Global Identification of C2 Domain Contain Proteins And Characterization Of Genetic Variations For Involved Abiotic Stress Tolerance In Rice (*Oryza sativa* L.)

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Research Article

Keywords: Rice, C2 domain contain protein (OsC2DP), Abiotic stress, Haplotype, Phylogenetics, Expression pattern

Posted Date: July 12th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-618284/v1>

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Abstract

Background

C2DPs (C2 domain contain proteins) have been identified in different genomes that contain single or multiple C2 domains in their C or N-terminal, it possesses higher functional activity in the cell membrane between the cytoplasm and nucleus. Despite the identification of MCTPs and NTMC2s in rice, Arabidopsis, and cotton in a previous study, however, the *C2DP* gene family in rice has not been comprehensively studied, and the role of the *C2DP* gene in rice in response to abiotic stress is unclear.

Results

In this study, we identified 82 C2DPs in the rice genome and divided them into seven groups through phylogenetic analysis. Synteny analysis revealed that duplication events were either exhibited within the genome of rice or between the genome of rice and other species. Through the analysis of *cis*-acting elements in promoters, expression profiles, and qRT-PCR results, the functions of *OsC2DPs* were found to be widely expressed in diverse tissues and were extensively involved in phytohormones and abiotic stress in rice. Prediction of the miRNA targets of *OsC2DPs* revealed that some of the homolog genes were regulated by consistent miRNAs and may carry out redundancy function. Notably, *OsC2DP50/51/52* as a co-tandem duplication exhibited similar expression variations and involved the coincident miRNA-regulation pathway. Moreover, the results of SNP genotyping and haplotype analysis revealed that *OsC2DP17*, *OsC2DP29*, and *OsC2DP49* possessed diverse haplotypes for impacted cold tolerance owing to genomic variations.

Conclusions

These findings provide a comprehensive sight for characterized *OsC2DPs* in rice and their roles for abiotic stress. Further, the genetic variation supports the theoretical reference for molecular breeding in rice.

Background

Ca is a necessary factor for plant development as it serves as a signaling factor for transduction functions in multiple physiological processes [1]. Ca maintains the steadiness of the cell wall, membrane, and membrane-binding protein and improves the resistance to diverse abiotic and biotic stresses in plant cells, thereby triggering multiple signaling pathways [2, 3]. Ca-binding protein is a kind of protein for specific binding to the intracellular free Ca²⁺, the signaling transduction function made by binding for each other. To date, 3 major Ca-binding proteins, namely calmodulins [4], Ca-dependent protein kinase [5], calcineurin B-like proteins [6], have been identified as well-known Ca²⁺ sensors.

C2DPs, a type of Ca-dependent protein kinase, are highly conserved long term. The typical C2 domain consists of approximately 135 amino acids and was given the name, PKC-C2 (PFAM: PF00168), in a previous study [7]; the other two branches of the C2 domain super family include the PI3K-C2 (PFAM: PF00792) and PTEN-C2 domains (PF10409). Most PKC-C2 proteins are involved in Ca²⁺-dependent interactions with the membrane and most previous reports discussed PKC-C2 [8]. Generally, C2DPs contain at least one C2 domain, with or without transmembrane domains in the C-terminus of the protein. However, several C2 domains are frequently coupled to other enzymatic domains, such as the phospholipase domain [9, 10], synaptotagmin domain [11], and phosphatidylinositol domain [12], which bind to the membrane or point to other functions. With the development of protein structure, a set of multiple C2 domains and transmembrane region proteins were identified as branches of C2DPs [13], which contained 3–4 C2 domains at the N-terminus and 1–4 transmembrane regions at the C terminus. In membrane transport proteins, multiple C2 domains have been found to have different but conserved sequences, suggesting the formation of more biological functions due to the cooperation or crosstalk between multiple C2 domains [14].

Related studies on C2DP started from a long time before in eukaryon. The first characterized C2 domain was found in mammalian cells, the animal PKC contained 3–4 conserved domains, named C1, C2, C3, and C4, respectively. Interestingly, C1, C3, and C4 exist in all PKCs; only the C2 domain exists in Ca²⁺-dependent PKC, indicating a synergistic relationship between the Ca²⁺ and C2 domains [15]. The function of the C2 domain has also been reported [16, 17]. The C2 domain mediates the transduction mechanism whereby peptides derived from PKC modulate protein-protein interactions within PKC and the PKC binding proteins [18]. The peptides derived from the C2 domain were also identified as two branches, isozyme-selective activator and selective inhibitor of epsilon PKC, which play important roles in the prevention and protection of cardiac and brain ischemic damage, diabetes complications, and transplanted hearts [19].

The functions of C2DPs have also been reported in plants within the last 20 years [20, 21, 22]. In *Arabidopsis thaliana*, gene *SYT1*, which belongs to Synaptotagmins, was identified by the affected development of the cell. Synaptotagmins constitute a family by characterized of N-terminal transmembrane region and two C2 domains at the C-terminal. *SYT1*, which is widely expressed and is located in the plasma membrane, has all the characterized domains of Synaptotagmins. However, when the function of *SYT1* is repressed, the plasma membrane has reduced integrity, which decreases cell viability [11]. The *QKY* gene has four predicted C2 domains and is the first characterized MCTP in *Arabidopsis*. *qky* mutant plants showed defects in the development of the outer integument, floral organ, and stem, and impacted the growth of floral meristem and root hair. *QKY* can bind with the receptor-like kinase, *SUB*, to coordinate the regulation of intercellular communication and organ development [23]. In rice, *OsERG1*, which is the first reported C2DP, expression of *OsERG1* increased following treatment of *Magnaporthe grisea* infection and Ca²⁺ ions, the subcellular localization from the cytoplasm to the cell membrane, and participates in the plant defense signaling pathway [24]. *OsSMCP1*, a Ca²⁺-dependent membrane-targeting domain protein, contains a single C2 domain. Overexpression of *OsSMCP1* enhances plant tolerance to salt, drought, osmotic, and oxidative stress, and causes improved resistance to *Pseudomonas syringae* [25]. *OsC2DP* (LOC_Os09g39770) is also significantly involved in salt tolerance in rice. The *OsC2DP* CRISPR/Cas9 knockout mutant line was found to be more sensitive to salt stress than WT, and salt treatment caused the translocation of *OsC2DP* from the cytoplasm to the cell membrane [26], similar to the shift identified for the protein, *OsERG1* [24]. To date, gene family analysis of the C2 domain has only been reported for few species, in Arabidopsis and cotton, the study revealed

results for MCTPs, a branch of the C2DP family, divided into five subfamilies according to the phylogenetic tree [27, 28]. In rice, a comprehensive analysis of NTMC2s revealed 13 family members divided into six subfamilies according to phylogeny and motif constitution [29]. Therefore, the *C2DP* gene family of rice requires further comprehensive analysis to identify the relationships and functions of the members.

In this study, 82 family members of the C2DP family were identified in rice, named the *Osc2DP* gene family. In addition, seven subgroups were identified based on homologous relationships and conserved domain structures. Our results provide a theoretical basis for the future characterization of the roles of *Osc2DPs* in abiotic stress in rice.

