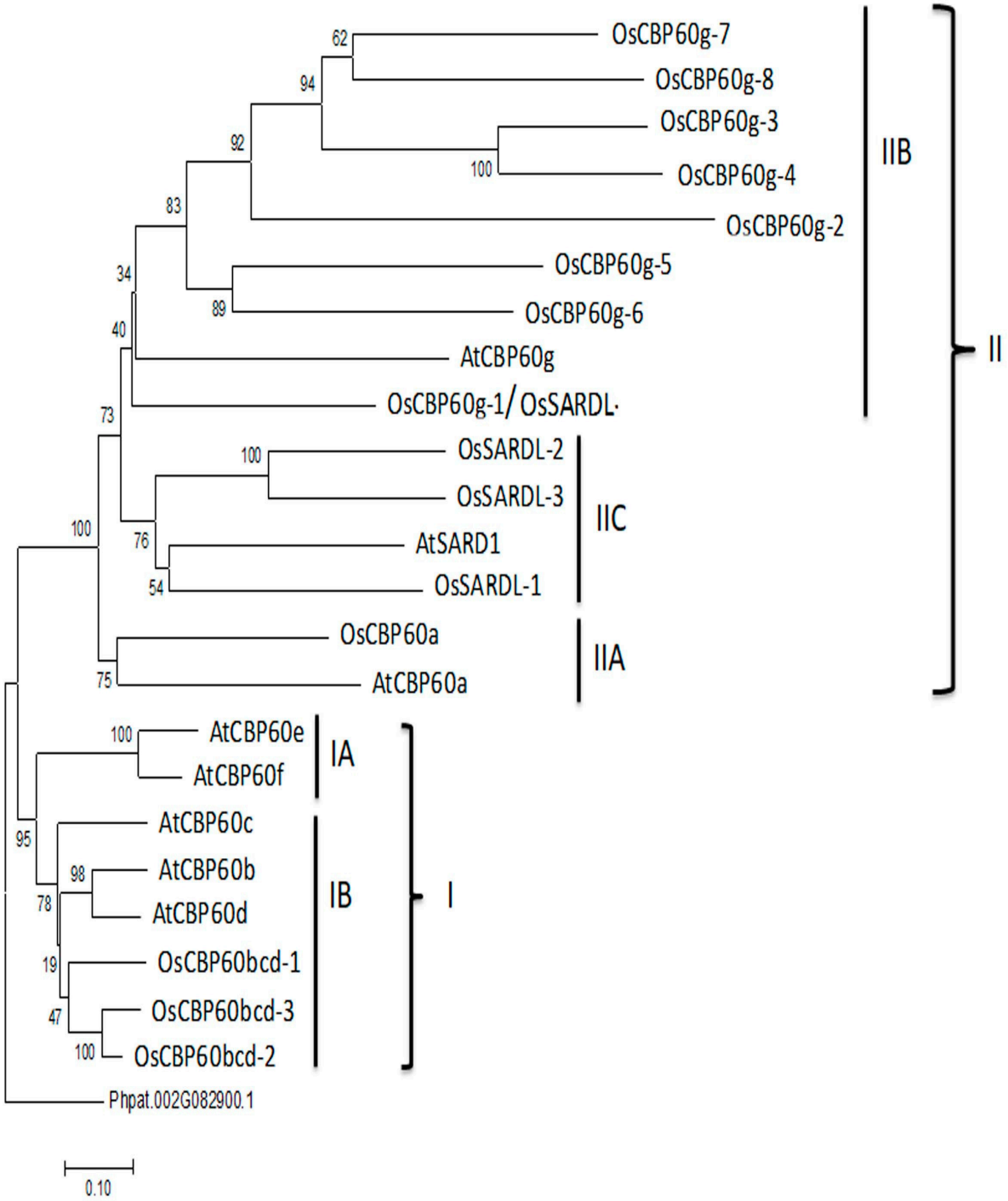


Figure 1. Phylogenetic tree showing relationships of the CBP60 proteins of rice and Arabidopsis. AtCBP60 and OsCBP60 sequences were obtained from TAIR and Phytozome 11, respectively. Phylogenetic tree was generated by the neighbor-joining method using MEGA7 software after alignment of the CBP60 sequences using MUSCLE. The tree was rooted with the moss homologue Phpat.002G082900. Bootstrap values from 500 replicates are shown at each node. The scale bar indicates 0.1 amino acid substitutions per site. Clade I and II and their sub-clades are indicated on the right.

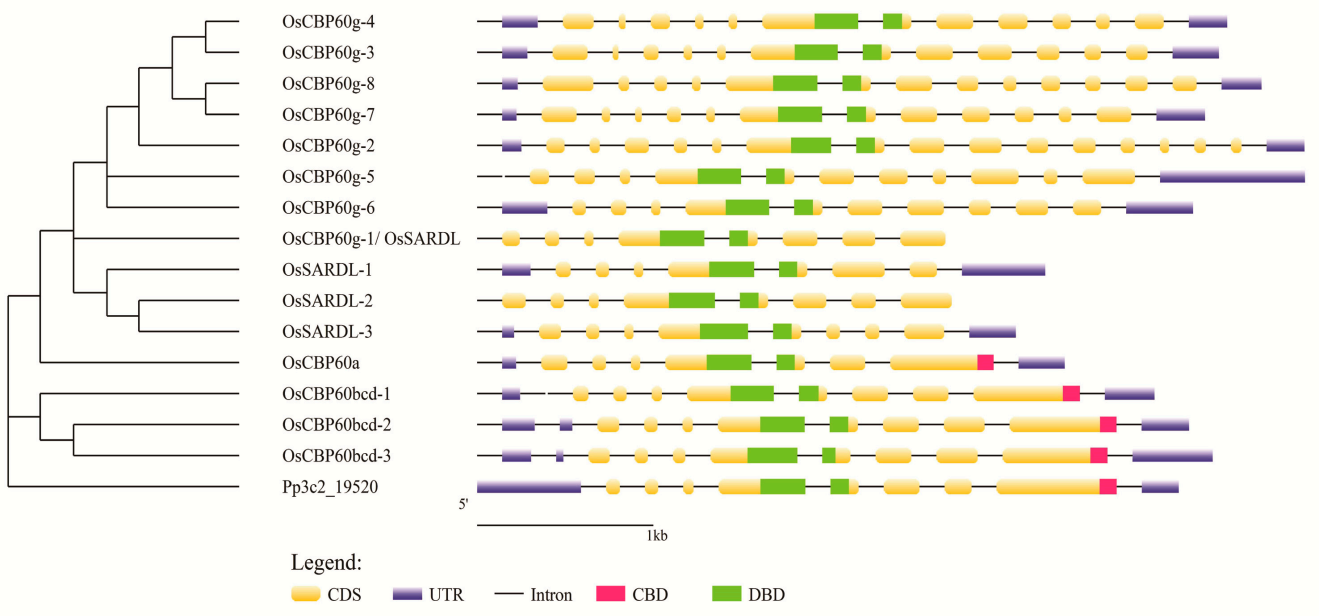


Figure 2. Gene structure analysis comparing the exon-intron structure, and the position of predicted Calmodulin-binding domain (CBD) and DNA-binding domain (DBD) encoding regions of rice OsCBP60 proteins, with that of a root sequence from Physcomitrella patens. The figure was created using the Gene Structure Display Server (GSDS 2.0).

3.2. Predicted CaM- and DNA-Binding Regions in OsCBP60 Proteins

CBP60 proteins, in general, function as CaM-regulated transcription factors and most members of the AtCBP60 family contain a CaM-binding domain (CBD) [28,45]. We first examined amino acid similarities between OsCBP60 and AtCBP60 proteins to tentatively identify CBD and DNA-binding domains (DBDs) in OsCBP60 proteins. Since the prediction of CBD is quite difficult as it does not have a signature sequence, the CBD in OsCBP60s was also predicted using two tools, Calmodulin target database and CaMELS (Table 1 and Supplementary Table S2).

Based on the high interaction site prediction scores through CaMELS and amino acid similarities with AtCBP60s, a CBD was identified in the C-termini of each OsCBP60bcd-1, 2 and 3 (Supplementary Table S2). The predicted CBDs in OsCBP60bcd-1, 2 and 3 showed a high level of conservation with each other as well as with AtCBP60b, c, d, e and f (Figure 3). Based on the search in Calmodulin target database, the CBD in OsCBP60a was predicted to be localised at the C-terminus of the protein, similar to the position identified in AtCBP60a (Table 1). Since the predicted CBD in OsCBP60a showed moderate amino acid similarity with the CBD in AtCBP60a (Figure 4a), the characteristic basic amphiphilic alpha helix found in CBDs [46], was identified by the Heliquest algorithm (Figure 4b). The positively charged amino acids in both proteins lie on one face of the helix while the hydrophobic residues lie on the other face, indicating that this region could serve as CBD in both proteins. Similar to AtSARD1 [26,28], a CBD was not predicted in the OsSARDL proteins (Table 1). Unlike the usual C-terminal localisation of CBD in most AtCBP60s, the CBD in AtCBP60g was identified in the N-terminus [26,31]. It is to be noted that the N-terminal localised CBD in AtCBP60 was identified through experimentation and not on the basis of sequence analysis. A CBD could not be identified in the OsCBP60g proteins. The rice proteins within this clade have highly variable N-terminal sequences with little or no homology to each other or to AtCBP60g (data not shown). For the same reason, Val-29 that is required for the binding of AtCBP60g to CaM [47], could not be identified in OsCBP60g proteins. Based on this information it is tentatively concluded that the OsCBP60g proteins lack a CBD.

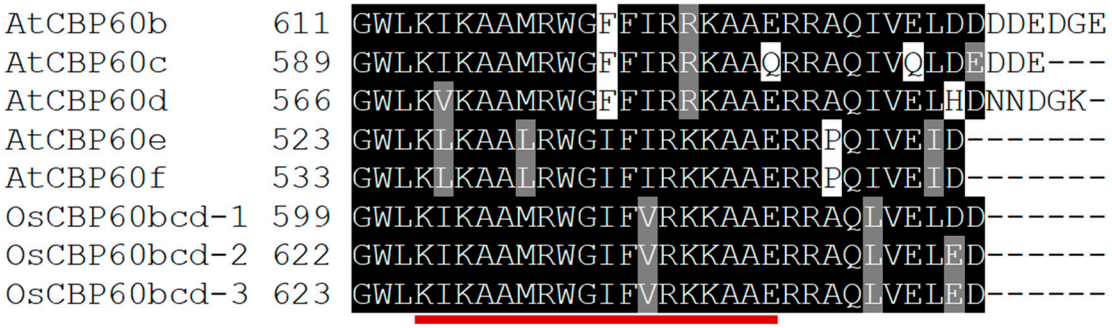
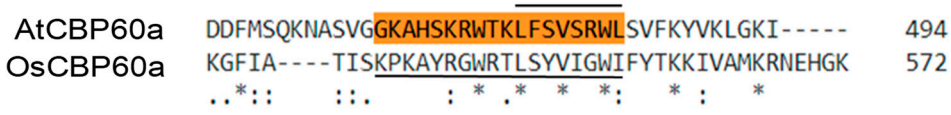
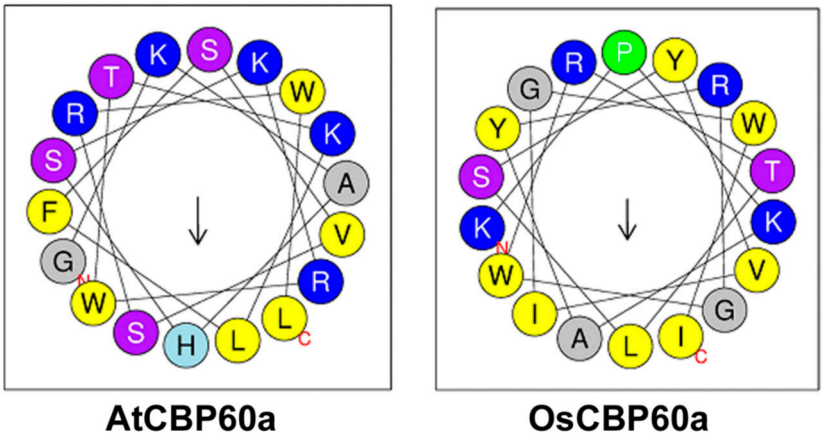


Figure 3. Multiple sequence alignment of the predicted CBDs of AtCBP60b, c, d, e, and f and their putative rice orthologs OsCBP60bcd -1, 2, and 3. The alignment was carried out in CLUSTAL Omega and displayed using Boxshade. The underlined sequence indicates the CBD in Arabidopsis proteins. Black shading indicates residues conserved in over 50% of the sequences; grey shading indicates semi-conservative substitutions while no shading indicates the lack of any similarity.



(**a**)



(**b**)

Figure 4. Identification of a CBD in OsCBP60a. (**a**) Multiple sequence alignment (Clustal Omega) of the C-terminus localised CBD of AtCBP60a with the loosely conserved region in the C-termius of OsCBP60a protein. Identical amino acids are indicated by a star, strongly conserved groups by double dots, and weakly conserved groups by dots. The highlighted region in the CBD of AtCBP60a is predicted to form the amphipathic helix shown in (**b**). The overlined residues in AtCBP60a contribute to CBD as determined by mutagenesis [25], while the underlined region in OsCBP60a indicates the predicted CBD. (**b**) Prediction of amphipathic helix in AtCBP60a and OsCBP60a by the Heliquest algorithm. Both proteins show the presence of an amphiphilic alpha helix with majority of positively charged residues lying on one face of the helix.

The DBD in CBP60s is present within the highly conserved region in these proteins (Figure 5) and, to date, has been experimentally verified only in AtCBP60g and AtSARD1 [47]. A comparison of the previously identified DBD in AtCBP60g and AtSARD1 with the rice proteins indicated that while this region is most conserved amongst all OsCBP60, OsSARDL-1, 2, and 3 and OsCBP60g proteins exhibit high level of conservation with their Arabidopsis counterparts (Figure 5). Of the latter group, the DBDs in OsCBP60g-

1/OsSARDL, OsCBP60g-5 and OsCBP60g-6 showed high degree of sequence similarity to the DBDs of AtSARD1 and AtCBP60g. Localisation predictions by BaCelLo indicated nuclear localisation for all OsCBP60 proteins with the exception of OsCBP60g-4 (Table 1), although an alternative prediction program CELLO (http://cello.life.nctu.edu.tw/) (accessed on 30 October 2021) located OsCBP60g-4 also in the nucleus. These results suggest that OsCBP60 function as transcription factors like their Arabidopsis orthologs.