Results

Identification, polygenetic, and sequence analyses of *Osc2DPs*

To identify the *Osc2DP* gene family members in rice, a BLAST search was performed for the rice genome using the HMM model (PF00168) and the C2-domain reconfirmed in the InterPro and SMART databases. As a result, 82 *Osc2DPs* were identified by filtering an E-value $< 1e^{-5}$ (Additional file 1: Table S1). Whole *Osc2DPs* were found to have different basic characteristics (Table 1). Further, the genome DNA length ranged from 784 bp (*Osc2DP67*) to 13170 bp (*Osc2DP62*), with an average of 4484 bp, while the cds length ranged from 432 bp (*Osc2DP20*) to 6354 bp (*Osc2DP80*), with an average of 1875 bp. The physical properties were also revealed to be widespread (Table 1). The pI of *Osc2DPs* ranged from 4.22 (*Osc2DP36*) to 11.23 (*Osc2DP12*), with an average of 7.13; 46 members had pI < 6 while 31 members had pI > 8 . Further, the Mw of *Osc2DPs* ranged from 8.847 kD (*Osc2DP54*) to 227.943 kD (*Osc2DP80*), with an average of 65.62 kD. The chromosome distribution results showed that *Osc2DPs* were present in each chromosome (Additional file 2: Fig. S1), with most chromosomes existing in many members, except chr08, chr10, and chr11. Interestingly, there were few gene clusters in the closed position, such as *Osc2DP7* and *Osc2DP8* in chr01, and *Osc2DP67* and *Osc2DP68* in chr07. Further, a larger cluster was identified in chr06, which included *Osc2DP50* to *Osc2DP57*, implying that unknown tandem or segmental duplications might exist in a few regions. We also analyzed the prediction of subcellular localization of *Osc2DPs* and found that the *Osc2DPs* performed functions in multiple positions, including nuclear, chloroplast, cytoplasmic, membrane, and mitochondrial (Additional file 1: Table S2), and most members were found to gather in the cytoplasm and nucleus (Additional file 2: Fig. S2).

Table 1
Gene list and information for OsC2DP gene family

Gene Symbol	RAP-ID	MSU-ID	Chr.	Start	End	Gene Length	Cds Length	PI	MW
OsC2DP1	Os01g0128800	LOC_Os01g03820	Chr1	1597486	1604610	7125	1956	7.71	66142.35
OsC2DP2	Os01g0172400	LOC_Os01g07760	Chr1	3724314	3729284	4971	2439	5.6	92219.32
OsC2DP3	Os01g0242600	LOC_Os01g14050	Chr1	7862001	7867812	5812	2025	5.82	75308.13
OsC2DP4	Os01g0369500	LOC_Os01g27190	Chr1	15160423	15161640	1218	1020	6.83	31498.49
OsC2DP5	Os01g0587300	LOC_Os01g40480	Chr1	22868546	22872149	3604	3243	7.42	119356.5
OsC2DP6	Os01g0819150	LOC_Os01g60340	Chr1	34902303	34906955	4653	1563	8.49	50328.86
OsC2DP7	Os01g0819300	LOC_Os01g60350	Chr1	34908079	34912602	4524	1548	5.75	58441.86
OsC2DP8	Os01g0841700	LOC_Os01g62430	Chr1	36135811	36137295	1485	570	6.04	17735.82
OsC2DP9	Os01g0853800	LOC_Os01g63470	Chr1	36770986	36772435	1450	984	9.1	35355.71
OsC2DP10	Os01g0934100	LOC_Os01g70790	Chr1	40973255	40974782	1528	1077	8.71	36238.43
OsC2DP11	Os01g0951100	LOC_Os01g72230	Chr1	41882488	41886410	3923	1146	8.52	42706.69
OsC2DP12	Os02g0120200	LOC_Os02g02790	Chr2	1061877	1066213	4337	2733	11.23	99144.34
OsC2DP13	Os02g0198300	LOC_Os02g10480	Chr2	5505016	5508425	3410	963	5.51	35250.87
OsC2DP14	Os02g0199800	LOC_Os02g10630	Chr2	5586939	5593645	6707	3336	8.18	123146.8
OsC2DP15	Os02g0313700	LOC_Os02g20970	Chr2	12398421	12404925	6505	2217	6.07	80965.85
OsC2DP16	Os02g0327000	LOC_Os02g22130	Chr2	13173639	13176606	2968	498	5.95	18547.61
OsC2DP17	Os02g0448400	LOC_Os02g25060	Chr2	14529662	14537548	7887	1617	6.36	61026.85
OsC2DP18	Os02g0521300	LOC_Os02g32160	Chr2	18994014	19001080	7067	1755	5.26	63830.39
OsC2DP19	Os02g0605400	LOC_Os02g39280	Chr2	23720437	23726180	5744	1578	9.15	50703.85
OsC2DP20	Os02g0640000	LOC_Os02g42710	Chr2	25684044	25687149	3106	432	4.56	15775.67
OsC2DP21	Os02g0663900	LOC_Os02g44490	Chr2	26924717	26927173	2457	2340	9.12	88984.9
OsC2DP22	Os02g0665100	LOC_Os02g44560	Chr2	27009020	27010017	998	666	9.15	25491.47
OsC2DP23	Os02g0722450	LOC_Os02g49070	Chr2	29997568	30002813	5246	999	5.58	36843.03
OsC2DP24	Os02g0815100	LOC_Os02g57000	Chr2	34920542	34921736	1195	858	9.82	30640.46
OsC2DP25	Os02g0816000	LOC_Os02g57090	Chr2	34958012	34961608	3597	3000	9.42	110780.7
OsC2DP26	Os02g0829200	LOC_Os02g58230	Chr2	35643004	35648165	5162	1977	6.54	72271.9
OsC2DP27	Os03g0119100	LOC_Os03g02740	Chr3	1027577	1031646	4070	2715	7.04	100864.2
OsC2DP28	Os03g0194100	LOC_Os03g09840	Chr3	4894522	4897057	2536	1209	4.54	43864.28
OsC2DP29	Os03g0251600	LOC_Os03g14700	Chr3	7989864	7994679	4816	1815	6.14	65065.62
OsC2DP30	Os03g0289300	LOC_Os03g18010	Chr3	10025401	10027931	2531	1455	6.04	67382.87
OsC2DP31	Os03g0391400	LOC_Os03g27370	Chr3	15673720	15679214	5495	2556	8.31	93550.93
OsC2DP32	Os03g0652000	LOC_Os03g44890	Chr3	25326521	25330063	3543	3165	8.73	115492
OsC2DP33	Os03g0840800	LOC_Os03g62410	Chr3	35350196	35354897	4702	2544	7.63	60988.55
OsC2DP34	Os04g0472900	LOC_Os04g39680	Chr4	23640853	23643655	2803	2568	9.51	88152.69
OsC2DP35	Os04g0476600	LOC_Os04g40070	Chr4	23859220	23863737	4518	2121	5.88	79244.91
OsC2DP36	Os04g0531100	LOC_Os04g44870	Chr4	26558810	26561326	2517	435	4.22	15868.54
OsC2DP37	Os04g0644900	LOC_Os04g55220	Chr4	32826758	32831122	4365	1728	6.99	65016.4
OsC2DP38	Os04g0682100	LOC_Os04g58570	Chr4	34824425	34826306	1882	438	4.58	16107.97
OsC2DP39	Os04g0683800	LOC_Os04g58720	Chr4	34915791	34920089	4299	3036	9.1	114912.4
OsC2DP40	Os04g0691800	LOC_Os04g59520	Chr4	35398604	35402604	4001	3066	9.31	113276.4
OsC2DP41	Os05g0127200	LOC_Os05g03610	Chr5	1535708	1541571	5864	1797	5.69	67052.3