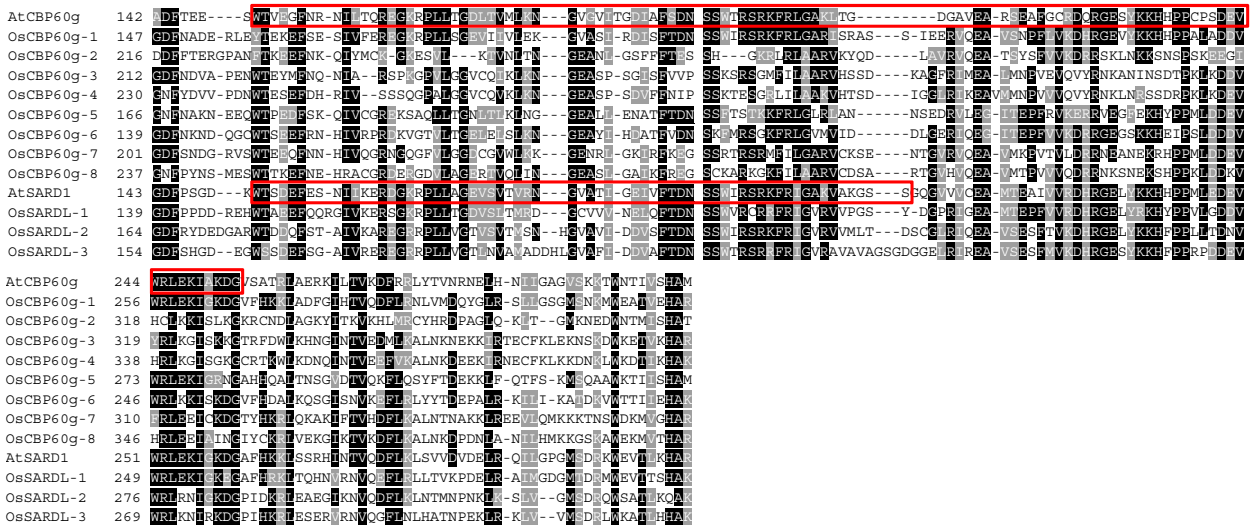


Figure 5. Multiple sequence alignment of the DBDs of AtCBP60g and AtSARD1 proteins with similar regions of OsCBP60g and OsSARDL proteins. The boxed residues indicate the DBD in AtCBP60g and AtSARD1 [47]. The alignment was carried out in CLUSTAL Omega accessed and displayed using Boxshade. Black shading indicates residues conserved in over 50% of the sequences; grey shading indicates semi-conservative substitutions; and the unshaded residues show no similarity.

3.3. OsCBP60 Gene Expression Changes in Response to Pathogen Infection

Since the role of *AtCBP60g* and *AtSARD1* in immunity is well documented [26,27,31,47], we studied the expression of *OsCBP60* genes in rice seedlings infected with *M. oryzae* and *X. oryzae*. *OsPR1a*, a marker gene for defense response in rice, was upregulated at all-time points (12, 24 and 48 h) post infection with *M. oryzae* and *X. oryzae*, serving as a positive control in this analysis (Figures 6 and 7). Genes that were highly upregulated at 12 h post infection with *M. oryzae* included *OsCBP60g-5* (~85-fold induction), followed by *OsCBP60g-8* (~73-fold induction) and *OsCBP60g-6* (~45-fold induction), while those expressed at the highest level at 48 h were *OsCBP60g-7* (~39-fold induction) followed by *OsCBP60g-8* (~32-fold induction) and *OsCBP60a* (~27-fold induction) (Figure 6). For *OsCBP60g-5*, *6*, *8*, the initial high induction at 12 h was reduced by 48 h of *M. oryzae* infection. It is possible that *OsCBP60g-5*, *6*, *8*, similar to *AtCBP60g* [48], respond to the initial Ca^{2+} signal that is generated during plant-pathogen interaction.

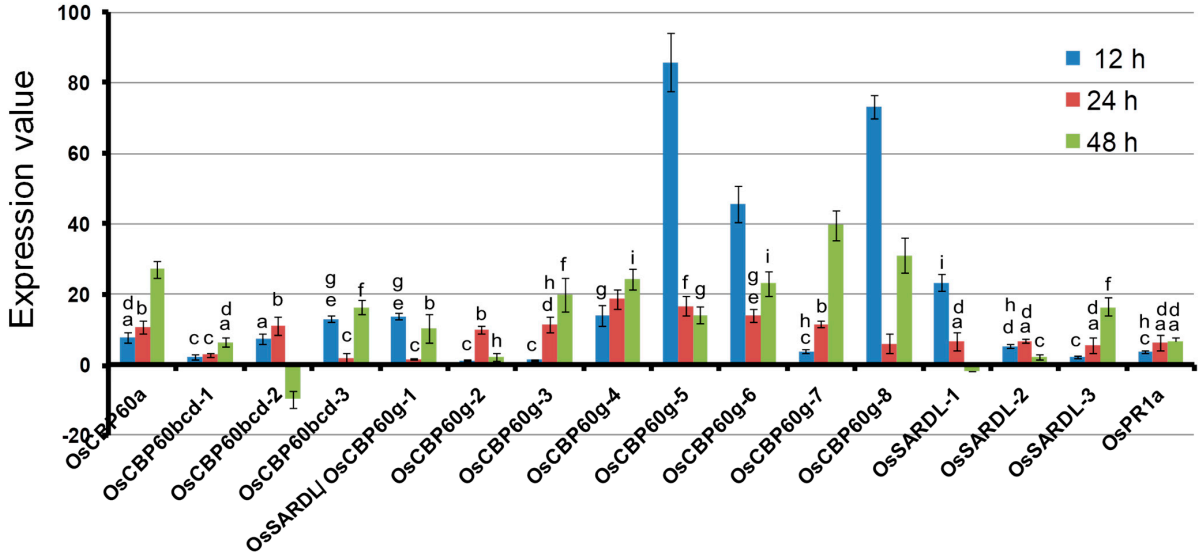


Figure 6. Transcriptional response of *OsCBP60* genes in rice seedlings infected with *M. oryzae*. Rice seedlings were infected with the spores of *M. oryzae* and transcript levels were analyzed by qRT-PCR at 12, 24 and 48 h post infection. Relative transcript abundance (expression value) is expressed as fold-change relative to the mock treatment. *OsPR1a* expression served as a positive control. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

Genes that were highly upregulated at 12 h post infection with *X. oryzae* pv. *oryzae* included *OsCBP60g-3* (~25-fold induction) followed by *OsCBP60g-7* (~19-fold induction), *OsCBP60bcd-2* (~13-fold induction), while those expressed at the highest level at 48 h were *OsCBP60a* (~28-fold induction), followed by *OsCBP60g-7* (~24-fold induction) and *OsSARDL-1* (~19-fold induction) (Figure 7). In case of *OsCBP60a*, *OsSARDL-1*, *OsCBP60g-4* and *OsSARDL-3*, a steady increase in expression was observed from 12–48 h.

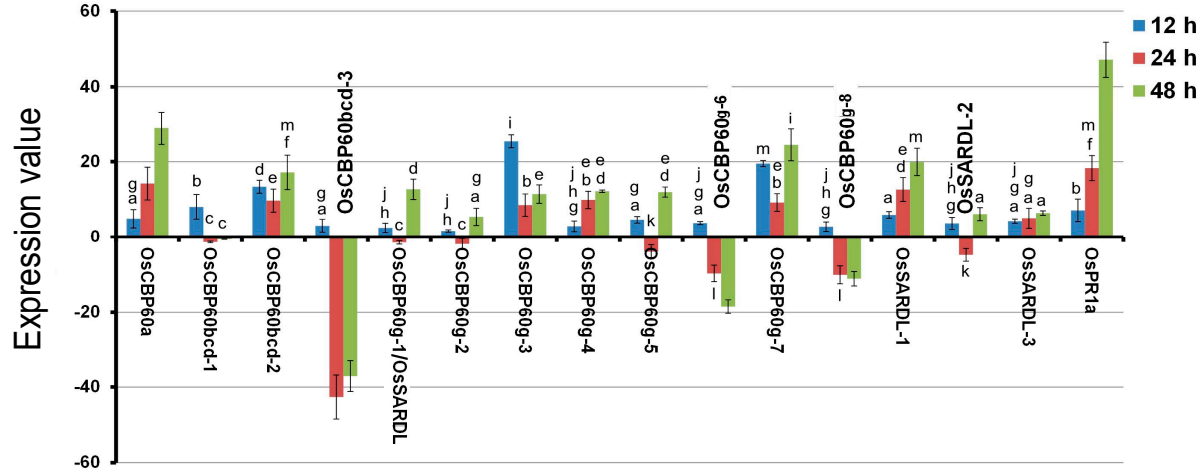


Figure 7. Transcriptional response of *OsCBP60* genes in rice seedlings infected with *X. oryzae* pv. *oryzae*. Rice seedlings were infected with *X. oryzae* and transcript levels were analyzed by qRT-PCR at 12, 24 and 48 h post infection. Relative transcript abundance (expression value) is expressed as fold-change relative to the mock treatment. *OsPR1a* expression served as a positive control. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

OsCBP60g-3, *4*, *7*, *OsCBP60a* and *OsSARDL-3*, all belonging to immunity cluster, were upregulated at all the time points studied for both pathogens. *OsCBP60g-6*, *8* and *OsCBP60bcd-3* had opposing expression patterns in response to the bacterial and the fungal pathogens. The reason for these observations may lie in the fact that rice interactions with the two pathogens have both distinct as well as shared defense responses [49,50].

3.4. OsCBP60 Gene Expression Changes in Response to Phytohormones

Typically, SA and JA are considered to play key roles in plant defense responses [6,38,51]. The involvement of SA and JA in rice defense responses against *M. oryzae* and *X. oryzae* was strongly endorsed through extensive global gene expression analyses [50]. A growing body of evidence supports a role of BR in plant disease resistance [6,10]. We studied the expression of *OsCBP60* genes, along with the expression of the defense marker gene *OsPR1a*, in rice seedlings treated with SA, JA and 24-epibrassinolide (EBR), a BR.

With the exception of *OsCBP60g-5*, all other *OsCBP60s* were upregulated to different levels in response to SA treatment (Figure 8). *OsCBP60g-4* was maximally induced by SA (28 to 41-fold) at all-time points studied, followed by *OsCBP60a* (Figure 8). The induced expression of several *OsCBP60s* in response to *M. oryzae*, *X. oryzae* and SA treatment supports a role for these proteins in disease resistance in rice.

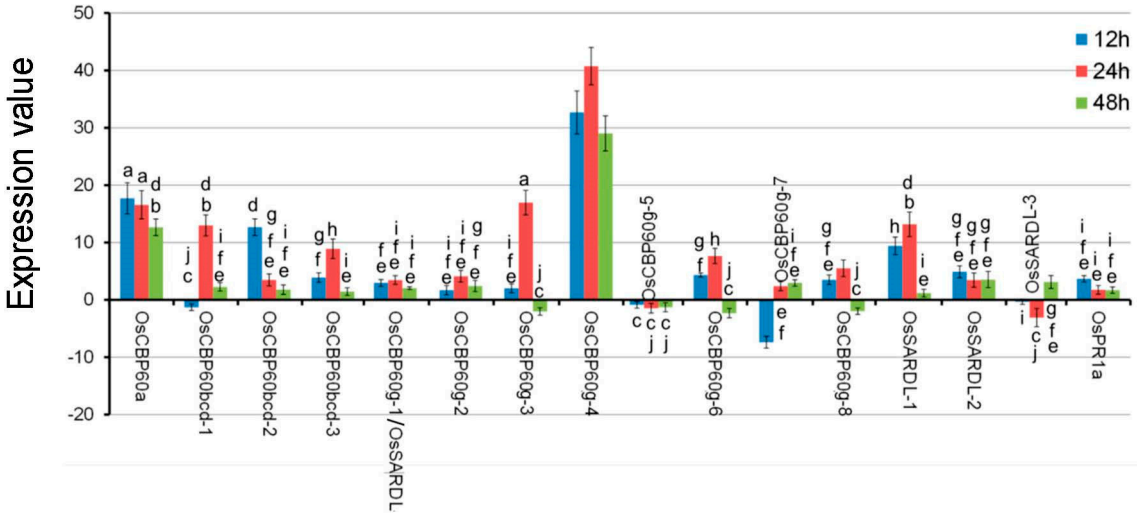


Figure 8. Transcriptional response of *OsCBP60* genes in rice seedlings treated with salicylic acid (SA). Rice seedlings were treated with SA (3 mM sodium salicylate) and transcript levels were analyzed by qRT-PCR at 12, 24 and 48 h post treatment. Relative transcript abundance (expression value) is expressed as fold-change relative to the mock treatment. *OsPR1a* expression served as a positive control. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

JA induced both up and down -regulation of *OsCBP60* genes with *OsCBP60g-6* and *OsSARDL-2* showing maximum induction at ~ 23- and 20- fold, respectively, at 24 h post treatment. By contrast, *OsCBP60g-5* and *OsCBP60g-4* were downregulated by ~ 18 and 12-fold, respectively (Figure 9). The down-regulation by JA of *OsCBP60g-4* and other OsCBP60 genes that were upregulated by SA is reminiscent of the antagonistic interactions between SA and JA signaling pathways.

In EBR-treated rice samples, all 15 *OsCBP60* genes were upregulated to different levels (Figure 10). Maximum upregulation was observed for *OsCBP60g-3* (~15-fold) followed by *OsCBP60g-4* (~9-fold) and *OsCBP60g-7* (~9-fold). These genes also showed prominent upregulation by pathogens (Figures 6 and 7), and the former two by SA (Figure 8). Of the SARD-like genes, *OsSARDL-1* was upregulated by pathogens, SA and EBR. Interestingly,

four *OsCBP60s*, namely *OsCBP60bcd-1*, *3* and *OsCBP60g-6*, *7*, were upregulated in both JA and EBR -treated rice seedlings (Figures 9 and 10).