Gene Symbol	RAP-ID	MSU-ID	Chr.	Start	End	Gene Length	Cds Length	PI	MW
OsC2DP42	Os05g0149100	LOC_Os05g05650	Chr5	2806163	2808004	1842	840	9.42	28864.57
OsC2DP43	Os05g0171000	LOC_Os05g07880	Chr5	4255763	4260333	4571	2475	6.13	76484.67
OsC2DP44	Os05g0370600	LOC_Os05g30750	Chr5	17812073	17816465	4393	2325	9.21	89345.46
OsC2DP45	Os05g0373300	LOC_Os05g30970	Chr5	17983282	17989911	6630	1791	5.3	59904.44
OsC2DP46	Os05g0382000	LOC_Os05g31720	Chr5	18466963	18470649	3687	1188	5.91	44599.41
OsC2DP47	Os05g0429700	LOC_Os05g35480	Chr5	21080988	21084058	3071	2415	9.5	90463.05
OsC2DP48	Os06g0223800	LOC_Os06g11990	Chr6	6401419	6407785	6367	3378	5.27	105945.3
OsC2DP49	Os06g0297800	LOC_Os06g19400	Chr6	11046380	11054423	8044	3114	5.45	115810
OsC2DP50	Os06g0604200	LOC_Os06g40170	Chr6	23908918	23913797	4880	2499	5.92	92513.68
OsC2DP51	Os06g0604300	LOC_Os06g40180	Chr6	23921940	23924921	2982	2529	5.89	93236.21
OsC2DP52	Os06g0604400	LOC_Os06g40190	Chr6	23928702	23932372	3671	2454	6.56	91318.9
OsC2DP53	Os06g0607900	LOC_Os06g40570	Chr6	24187249	24192151	4903	3012	9.04	40376.51
OsC2DP54	Os06g0609450	LOC_Os06g40704	Chr6	24264404	24268015	3612	966	9.59	8847.43
OsC2DP55	Os06g0614000	LOC_Os06g41090	Chr6	24555313	24557973	2661	2475	9.24	92947.99
OsC2DP56	Os06g0638900	LOC_Os06g43190	Chr6	25963873	25967411	3539	927	8.57	34271.57
OsC2DP57	Os06g0685300	LOC_Os06g47130	Chr6	28558292	28564762	6471	1623	9.15	11160.86
OsC2DP58	Os07g0108400	LOC_Os07g01770	Chr7	452093	460151	8059	1650	9.07	18645.64
OsC2DP59	Os07g0108500	LOC_Os07g01780	Chr7	457738	460218	2481	486	5.18	18121.8
OsC2DP60	Os07g0165100	LOC_Os07g07070	Chr7	3485498	3489389	3892	3114	9.1	89297.46
OsC2DP61	Os07g0260400	LOC_Os07g15680	Chr7	9105495	9112919	7425	2517	6.84	94957.94
OsC2DP62	Os07g0409100	LOC_Os07g22640	Chr7	12747509	12760678	13170	1548	8.51	55377.37
OsC2DP63	Os07g0483500	LOC_Os07g30020	Chr7	17687049	17692496	5448	3036	8.85	116382.9
OsC2DP64	Os07g0500300	LOC_Os07g31720	Chr7	18840247	18842537	2291	522	6.51	18916.74
OsC2DP65	Os07g0501700	LOC_Os07g31830	Chr7	18911787	18914891	3105	501	5.71	18739.53
OsC2DP66	Os07g0585000	LOC_Os07g39620	Chr7	23746456	23748732	2277	1338	4.55	46052.94
OsC2DP67	Os07g0670200	LOC_Os07g47390	Chr7	28339687	28340470	784	699	9.32	24564.25
OsC2DP68	Os07g0670300	LOC_Os07g47400	Chr7	28344133	28345412	1280	933	9.43	32738.44
OsC2DP69	Os07g0694000	LOC_Os07g49330	Chr7	29545825	29548981	3157	1797	6.15	67783.01
OsC2DP70	Os08g0300200	LOC_Os08g20544	Chr8	12331526	12342183	10658	1269	5.29	61544.93
OsC2DP71	Os08g0492400	LOC_Os08g38440	Chr8	24326030	24332435	6406	2850	5.37	105788.6
OsC2DP72	Os08g0562600	LOC_Os08g44850	Chr8	28170358	28172330	1973	876	4.77	30344.58
OsC2DP73	Os09g0251800	LOC_Os09g07800	Chr9	3949844	3953272	3429	516	5.6	19221.11
OsC2DP74	Os09g0421300	LOC_Os09g25390	Chr9	15222652	15226444	3793	2454	6.02	89964.53
OsC2DP75	Os09g0516900	LOC_Os09g34130	Chr9	20146860	20154260	7401	1839	5.88	67647.43
OsC2DP76	Os09g0538800	LOC_Os09g36770	Chr9	21213298	21223349	10052	1620	6.33	61417.39
OsC2DP77	Os09g0543100	LOC_Os09g37100	Chr9	21391774	21397949	6176	2565	6.51	95117.24
OsC2DP78	Os09g0571200	LOC_Os09g39770	Chr9	22805610	22807049	1440	873	5.97	30468.78
OsC2DP79	Os10g0524400	LOC_Os10g38060	Chr10	20377031	20386096	9066	3141	7.29	116544.8
OsC2DP80	Os11g0183800	LOC_Os11g08090	Chr11	4233460	4243344	9885	6354	5.42	227943.6
OsC2DP81	Os12g0187500	LOC_Os12g08670	Chr12	4397152	4400640	3489	2307	6.43	129412.6
OsC2DP82	Os12g0562400	LOC_Os12g37560	Chr12	23048705	23053324	4620	1773	8.97	22230.7

To further detect the diverse functions of OsC2DPs, polygenetic relationships and gene and domain structures were analyzed; the results are shown in Fig. 1 and Fig. 2. The NJ-Tree showed that 82 members were divided into seven groups, among which, most (19 members) gathered in Group-III and the least (3

members) gathered in Group-IV (Fig. 1). In the structure analysis, the C2 domains were consistently identified, and single or multiple C2 domains were consistently present in each family member. The members of the seven groups had diverse structures, and the structure effectively supported the grouping results of the polygenetic tree (Fig. 2). In Group-I, 12 of the 13 members contained two Phospholipase D/Transphosphatidylase domains and a single Phospholipase D C-terminal domain. In particular, only OsC2DP12 showed other types and had distant relationships with other group members. Further, the results of multiple sequence alignment showed that this domain was highly conserved among the members (Additional file 2: Fig. S3). Group-II and Group-V showed similar domain structures (only a single C2 domain was found). However, most members of Group-II had a simple gene structure that contained one exon; Group-V members showed opposite results with multiple exon regions. Group-III had 19 members with one cluster of polygenetic tree, based on the mixture structure, we further divided them into three classes (A, B, and C); Class-C is a special class that showed a simple gene structure and a Phosphoribosyltransferase C-terminal. The results of multiple sequence alignment revealed a high homology between members of this class (Additional file 2: Fig. S4). In Group-VI and Group-VII, there displayed similar groupings divided into three classes (A, B, and C) according to the diverse domain structure. Further, in Group-VI Class-A and Class-C, there were single and two highly conserved domains, respectively. Synaptotagmin, SMP domain in Class-A (Additional file 2: Fig. S5); Phosphatidylinositol-specific phospholipase C, X domain and Phospholipase C, phosphatidylinositol-specific, Y domain in Class-C (Additional file 2: Fig. S6).