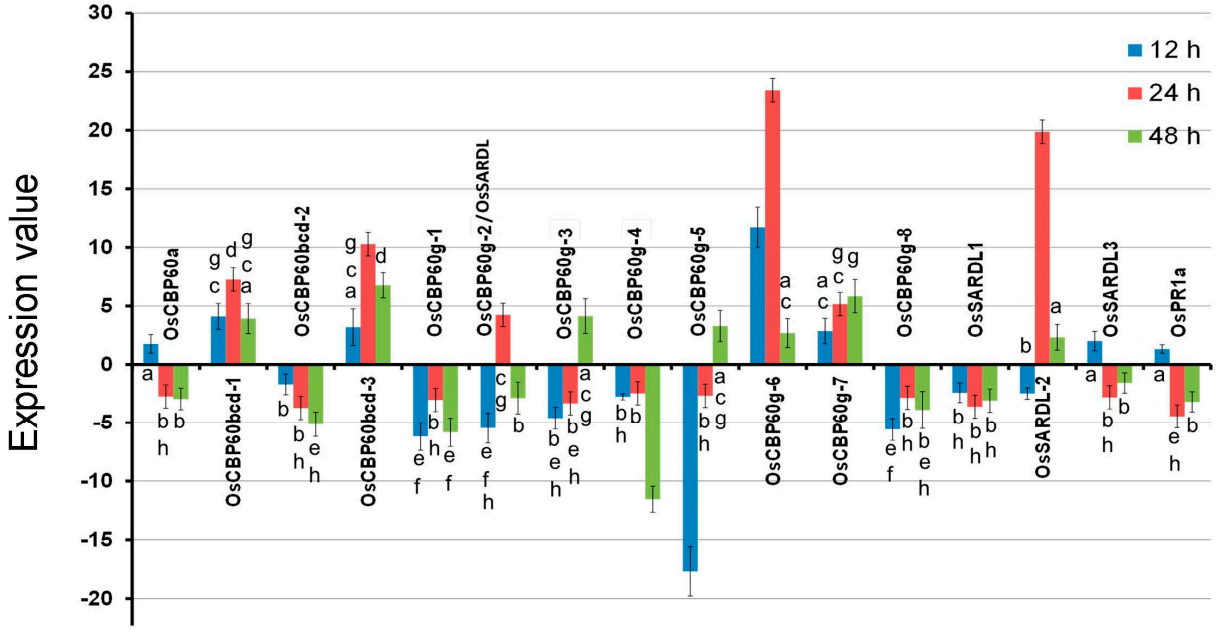


Figure 9. Transcriptional response of *OsCBP60* genes in rice seedlings treated with jasmonic acid (JA). Rice seedlings were treated with 100 µM JA and transcript levels were analyzed by qRT-PCR at 12, 24 and 48 h post treatment. Relative transcript abundance (expression value) is expressed as fold-change relative to the mock treatment. *OsPR1a* was used as a control. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

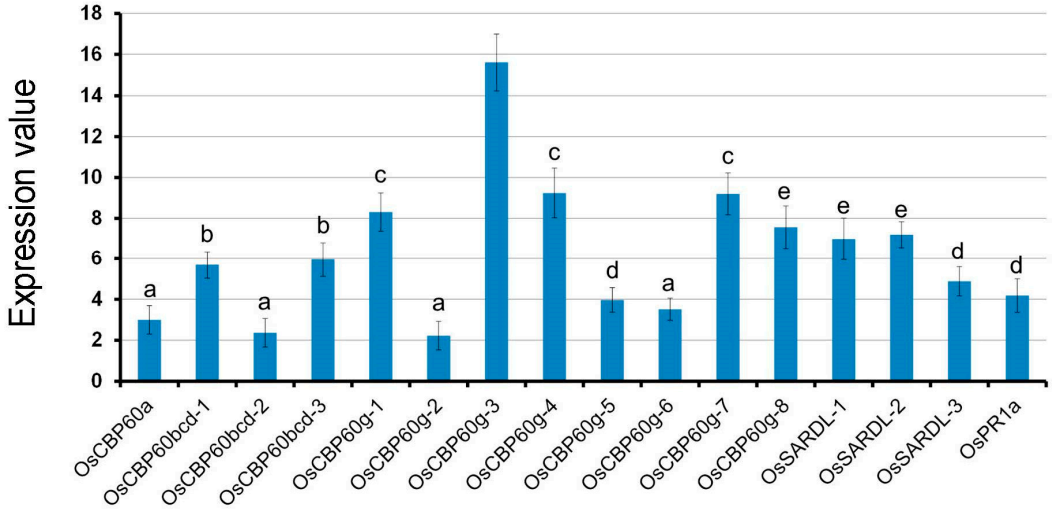


Figure 10. Transcriptional response of *OsCBP60* genes in rice seedlings treated with brassinosteroid. Rice seedlings were grown for 15 days on MS medium supplemented with 1 µM EBR. Relative transcript abundance (expression value) is expressed as fold-change relative to the mock treatment. Since EBR enhanced *AtPR1* expression [52], *OsPR1a* expression was studied as a probable positive control. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

3.5. Analysis of Transcription Factor Binding Sites in Putative Promoter Regions of CBP60 Genes

In recent years, transcription factors (TFs) that regulate the expression of *AtCBP60g* and *AtSADR1* have been identified. These include bZIP transcription factor family proteins TGACG-BINDING FACTOR 1 (TGA1) and TGA4 as positive regulators of both genes [53], and WRKY70 and CAMTA3 as negative regulators of AtSARD1 [54,55]. We identified TGACG motif as binding site for TGA1/TGA4, W-box [(T)TGAC(C/T)] for WRKY70, and the CAMTA [(A/C/G)CGCG(C/G/T)] motif in putative promoters regions (1000 bp and 2000 bp upstream of transcription start site) of Arabidopsis and rice *CBP60* genes using PlantPAN 3.0 platform. In the 2000 bp upstream regions of Arabidopsis genes, 14 CAMTA-binding sites were identified in *AtSARD1*, followed by six sites in each *AtCBP60c* and *AtCBP60e* and three sites in *AtCBP60g* (Supplementary Table S3). In rice genes, maximum number of CAMTA-binding sites were identified in the putative promoter region of *OsCBP60g-3* (12 sites) followed by *OsSARDL-1* (nine sites), *OsCBP60g*-1/*OsSARDL* (six sites), *OsCBP60g*-4 (six sites), *OsCBP60bcd-3* (five sites), *OsCBP60g-6* (five sites), and *OsCBP60g-7*, *OsCBP60g-8* and *OsCBP60bcd-2* (four sites) (Supplementary Table S3).

Maximum number of TGACG motif (42) was identified in *AtSARD1*, followed by 13 sites in *AtCBP60g*, nine sites in *AtCBP60f*, six sites in *AtCBP60a* and two sites in *AtCBP60d* in the 2000 bp upstream regions of Arabidopsis genes (Supplementary Table S3). In rice genes, maximum number of TGACG motif were identified in the putative promoter region of *OsCBP60g*-3 (13 sites) followed by *OsCBP60g*-1/*OsSARDL* (eight sites), *OsCBP60a* (seven sites), *OsCBP60g*-5 (seven sites) and *OsCBP60g*-6 (five sites). The maximum number of W-box site was observed in *AtCBP60e* followed by *AtCBP60g*, *AtCBP60f*, *AtCBP60c*, *AtCBP60d*, *AtSARD1*, *AtCBP60a* and *AtCBP60b*. In rice genes, maximum enrichment of W-box was observed in *OsCBP60g-3* (35), followed by *OsCBP60g*-4 (26), *OsCBP60g*-2 (23), *OsCBP60g*-6 (23), *OsSARDL-1* (20), *OsCBP60g*-5 (13) and *OsSARDL-3* (10). While the number of TF binding sites identified here is an overrepresentation, the identification of these sites mainly in *OsCBP60g* and *OsSARD-Like* genes indicates that the TFs involved in the regulation of these genes in rice may be similar to those identified in Arabidopsis.

3.6. Analysis of BZR1/BES1-Binding Sites in Putative Promoter Regions of OsCBP60 Genes

BR plays important roles in disease resistance [56] and works in part via Ca^{2+}/CaM signalling to mount a BR response [57–59]. This together with the observation that EBR could induce *OsCBP60* expression (Figure 10) led us to search for binding sites of BZR1 and BES1 in the putative promoter regions of *OsCBP60* genes. BZR1 and BES1 are the main TFs of the BR signalling pathway that are involved in mounting a BR response [34,35]. The search for BES1-binding site (CANNTG) and BZR1-binding site (*CGTG*(*T/C*)*G* identified maximum enrichment of BES1 sites in *OsSARDL-1* (14 sites) followed by *OsCBP60a* (13 sites), *OsCBP60g-4* (12 sites) and *OsCBP60bcd-1*, *2*, *3* (11 sites), and relatively lesser number of BZR1 sites in *OsCBP60g-2* (2 sites) and a single site in *OsSARDL-1*, *2*, *OsCBP60g-1/OsSARDL*, *3*, *4*, *8* and *OsCBP60bcd-3*. BES1-binding sites were also observed in promoter regions of *AtCBP60e* (12 sites), *AtCBP60f* (10 sites), *AtCBP60d* (eight sites), *AtCBP60g* (five) and *AtSARD1* (five) (Supplementary Table S3). BZR1-binding sites were identified only in *AtCBP60a* (3 sites).

3.7. Promoter Mining of OsCBP60g-4

OsCBP60g-4 was upregulated in response to infection with both *M. oryzae* and *X. oryzae* (Figures 6 and 7) and was strongly induced by SA (Figure 8) and to a lesser extent by EBR (Figure 10), suggesting a probable role in disease resistance. We further tested the expression of *OsCBP60g-4* in *M. oryzae* and *X. oryzae* resistant and susceptible rice genotypes. Analysis of *OsCBP60g-4* expression in leaf tissue of Rajendra Kasturi (an indica rice cultivar susceptible to rice blast), and IC-346004 (resistant to rice blast, containing R-genes *Piz-5*, *Pi-9*, *Pitp(t)*, *Pi-1*, *Pi-33*, *Pi-b*, *Pi27(t)*, *Pi-ta*) [60] seedlings grown under controlled conditions, identified ~3-fold higher expression of the gene in IC-346004 as compared to Rajendra Kasturi (Figure 11a). Additionally, *OsCBP60g-4* was found to be maximally upregulated

in the resistant genotype IRBB55, followed by the moderately resistant Tetap, at 6 h, 12 h and 48 h post infection with *X. oryzae* (Figure 11b). By contrast, in the susceptible genotype [Pusa Basmati-1 (PB-1)], the expression of *OsCBP60g-4* was downregulated at 12 h and 24 h post infection.

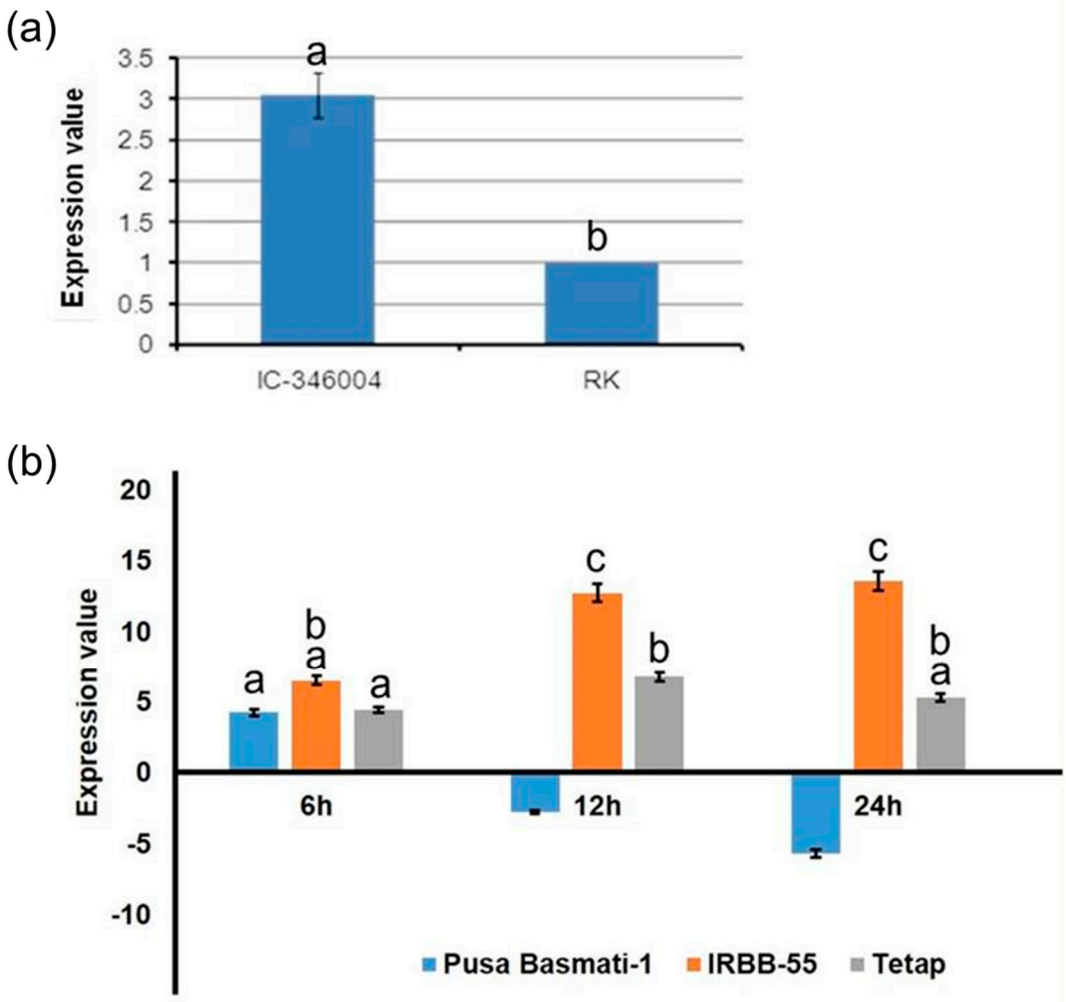


Figure 11. (**a**) Transcriptional response of *OsCBP60g-4* in uninfected leaves of IC-346004 and Rajendra Kasturi rice seedlings grown under controlled conditions. Relative transcript abundance (Expression value) of *OsCBP60g-4* is expressed as fold-change in IC-346004 relative to Rajendra Kasturi. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Different lower-case letters indicate statistically significant differences ($p < 0.05$) by one-way ANOVA-protected LSD test. (**b**) Transcriptional response of *OsCBP60g-4* in leaves of rice genotypes Pusa Basmati-1, IRBB-55 and Tetap infected with *X. oryzae* pv. *oryzae* at 6, 12 and 24 h post infection. Relative transcript abundance (Expression value) is expressed as fold-change relative to the mock treatment. Results are representative of three independent experiments. Error bars represent standard error (SE) of mean for three replicates. Superscripts with the same letter are not significantly different ($p < 0.05$) by one-way ANOVA-protected LSD test.

To putatively identify regulatory single nucleotide polymorphisms (SNPs) affecting gene expression due to change/modification in TF binding sites (TFBSs), we mined the promoter sequences of *OsCBP60g-4* from a susceptible and a resistant to *M. oryzae* rice genotype. The 1000 bp upstream regions of *OsCBP60g-4* from Rajendra Kasturi, and from rice germplasm accession IC-346004, were sequenced. The sequences were compared with the reference sequence of Nipponbare (AP014967.1 obtained from NCBI). Multiple sequence alignments identified 12 SNPs of which seven SNPs were associated with known

TF binding sites (Figure 12). Two SNPs which change the binding site of EIN3, a TF involved in ethylene signalling [61], were identified in the sequence of the susceptible cultivar Rajendra Kasturi. Other transition and transversion mutations alter B3 and NF-YB -binding sites, leading to the formation of potential TALE homeodomain and B3 binding sites, respectively, in the Rajendra Kasturi sequence (Figure 12). Comparison between the reference and resistant genotype IC-346004 sequences showed loss of two TCP-binding sites with formation of potential homeodomain and other TF -binding sites in the IC-346004 sequence, and potential conversion of an EIN3 site to B3-binding site (Figure 12). While these results do not provide evidence of a link between the higher expression of OsCBP60g-4 in the resistant IC-346004 vs. susceptible Rajendra Kasturi (Figure 11a) and the SNPs identified (Figure 12), they providde impetus for detailed functional analysis of OsCBP60 genes and their promoters to understand the significance of this gene family in biotic and abiotic stress tolerance.