These results suggest that OsC2DPs as a widely gene family and performed diverse roles in plants; Members with a close relationship might possess similar functions; and diverse domains also involve differentiation in each group. Notably, the most conserved phospholipase-related domains were found in OsC2DPs, implying that they might be involved in abiotic stress, ion transporters, or exchange functions.

Synteny analysis and Ka/Ks ratio of OsC2DPs in rice and others genome

The analysis of tandem or segmental duplications could explain the derivation of gene family duplication events. According to a previous study, alignment ratios greater than 70% were identified as duplications while gene pairs within the closed region (100 kb) were selected as tandem duplications. The results are shown in Fig. 3, red color and other color lines represent tandem and segmental duplications, respectively. Six tandem duplications (OsC2DP6/7, OsC2DP50/51, OsC2DP51/52, OsC2DP50/52, OsC2DP58/59, and OsC2DP67/68) and eight segmental duplications (OsC2DP11/46, OsC2DP2/43, OsC2DP10/67, OsC2DP20/36, OsC2DP13/54, OsC2DP14/53, OsC2DP30/69, and OsC2DP72/78) were found in the rice genome. Interestingly, three genes, OsC2DP50/OsC2DP51/OsC2DP52, involved a co-duplication event in a narrow region (19.784 kb), implying that these genes might have the same function or signaling pathway, or possess a redundancy effect in the regulation process. We also analyzed the duplication events of C2DPs between rice and other popular crop genomes. A total of 96, 100, 93, 82, 187, and 302 C2DPs were identified in Arabidopsis, Barley, Maize, Sorghum, Soybean, and Wheat genomes, respectively; the results are shown in Additional file 2: Fig. S7. Among these, Arabidopsis and wheat showed the most distant and close relationship with rice, with 8 and 176 duplications, respectively (Additional file 2: Fig. S7A and F). A total of 53, 83, 74, and 26 duplications were found between rice and barley, maize, sorghum, and soybean (Additional file 2: Fig. S7B-E). Such finding indicates that the OsC2DP gene family has expanded and evolved through genome duplication in rice or infusion in other crops.

In genetic studies, the Ka/Ks ratio represents the comparison of non-synonymous substitution rate (Ka) and the synonymous substitution rate (Ks) of duplications. Therefore, the Ka/Ks ratio could determine whether there is selective pressure for this gene pair [30]. In previous results, we identified duplications in rice and other genomes; thus, we calculated these duplications Ka/Ks ratio for understanding the evolutionary model for OsC2DPs. All Ka/Ks ratios of the tandem and segmental duplications were less than 1 (Table 2), suggesting that these duplications involved purify (negative) selection. The duplication results of Arabidopsis, barley, maize, sorghum, soybean, and wheat were similar to those of intra rice, with Ka/Ks ratios less than 1 (Additional file 1: Table S3-S8), involved purify (negative) selection. Notably, there was one tandem duplication (OsC2DP58/59) with a Ka/Ks ratio of 0.905143 (Table 2), which is close to 1 as the neutral evolution. Another gene pair (OsC2DP67- TraesCS2A02G115700) had a Ka/Ks ratio of 1.0688 in the rice-wheat duplications (Additional file 1: Table S8), suggesting that these homologs involved positive selection. The average Ka/Ks ratio in tandem duplications (0.561) was found to be greater than that in segmental duplication (0.263) and in the comparison of other genomes (0.076, 0.248, 0.205, 0.236, 0.114, 0.255), implying that tandem duplications of intra-rice were more likely to involve positive selection in the evolutionary process.

Table 2. Synteny analysis for OsC2DP gene family intra rice and rice genome

Tandem Duplication										Ka	Ks
Gene Symbol	Gene ID	Chr	Start	End	Gene Symbol	Gene ID	Chr	Start	End		
OsC2DP6	LOC_Os01g60340	Chr1	34902303	34906955	OsC2DP7	LOC_Os01g60350	Chr1	34908079	34908079	0.15904	0.3
OsC2DP50	LOC_Os06g40170	Chr6	23908918	23913797	OsC2DP51	LOC_Os06g40180	Chr6	23921940	23921940	0.09049	0.1
OsC2DP51	LOC_Os06g40180	Chr6	23921940	23924921	OsC2DP52	LOC_Os06g40190	Chr6	23928702	23928702	0.16077	0.4
OsC2DP50	LOC_Os06g40170	Chr6	23908918	23913797	OsC2DP52	LOC_Os06g40190	Chr6	23928702	23928702	0.16616	0.4
OsC2DP58	LOC_Os07g01770	Chr7	452093	460151	OsC2DP59	LOC_Os07g01780	Chr7	457738	457738	0.00405	0.0
OsC2DP67	LOC_Os07g47390	Chr7	28339687	28340470	OsC2DP68	LOC_Os07g47400	Chr7	28344133	28344133	0.40704	0.6
Average										0.16459	0.3

Segmental Duplication										Ka	Ks
Gene Symbol	Gene ID	Chr	Start	End	Gene Symbol	Gene ID	Chr	Start	End		
OsC2DP11	LOC_Os01g72230	Chr1	41882488	41886410	OsC2DP46	LOC_Os05g31720	Chr5	18466963	18470649	0.26074	1.1
OsC2DP2	LOC_Os01g07760	Chr1	3724314	3729284	OsC2DP43	LOC_Os05g07880	Chr5	4255763	4260333	0.11203	5.5
OsC2DP10	LOC_Os01g70790	Chr1	40973255	40974782	OsC2DP67	LOC_Os07g47390	Chr7	28339687	28340470	0.40741	0.6
OsC2DP20	LOC_Os02g42710	Chr2	25684044	25687149	OsC2DP36	LOC_Os04g44870	Chr4	26558810	26561326	0.1123	0.5
OsC2DP13	LOC_Os02g10480	Chr2	5505016	5508425	OsC2DP54	LOC_Os06g40704	Chr6	24264404	24268015	0.15437	0.8
OsC2DP14	LOC_Os02g10630	Chr2	5586939	5593645	OsC2DP53	LOC_Os06g40570	Chr6	24187249	24192151	0.22964	0.8
OsC2DP30	LOC_Os03g18010	Chr3	10025401	10027931	OsC2DP69	LOC_Os07g49330	Chr7	29545825	29548981	0.17977	1.1
OsC2DP72	LOC_Os08g44850	Chr8	28170358	28172330	OsC2DP78	LOC_Os09g39770	Chr9	22805610	22807049	0.16323	0.4
Average										0.20244	1.3