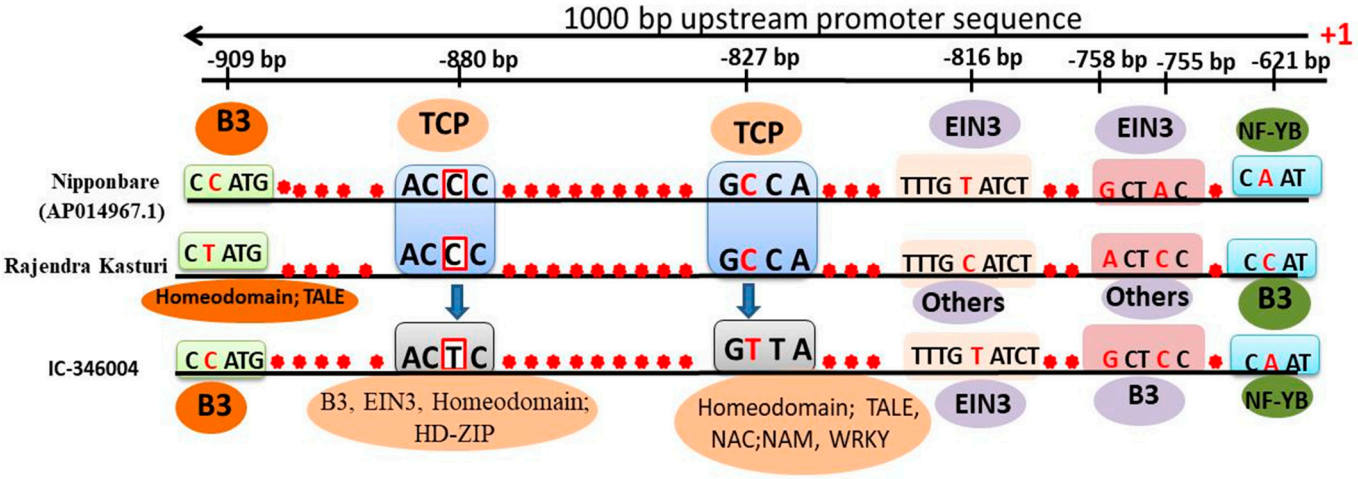


Figure 12. In silico analysis of the putative promoter regions of *OsCBP60g-4* in *M. oryzae* resistant and susceptible rice genotypes. A 1000 bp upstream region of *OsCBP60g-4* gene from each genotype, Rajendra Kasturi (indica rice genotype susceptible to *M. oryzae*) and IC-346004 (indica rice accession resistant to *M. oryzae*), was amplified and sequenced. Multiple sequence alignment of reference sequences (Nipponbare; AP014967.1) available in NCBI was performed to identify SNPs. The SNPs associated with TF binding sites are highlighted in different colours and shown in circles. The red colour stars (*) indicate conserved residues in both sequences.

4. Discussion

In plant immune systems, the immunogenic elicitors (PAMPs, MAMPs) arising from pathogens are recognised by receptor proteins localised at the plasma membrane, which then trigger cytosolic signalling events [62]. The increases in cytosolic Ca^{2+} within minutes of elicitor recognition is an early event in the signalling cascade that leads to the immune response [17,63,64]. In Arabidopsis leaves a significant increase in Ca^{2+} was seen to occur within two hours of infection with an avirulent bacteria [65]. The spatiotemporal patterns of Ca^{2+} changes at the cellular and tissue levels form the Ca^{2+} signatures, which are decoded by CBPs. CaM is the most researched Ca^{2+} sensor which can bind to TFs and regulate their activities, and it has been linked to SA-mediated response to pathogens [66]. One of the links between Ca^{2+}/CaM and SA has been through CaM transcription activators (CAMTAs), which negatively regulate SA biosynthesis [55]. SA has been known since long to play a critical role in plant immunity [67]. SA levels increase both locally and systemically in response to pathogen infection, which sets in train the SA signalling-induced gene expression and immunity.

Another gene family, *CBP60*, linked with Ca^{2+}/CaM and SA was identified in Arabidopsis. Members of this family, AtSARD1 and AtCBP60g, function as TFs and directly regulate the expression of *isochorismate synthase 1* (*ICS1*), which encodes a crucial enzyme in

SA biosynthesis [47]. The expression of *AtSARD1* and *AtCBP60g* is repressed by CAMTAs, specifically by CAMTA3, leading to inhibition of SA synthesis [55]. Recent reports have also linked additional members of the AtCBP60 family, AtCBP60a and AtCBP60b with immune responses in Arabidopsis [25,29], highlighting the importance of the Ca^{2+}/CaM-regulated CBP60 family in plant immunity.

PAMP induced Ca^{2+} influx is critical for disease resistance in rice [68], and a large number of Ca^{2+} sensors, including CaM and CaM-like (CML) proteins have been identified in the rice genome [69,70]. However, there is little information on CBPs in rice. A CAMTA gene, *OsCBT*, was isolated from a rice cDNA library constructed from fungal elicitor-treated rice suspension cells [71]. *OsCBT* was shown to be a negative regulator of defense-related gene expression [72]. A genome wide analysis of the *CAMTA* gene family in rice identified seven *CAMTA* genes, and, in silico analysis of their expression indicates potential functions in abiotic and biotic stress responses [73]. Very recently, Wang et al. [74] reported a CBP60 gene family of 19 members in the rice cultivar Zhonghua 11 (ZH11). In this study upregulation in response to chitin and pathogen infection of only a subset of genes was seen in rice seedlings grown from $CaCl_2$-pretreated seeds.

In view of the importance of *AtCBP60* genes in plant immunity [25,27,29,75], we carried out a search of *CBP60* genes in rice. The 15 OsCBP60s candidates in rice showed an expanded and diversified immunity clade II but a reduced clade I comprising only three gene members (*OsCBP60bcd1-3*) as opposed to five members (*AtCBP60b-f*) in Arabidopsis. The immunity clade in rice maintained the previously identified three subfamilies—*CBP60a*, *SARD1* and *CBP60g* [31]. Similar to AtSARD1 [26], OsSARD-like proteins lack a CBD, and similar to AtCBP60a, which is demonstrated to bind CaM [25], OsCBP60a appears to contain a CBD at its C-terminus (Figure 4). Unlike AtCBP60g, which contains a CBD at its N-termius [28], a CBD could not be identified in the highly expanded OsCBP60g subfamily. This raises the question of whether the OsCBP60g subfamily is evolving for functions unrelated to immunity or whether the immunity-related functions of this subfamily are independent of regulation by Ca^{2+}/CaM. Although experimental verification is required to address the CaM binding ability of OsCBP60g proteins, the high level induction of *OsCBP60g-3*, *4*, *5*, *7* in response to pathogen infection (Figures 6 and 7) strongly suggests that these proteins are involved in rice defense responses against pathogens. An extensive phylogenetic analysis of CBP60 members in 247 diverse land plant species led Zheng et al. [31] to suggest that either CaM-binding is not essential for CBP60g proteins for their immunity-related functions or that a CaM-binding adapter protein works with these proteins to regulate their immune-related activities. The identification of three OsSARD-like and eight OsCBP60g proteins in rice (Figure 1) is also in agreement with the observations made in the study of diverse angiosperm species, which concluded that the immune regulator subfamilies are evolving rapidly, likely due to pressure from fast evolving pathogens [31].

In Arabidopsis, AtCBP60g and AtSARD1 were experimentally shown to be located in the nucleus and to bind to gene promoters [47], and AtCBP60b to be recruited to the *AtSARD1* promoter region [75]. Two sets of analyses made with OsCBP60 proteins in this study suggest that the rice proteins also function as TFs; (1) multiple sequence alignment indicated the presence and conservation to varying degrees of a DBD in OsCBP60g and OsSARD-like proteins, and (2) in silico localization analysis indicated that all *OsCBP60s* are localized to the nucleus.

In signalling events that mediate rice innate immunity, the host sensors transduce the signal through mitogen-activated protein kinase (MAPK) cascades and TFs to activate the expression of numerous genes, including *pathogenesis-related* (*PR*) genes [76–78], which are a hallmark component of innate immunity system in plants [79,80]. *OsPR1a* was strongly induced by *X. oryzae* with >45-fold induction at 48 h post pathogen infection (Figure 7), but to a much lower level by the fungal pathogen *M. oryzae* (Figure 6). This difference in the expression levels of *OsPR1a* in response to the two pathogens may lie in distinct temporal regulation of the gene by different signals. Previously, a study of *OsPR1a* expression at

36 h post infection, led the authors to conclude that *OsPR1a* is a rapid response gene to blast fungus infection [77]. The same study also reported *OsPR1a* expression to be induced by SA but suppressed by JA. We found *OsPR1a* expression to follow the same trend in response to SA and JA (Figures 8 and 9), which authenticates the expression patterns we obtained for *OsCBP60* genes.

The consistent upregulation of gene members belonging to the immunity subfamilies, albeit to different levels, in response to pathogens and SA strongly suggests that the *OsCBP60* family is involved in rice defense responses. For example, *OsCBP60a*, *CBP60g-3*, *-4*, and *OsSARDL-1* were induced by all three signals—fungal and bacterial pathogens and SA. Based on SA levels and gene expression in Arabidopsis mutants *cpb60g* and *sard1*, it was hypothesised that *AtCBP60g* plays a more significant role in the early defense response of the plant, while *AtSARD1* functions at a later time [26]. The strong induction of *OsCBP60g-5*, *-6* and *-7* in response to *M. oryzae* infection and that of *OsCBP60g -3* and *-7* in response to *X. oryzae* at 12 h, as compared to the relatively lower level expression of *SARD-like* genes, could be taken to support the above stated hypothesis, but experimental verification will be required in the future to fully understand the co-operation between the OsCBP60g and OsSARD-like subfamilies.

SA and JA have both been shown to play roles in rice basal defense against fungal and bacterial pathogens [81]. However, there is little understanding of the role of SA biosynthesis in rice. Relative to Arabidopsis, rice accumulates high basal levels of SA, which do not undergo any significant changes in response to pathogen attack [82]. Nevertheless, a positive correlation exists between endogenous SA levels and the intensity of PAMP-triggered immunity, and mild increases in SA levels in rice confer broad range resistance without yield penalty [83]. Considering that pathogen-induced SA biosynthesis is co-ordinately regulated by AtCBP60g and AtSARD1 in Arabidopsis [27], how significant is the role of members of the expanded OsCBP60g and OsSARD-like subfamilies in SA biosynthesis is a question that will need to be addressed in the future.

The evidence for the growth hormone BR to play critical roles in plant immunity mechanisms continues to grow [10,84,85], including in rice [86]. BR regulates SA and JA biosynthesis and interacts with SA and JA signalling pathways [52,87–89]. The collective contribution and timing of these hormones during plant–pathogen interactions are crucial to determining plant immunity. The *OsCBP60* genes responded to all three phytohormones. The upregulation of *OsCBP60g-4*, *3*, *OsCBP60a* and *OsSARDL-1* in order of fold-increases by SA, and the downregulation of the same and other *OsCBP60* genes by JA is in line with the previously reported antagonistic interaction between SA and JA pathways [90]. The strong induction of *OsCBP60g-6* and *OsSARDL-2*, followed by that of *OsCBP60bcd-1*, *3* by JA suggests that some members of the genes family may have evolved for conferring immunity via JA controlled defense signatures [91].

All *OsCBP60* genes were found to be upregulated by EBR with highest induction in the following order: *OsCBP60g-3*, *4*, *7*, *1*. Since the treatment with EBR was for 15 days, these expression patterns are likely to result from both primary and secondary responses of BR, with the latter likely involving SA and JA signalling. The presence of probable BES1- and BZR1-binding sites in the putative promoter regions of different *OsCBP60* genes suggests that at least some of the genes may be direct targets of the two BR TFs.

In summary, the expanded and diversified CBP60 family in rice appears to be involved in immune system of this plant species through functioning as TFs whose activities may or may not be controlled by Ca^{2+}/CaM. The combinatorial regulation of gene family members may be required to generate diverse expression patterns that may be effective against the several pathogens of rice.

5. Conclusions

A growing body of evidence suggests that the CBP60 family of proteins plays key roles in plant immune responses. The present study identified a 15 member *CBP60* gene family in rice. The immunity-related clade II in rice comprises of 12 genes (*Os-*

CBP60a, *OsCBP60g-1/OsSARDL*, *OsCBP60g-2-8* and *OsSARDL1-3*) as opposed to three genes (*AtCBP60a*, *AtCBP60g* and *AtSARD1*) in Arabidopsis, while the number of clade I genes in rice were reduced to three (*OsCBP60bcd-1-3)* from the five genes identified in Arabidopsis (*AtCBP60b-f*). In contrast to AtCBP60g, which has a CBD at its N-terminus, a CBD could not be identified in the OsCBP60g subfamily proteins. Although CaM-binding ability of OsCBP60g proteins needs experimental verification, it is possible that this group of proteins have evolved to function independently of Ca^{2+}/CaM regulation. The up-regulation of several members of the *OsCBP60g* subfamily, as well as of *OsCBP60a* and *OsSARD-like* in response to bacterial and fungal pathogens and SA and BR strongly suggests that *OsCBP60* genes are involved in immune-related activities in rice. The significant expansion of immunity clade II, specifically of the *OsCBP60g* subfamily, suggests that the *OsCBP60* gene family may be evolving for confering broad spectrum immunity in rice.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123060/s1, Supplementary Table S1: List of primers used for qRT-PCR analysis; Supplementary Table S2: Prediction of CBD in OsCBP60s; Supplementary Table S3: Analysis of transcription factor binding sites (TFBSs) in putative promoter sequences of *OsCBP60s* and *AtCBP60s*. The 1000 bp and 2000 bp upstream sequences of rice and Arabidopsis *CBP60* genes were retrieved from Phytozome and TAIR, respectively. The CAMATA, TGACG and W-box binding sequences were identified using Plant PAN 3 software. The BES1 and BZR1 TFBSs were identified manually.

Author Contributions: B.D.P. and P.K. designed the research. D.K. and B.D.P. carried out research and analysed the data. H.M.N. generated the phylogenetic tree and helped in bioinformatics analysis. S.S. and B.D.P. did statistical analysis. B.D.P., P.K., D.K. and S.S. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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