Expression profile analysis of OsC2DPs in rice tissues

The change in transcriptional level of genes could better explain the vitality of a gene in diverse tissues and growth stages. To determine the specific function of the gene at spatiotemporal variations, we analyzed the transcriptome data for the detected expression of OsC2DPs during different tissues and stages in rice. Diverse expression patterns were observed in the OsC2DPs (Fig. 4). For example, OsC2DP16/40/67/81/etc. showed higher expression in EN (1–3) and ML, suggesting that these genes function in later mature stages. OsC2DP29/33/43/74/4/59/30/etc showed the highest expression in HP, suggesting that these genes may perform major functions in HP, specially. Additionally, such as gene OsC2DP47/39/44 or OsC2DP37/11/80/54, had higher expression in BP (1–4) while OsC2DP51/41/50/64/etc had lower expression in EN (1–3) (Fig. 4); these diverse expression patterns indicate that some genes involved group cluster, might be performed functions by cooperated with cluster genes. Based on this conclusion, we further analyzed the expression profiles of tandem and segmental duplications. OsC2DP50/51/52 showed three genes as co-tandem duplications that contained similar expression variations across the entire growth stage in rice (Additional file 2: Fig. S8A). OsC2DP58/59 showed differences at an earlier stage but maintained consistent expression variations in HL to SP (Additional file 2: Fig. S8B). Further, there were no significant similarity in phenomena in OsC2DP67/68 (Additional file 2: Fig. S8C). In segmental duplications, there were two gene pairs; the two gene pairs showed completely similar and partially similar expression variations, respectively (Additional file 2: Fig. S9). Among these, OsC2DP14/53 and OsC2DP72/78 showed similar expression variations across the whole growth stage (Additional file 2: Fig. S9D and H). OsC2DP11/46 was similar from the BP4 to EN stage (Additional file 2: Fig. S9A) while OsC2DP13/54 showed similar variations from GS to BLS2 and from HS to ML (Additional file 2: Fig. S9B).

Taken together, OsC2DPs were found to be widely expressed in whole tissues and growth stages. Further, a diverse expression pattern was observed in the gene cluster, suggesting that duplications were highly possible involved the same functions and regulation pathways in special tissues and stages by cooperation expression variation.

Prediction and analysis of the*cis*-acting elements in promoter regions of OsC2DPs

Generally, the gene response to treatments or other functions would be achieved by changing the activity of the treatment-related *cis*-acting elements [31]. In this study, we analyzed the abiotic stress and phytohormone related *cis*-acting elements in the promoter region of OsC2PDs as the validated functions of OsC2DP gene family were related to abiotic and phytohormone; the results are shown in Additional file 2: Fig. S10 and detailed information is in presented in Additional file 1: Table S9. A total of seven types of abiotic stress responsive elements and five types of phytohormone responsive elements were identified: the cold-responsive element, LTR (CCGAAA); drought responsive element, MBS (CAACTG); salt responsive element, GT1GMSCAM4 (GAAAAA); heat responsive element, CCAATBOX1 (CCAAT); diverse light responsive elements, ACE (GACACGTATG), G-box (TACGTG), GT1-motif (GGTTAA), etc.; circadian element,

CAAAGATATC; and wound responsive element, WUN-motif (AAATTCCT). Phytohormone responsive elements, including ABA (ABRE: ACGTG/CACGTG), IAA (TGA-element: AACGAC and AuxRR-core: GGTCCAT), GA (TATC-box: TATCCCA and GARE-motif: TCTGTTG), SA (TCA-element: CCATCTTTTT), and MeJA (TGACG-motif: TGACG and CGTCA-motif: CGTCA) were found. After statistical analysis, a total of 923 light responsive elements and 390 MeJA responsive elements were found as the most abundant *cis*-acting elements in *Osc2DPs*. For each gene, the light-responsive element most enriched for *Osc2DP5* (28), *Osc2DP29* (22), *Osc2DP3* (20), and *Osc2DP64* (20), implied that the functions of these gene functions were possible involved in the light signaling pathway in rice. In addition, some genes contained most elements with multiple functions, such as *Osc2DP5* contained most elements related to salt and light; *Osc2DP29* contained most elements related to cold, salt, and light; and *Osc2DP28* contained most elements related to drought and heat. These results indicate that the *Osc2DP* gene family is widely involved in abiotic and phytohormone stress in rice.

Expression analysis of *Osc2DPs* involved abiotic and phytohormone stress

To further verify the function of genes in abiotic and phytohormone stress, we collected multiple RNA-seq data and performed qRT-PCR to detect the variation in transcriptional levels in *Osc2DPs*. First, the results of transcript profile revealed that under treatments of ABA, GA, IAA, cytokinin, SA, and JA, the family members exhibited diverse responses to phytohormones (Additional file 2: Fig. S11). For example, *Osc2DP6*, *Osc2DP9*, etc., these genes were consistently repressed by ABA, GA, IAA, and cytokinin; *Osc2DP15*, *Osc2DP73*, etc., these genes were consistently induced by these phytohormone; and *Osc2DP16*, *Osc2DP15*, etc., these genes had opposite expression changes under the treatments of SA and JA. In addition, among the three co-tandem duplications, *Osc2DP50/51/52* showed a consistent response to SA and JA, and only *Osc2DP50/51* remained consistent under treatments of ABA, GA, IAA, and cytokinin. These results suggest that *Osc2DPs* are divided into diverse patterns that are widely involved in the response to phytohormone stress; among these, the duplications might maintain similar functions or involve the same pathway for different stresses.

Subsequently, some abiotic-response genes were selected via previous *cis*-acting element analysis based on the major count of abiotic-related motifs. We performed qRT-PCR to verify the expression change of candidate abiotic-response genes; the results are shown in Fig. 5 and Additional file 2: Fig. S12. *Osc2DP8/29/17* expression was significantly induced by cold stress, *Osc2DP46/49/71* expression was repressed, and only *Osc2DP19* showed no obvious change (Fig. 5A). Under heat shock stress, *Osc2DP71/28/41* expression was induced and *Osc2DP19/79* was first repressed but subsequently recovered (Fig. 5B). Such findings suggest that *Osc2DP71* expression was induced by cold and repressed by heat, ultimately indicating that this gene as a temperature-sensitive factor. In addition, *Osc2DP8/9/28* expression was induced and *Osc2DP25* was repressed by drought stress (Fig. 5C). Under salt and alkaline stress, *Osc2DP29/5* expression was similarly induced by treatments, and *Osc2DP46/49/41* showed diverse variation in response to these two stresses (Fig. 5D and E). In plants, light response is a key signal for plant growth and both photosynthesis and circadian rhythms affect plant growth and development [32]. Thus, we performed qRT-PCR under light and dark conditions. All candidate gene expression levels showed significant variation in different treatments (Additional file 2: Fig. S12). *Osc2DP29/46* expression was induced and *Osc2DP5* was repressed under dark conditions compared to that under light conditions (Additional file 2: Fig. S12A, B and C). For *Osc2DP79/3/64*, circadian rhythms were evidently observed under normal (light) growth condition (Additional file 2: Fig. S12D, E and F). In contrast, under dark conditions, the circadian rhythms of *Osc2DP79/3* were disrupted, resulting in levelling out for expression (Additional file 2: Fig. S12D and E). *Osc2DP64* expression showed an opposite trend compared to that found under the normal (light) condition (Additional file 2: Fig. S12F). These results suggest that these *Osc2DPs* widely involved diverse abiotic stresses; *Osc2DP5/29/49/71* could respond to multiple stress treatments, possibly playing positive or negative roles in these functions.

Bioinformatics predictions and enrichment of *Osc2DPs*

In the last decade, miRNAs have been found in diverse plant species, influence plant growth development and change the survivability to biotic and abiotic stress [33], which are directly involved in the functions of miRNAs by cleavage or translation at the transcriptional level [34]. In this study, we analyzed the *OsmiRNAs-Osc2DPs* pathway and predicted the putative regulatory network using the miRNA database. A total of 167 unique potential *OsmiRNAs*-targets of *Osc2DPs* were identified, with miRNAs 19–24 nucleotides long (Additional file 1: Table S10), among these, functions of 146 unique targets as the cleavage and 21 unique targets as the translation were identified, suggesting that cleavage is the major function in the regulation of *OsmiRNAs*-targets of *Osc2DPs*. There were 28 *Osc2DPs* involved in one relevant network (Fig. 6) while the remaining 29 *Osc2DPs* were involved in fragmented networks (Additional file 2: Fig. S13). Among these, *Osc2DP71* possessed 11 potential targeted *OsmiRNAs*; genes *Osc2DP53/79/27/39/3* possessed 9/7/7/7/6 potential targeted *OsmiRNAs*, respectively; and *OsmiR2927*, *OsmiR5809*, *OsmiR5075*, and *OsmiR5833* contained six, five, six, and six potential targets, as the most and major *OsmiRNAs*-target in the *Osc2DP* gene family. In addition, subfamily specific targets were identified: Group-I members, *Osc2DP27/79/61* co-targeted by *OsmiR5830*; and Group-III members, *Osc2DP32/34/47/21* co-targeted by *OsmiR5833*. As expected, *Osc2DP50/51/52* was co-targeted by *OsmiR2927* as the co-tandem duplications, jointly targeted with Group-I members, *Osc2DP31/43/12* (Fig. 6). Additionally, we performed GO and KEGG enrichments analyses of the *Osc2DP* gene family. Results showed major functions involved membrane, plasma membrane, multiple metabolic process, phospholipase activity and ion binding of GO functions (Additional file 2: Fig. S14A). KEGG results showed that the major pathways were membrane and multiple metabolic processes (Additional file 2: Fig. S14B). Together, these results imply that *Osc2DPs* perform major functions in the cell membrane, possibly via ion combination and transportation.

SNP genotyping and haplotype analysis of *Osc2DPs*

In rice, Japonica and Indica, the major subspecies, contain different traits caused by many genotype variations; thus, we analyzed the SNP genotyping and abiotic-related haplotypes of *Osc2DPs*. First, we extracted all SNPs of the *Osc2DPs* promoter, UTR, exon, and intron regions from a ~ 1.85 million high-quality core collection re-sequence data. A total of 2,861 SNPs were selected based on diverse gene positions. SNP annotation results showed that 424 SNPs were located in promoters; 219 SNPs were located in UTR regions; and 223 SNPs were located in exon regions, 124 of which were synonymous and 99 were non-synonymous variants (Table 3). After removing the Adm variety, a PCA was carried out using *Osc2DPs* SNP data (only remained Jap and Ind varieties).

Results showed that PC1 explained 87.55% and PC2 explained 12.55% of the variation, and two subspecies varieties were separated into two groups (Additional file 2: Fig. S14C).

Table 3. Summary for SNPs in OsC2DPs

Variation type	Count	Variation in Exon	Count
Promoter variation	424		
Upstream variation	575		
Downstream variation	73		
Upstream & Downstream variation	265		
5'UTR variation	47		
3'UTR variation	172		
Intergenic variation	178		
Exonic variation	223	Synonymous variant	124
		Non-synonymous variant	99
Intronic variation	829		
ncRNA_exonic variation	75		

In the haplotype analysis, a set of cold-related phenotypes was associated with genotype data; this analysis was performed to identify the functional phenotype-related genotypes in different varieties. According to previous qRT-PCR results, OsC2DP17, OsC2DP29, and OsC2DP49 were selected as candidate genes for cold stress. For OsC2DP17, after filtering genotype data that SNPs contained missing or heterozygotes, eight SNPs were found in the intron, exon, and promoter regions (Fig. 7A). These SNPs formed four haplotypes for OsC2DP17 and the major varieties involved Hap1 and Hap4 (Fig. 7B), LD analysis results showed a strong LD relationship between each SNP pair (Fig. 7C). Haplotype network and variation analysis showed that there were two major groups, Hap1 and Hap2, which contained major Ind and Aus subspecies; Hap3 and Hap4 contained major Tej and Trj subspecies. Large genotype variations were also observed between these two groups (Fig. 7D). The association of phenotype-haplotype was analyzed, and a set of CT score (1–9 score) was used as the evaluation index. As shown in Fig. 7E and F, Hap1 and Hap2 showed significantly cold sensitivity (higher CT score) compared with Hap3 and Hap4, respectively, indicating that Hap1 and Hap2 conferred major sensitivities to cold tolerance in OsC2DP17. By comparing these SNPs, we found a key SNP, SNP 8, located at promoter – 1652 bp, which made a nucleotide change from T to C (named as -1652^T and – 1652^C). The SNP 8-haplotype analysis result showed that – 1652^T conferred major tolerance to cold stress compared with – 1652^C (P = 2.492E-11). Interestingly, through promoter analysis, we found that SNP 8 was located in the cold-related motif, MYCCONSSENSUSAT (CANNTG) (Fig. 7G) [35], and its mutant possibly led to a change in gene expression under cold stress.

For OsC2DP29 and OsC2DP49, six SNPs were selected in both genes by removing missing or heterozygote data, which were found in the intron and exon regions of OsC2DP29 and the promoter, intron, and exon regions of OsC2DP49, respectively (Additional file 2: Fig. S15A and B, Additional file 2: Fig. S16A and B). LD analysis of both genes revealed similar results to OsC2DP17, which had a strong LD relationship between each SNP pair (Additional file 2: Fig. S15C and Additional file 2: Fig. S16C). The haplotype network showed that the haplotypes of OsC2DP29 were divided into two groups: Hap1, Hap4, and Hap5, which contained the most Ind, Aus, and Adm varieties (Additional file 2: Fig. S15D); and Hap2 and Hap3, which included most Trj and Tej varieties. Such finding suggests that OsC2DP29 is a Jap-Ind specific genotyping gene. As expected, the phenotype-associated results showed that Hap3 and Hap2 had significantly lower CT scores than other Haps (Additional file 2: Fig. S15E), suggesting that the genotypes of Trj and Tej conferred stronger cold tolerance in this population, which also aligns with the general difference in temperature sensitivity between Indica and Japonica subspecies. Similar to the above analysis, through a comparison of each SNP, SNP3 caused a nucleotide change from A to T (named as 1894^A and 1894^T) located at 1894 bp of intron region was selected, due to this SNP genotypes of Hap2 and Hap3 resulted in a phenotypic difference. Thus, by analysing independent SNP haplotypes, 1894^A was found to show highly significant variations in CT score compared with 1894^T (Additional file 2: Fig. S15F), suggesting that this SNP may be a key and functional SNP for the genotyping of OsC2DP29 in cold tolerance. Similarly, five haplotypes of OsC2DP49 were roughly divided into Jap (Hap1, Hap2, and Hap3) and Ind (Hap4 and Hap5) groups (Additional file 2: Fig. S16D), only Hap3 and Hap4 contained mixture varieties. The phenotype-haplotype associated results showed that Hap1 and Hap2 had significantly lower CT score in populations (Additional file 2: Fig. S16E). A key SNP, SNP3, changed the nucleotide G to A (named as -316^G and – 316^A), which was located at -316 bp of the promoter region. The SNP-haplotype showed that – 316^A had a significantly lower CT score for OsC2DP49 (Additional file 2: Fig. S16F), similar to that of 1894^A in OsC2DP29.

These results suggest that OsC2DP17, OsC2DP29, and OsC2DP49 are highly possible involved in cold stress, diverse haplotypes were identified, which supported the potential theoretical foundation of the relationship between genotype variations and stress tolerance for present population in rice.

Discussion

Identification and polygenetic relationship of OsC2DPs in rice genome

Recently, gene family studies have been constantly reported. An extensive study of gene family analysis could support a comprehensive understanding of the biological functions of each family. For example, OsANTH3, a member of the AP180 N-terminal homology domain-containing protein, has been reported to be involved in pollen-related functions in rice [36]. Further, the *MAPKKK* gene family is involved in drought stress in cotton [37], which supports the wide prediction of gene function and theoretical foundation for homologs of evolutionary relationships. The C2 domain contain proteins, which show at least one C2 domain that acts as a lipid binding domain and a major sensor of diverse Ca^{2+} -mediated cellular processes [14]. Most C2DPs can perform functions and cooperate with other transmembrane domains, such as phospholipase domain and synaptotagmin [38], and maintain highly conserved characteristics in the evolutionary process. In the present study, we used a conserved HMM model, performed blast function in the rice protein database, and identified 82 OsC2DPs at each chromosome (Additional file 1: Table S1, Additional file 2: Fig. S1). Few gene clusters were also found in chromosomes 1, 6, and 7. A previous study showed that gene clusters produced tandem duplication events in the genome, implying that OsC2DPs might have duplication events in cluster regions. The prediction of subcellular localization showed OsC2DPs protein play a role throughout the cell (Additional file 1: Table S2), suggesting that involved multiple regulation network of growth development process. Interestingly, major proteins were located in the cytoplasm and nucleus (Additional file 2: Fig. S6); thus, we speculated that some proteins played roles as regulation transporters for shuttling between the nucleus and membrane. A similar function was also reported in a previous study, including the localization of the protein, OsRAN2, in the nucleus and cytoplasm, which could regulate the transport of proteins and RNA across the nuclear envelope and play a role in mitotic spindle and nuclear envelope (NE) assembly, overexpression of OsRAN2 could enhanced tolerance to salt stress in rice [39]. Another study reported that the protein, OsSAP1, could interact with the proteins, OsSAP11 and OsRLCK253, and that this interaction occurred in karyotheca, plasmalemma, and endonuclear, and these proteins also perform roles in drought and salt tolerance in plants [40]. Suggested that some members of OsC2DPs might display similar functions for nuclear-cytoplasmic shuttling, perform interaction function, and act in abiotic stress in rice. Polygenetic analysis revealed that a total of seven groups were divided into 82 family members according to domain and gene structure, and multiple transmembrane region domains were found in the different groups (Fig. 2). A previous study also reported similar polygenetic results for C2DPs; in Arabidopsis, the multiple C2 domain and transmembrane region proteins were reported [27], the members of rice homologs in a previous study were similar to members of group-III Class-C in the present study; and in cotton, rice homologs of MCTPs were found in a polygenetic study that is consistent with the present study [28]. In rice, previous study identified OsNTMC2 members were identified [29], OsNTMC2s contain a highly conserved N-terminal TM domain, SMP domain, and C-terminal C2 domain, which is consistent with Group-VI Class-A in the present study, validating the conservatism of each group and the accuracy of our analysis (Fig. 2).

Duplications events performed in OsC2DPs

Normally, duplications contain conserved domains or sequences that may involve similar functions in plants [41]. Among these, tandem duplications as major and commonly evaluated mechanisms for gene family expansion which produced novel genes and clusters into gene families, had an impact on a small number of genes, but had a significant contribution to gene family expansion [42]. In the present study, a total of 8, 53, 83, 74, 26, and 176 duplications were identified between rice and Arabidopsis, barley, maize, sorghum, soybean, and wheat (Additional file 2: Fig. S7, Tables S3-S8), implying the occurrence of duplication events and the potential genome expansion of OsC2DPs from other species, especially the wheat genome. In rice, a total of six tandem and eight segmental duplications were found (Fig. 3, Table 2), suggesting that these duplications might involve similar functions in rice; this hypothesis was also validated through further analysis. In the expression file data, the tandem duplications, *OsC2DP50/51/52* and *OsC2DP58/59*, showed a similar expression variation tendency with rice growth stages. Evidently, co-duplications *OsC2DP50/51/52* expression showed the same response to diverse phytohormones and involved the same potential regulation pathway as the miRNA-targets of miR2927 (Fig. 6), providing exact evidence for the predicted functions of co-duplications *OsC2DP50/51/52*. A previous study reported that tandem duplication events occurred more frequently than other events and formed large gene copies and allelic variations [43]. Tandem duplications are widely involved in the control of stress tolerance and membrane functions in rice and Arabidopsis [44, 45], or the transduction of the signaling pathways in legumes [46].

Elements prediction and expression analysis of OsC2DPs

Based on the prediction of *cis*-acting elements in the promoter regions of *OsC2DPs*, many phytohormone- and abiotic stress-related motifs were found (Additional file 2: Fig. S10), implying that OsC2DPs may be involved in phytohormones and abiotic stress. Furthermore, expression profile data of phytohormone treatments validated the previous hypothesis, and qRT-PCR was performed to identify expression variations under abiotic stress treatments in some putative functional genes, which suggested that OsC2DPs are involved in phytohormones and abiotic stress. In a previous study, some OsC2DPs functions were reported in transgenic plants, such as OsPBP1(LOC_Os04g44870) was named as OsC2DP36, involved Ca^{2+} + concentration-dependent phospholipid-binding activity and localized in the cytoplasm and nucleus, whereas shuttling at the plasma membrane by increased intracellular Ca^{2+} [47]. The rice no pollen gene (LOC_Os06g40570) was named OsC2DP53 and is responsible for the production and development of pollen, which might be related to Ca^{2+} and phosphoinositol signaling pathways depending on the C2 and GRAM domains [48]. OsC2DP (LOC_Os09g39770) was named OsC2DP78, a functional mutant that changed tolerance to salt, showed variations in Na^{+} concentration and $\text{K}^{+}/\text{Na}^{+}$ ratio [26]. Thus, except for members of OsNTMC2s and MCTPs included in previous studies, other identified OsC2DP family members that involved present study also widely performed diverse functions of growth development in rice.

Identification of potential variation alleles for future breeding

SNP genotyping was performed PCA analysis using the SNP data of OsC2DPs (Additional file 2: Fig. S14C). The significant Japonica-Indica polarization of OsC2DPs supports a potential future research direction. Further, diverse genotypes of allelic variation could produce different tolerances to abiotic stress in multiple subspecies [49, 50]. Haplotype analysis revealed that OsC2DP17, OsC2DP29, and OsC2DP49 possessed diverse alleles among the core collection population, which generated different cold tolerance for each variety (Fig. 7, Additional file 2: Fig. S15 and Additional file 2: Fig. S16). Interestingly, we identified key SNPs that significantly impacted tolerance in these candidate genes. The SNPs – 1652^T, 1894^A, and – 316^A were located in intron and promoter regions, we speculated that these SNPs might possess strong LD levels coordinated with crucial translation or functional domain region of each gene, thereby

producing a diverse phenotype in rice. In summary, these haplotypes and SNP-haplotypes are supported potential opportunities for enhanced related tolerance in future molecular design breeding.

Conclusions

In summary, we are provided first study to comprehensively characterize the C2DP gene family in rice. Herein, 82 members and multiple transmembrane domains were identified by phylogenetic analysis. The roles of the OSC2DP gene in abiotic stress were identified by bioinformatics prediction, transcript profile analysis, and qRT-PCR experiments. Further, the polarization genotypes between diverse rice subspecies and putative functional haplotypes were characterized by SNP genotyping and haplotype analysis. The findings of this study contribute to a better understanding of the functions of the C2 domain and future functional characterization of OsC2DPs.

Methods

Identification, chromosome distribution, and localization of OsC2DPs

In the present study, rice genome and protein sequences were downloaded from the Phytozome database (<http://www.phytozome.net>). To identify the OsC2DP gene family members in rice, a Hidden Markov model (PF00168) of the C2-domain was downloaded from the PFAM database that as triggered for blast in rice Proteomic sequence, with E-value threshold less than $1e^{-5}$ [51]. The filtered blast hits were submitted to the InterPro (<https://www.ebi.ac.uk/interpro/>) and SMART (<http://smart.embl-heidelberg.de/>) databases and were retrieved for the C2-domain. The diverse lengths of OsC2DPs were calculated using TBtools [52], and the physical properties (pI and Mw) were analyzed using the ExPASy website (https://web.expasy.org/compute_pi/). According to the prepared gene positions obtained from the rice genome file, chromosome distribution and visualization were performed using the MapGene2Chromosome V2 (http://mg2c.iask.in/mg2c_v2.0/) according to the official default procedure. To predict the subcellular localization of OsC2DPs, full-length proteins of OsC2DPs were submitted to the website tool, CELLO v.2.5: subCELLular LOcalization predictor [53], and the final localization results were determined by the major score of each position in every family member.

Polygenetic, structure and multiple sequence alignment analyses

To perform the polygenetic relationship of OsC2DPs, the full-length protein of OsC2DPs leaded into MEGA software [54]. Further, a polygenetic tree was constructed using NJ-tree functions with 1000-times bootstrap iterations, and a final plot of visualization was generated using tools on the iTOLs website (<https://itol.embl.de/>) [55]. To identify multiple structure of OsC2DPs, full-length protein sequences were submitted and analyzed using the PFAM database. Visualization was performed using each domain length and position in the website Gene Structure Display Server (<http://gsds.gao-lab.org/>) [56]. Gene structures were analyzed using the software, TBtools, and the rice gff3 file. For multiple sequence alignment, whole protein sequences of OsC2DPs were aligned using ClustalW 2.0 [57] software and visualized using the website, Sequence Manipulation Suite (<http://www.bioinformatics.org/>) [58], where a consistent color represents a highly conserved amino acid.

Synten and Ka/Ks analysis

To analyzed synteny in OsC2DPs, C2DPs in the other six plant species were identified using the same procedure that used in the identification of OsC2DPs. BLAST calculations were performed between the genome of rice and those of other species using the BLAST software [59]. Thereafter, multiple genome synteny analysis was performed using MCScanX software [60]. Among these, duplications were determined by an alignment ratio over 70% and blast ratio over 70% while tandem duplications were determined based on the involvement within a 100 kb region [61]. All duplications of C2DPs were filtered from whole genome duplications, and plots were visualized using Circos software using default parameters [62]. For Ka/Ks calculation, full-length protein sequences of all duplication C2DPs were aligned using ClustalW 2.0. Thereafter, Ka, Ks, and Ka/Ks ratios were calculated using the software, KaKs_Calculator 2.0, following the default procedure [63].

Expression profile analysis and prediction of *cis*-acting elements

For collected expression profile data, multiple RNA-seq analysis results were selected from the NCBI GEO database. The rice tissue-specific expression and duplication expression pattern data followed GSE19024 [64], treatments of ABA, GA, IAA, cytokinin, SA, and JA data followed GSE39429 and GSE37557 [65, 66]. After the OsC2DP expression data were filtered from these data profiles, the mean value and related fold change were calculated by comparison to the control (no treatment). Visualization of the heatmap was performed using the R program. Data were analyzed using log, row normalization, and cluster analysis. For prediction of *cis*-acting elements for OsC2DPs, all promoters of OsC2DPs were extracted using the software, TBtools, and the promoter was determined using 2000 bp upstream sequences of the transcription initiation site for each family member. Element prediction was performed using the database, PlantCARE [67], and the related motifs were classified according to database annotations.

Plant growth, treatment, and quantitative real-time PCR analysis

To validate the OsC2DP expression response to abiotic stress, common variety Nipponbare (IT003166) was selected for experiment, that was obtained from Rural Development Administration (RDA) National Institute of Agricultural Sciences (<http://genebank.rda.go.kr/initMain.do>). Plants were grown to 2 leaves stage seedlings using Yoshida growth solutions under normal growth conditions (28°C /26°C, 12h/12h). Thereafter, they were collected under treatments of cold (4°C), heat (42°C), drought (dry), salt (200 mM), and alkaline (0.15% Na₂CO₃) stress. For light response, Nipponbare plants were grown to 2 leaves stage and leaf samples were collected under dark and normal (light) conditions. The time points for collection were 0 h, 1 h, and 3 h for cold, heat shock, drought, salt, and alkaline stress; and 0 h, 12 h, 24 h, 36 h, and 48 h for light and dark treatments. Total RNA was extracted from the collected samples using the

RNeasy Plant Mini Kit (QIAGEN). All samples were treated with RNase-Free DNase (QIAGEN) to remove genomic DNA. Complementary DNA was synthesized using the SuperScript III Kit (Thermo). For primer design, each gene cds sequence was inputted and produced by the software, Primer5. The gene, *Actin*, served as the internal control to ensure corrected accuracy of results; the total list is shown in Additional file 1: Table S11. qRT-PCR was performed using SYBR Green PCR Kit (QIAGEN) and Rotor-Gene Q (QIAGEN) system, following the default production procedure. Each reaction was repeated three times; the mean value was calculated and the $-\Delta\Delta CT$ method was used to calculate the relative expression [68].

miRNA target prediction and enrichment analysis of OsC2DPs

To predict the potential miRNA targets of OsC2DPs, the full-length of the cds sequences of OsC2DPs was extracted and predicted using the miRNA database, psRNATarget [69]. The expectation threshold was set to 4 for filtered pseudo results, and visualization was performed using the Cytoscape software [70]. For gene ontology and KEGG enrichment, the PlantGSEA database (<http://bioinformatics.cau.edu.cn/PlantGSEA/>) was employed for OsC2DPs [71]. The results were filtered by adjust p-value less than 0.05, showing 14 major enrichment functional terms, and visualization was performed using the R program.

SNP genotyping and haplotype analysis

A core collection set of rice was employed in the present study. A total of 137 varieties containing multiple Jap, Ind, and Adm were collected from 28 countries [72]. Approximately 1.85 million high-quality SNPs were filtered from raw re-sequencing data, following a previous study [73]. For SNP genotyping of OsC2DPs, all SNPs located at the promoter, UTR, intron, and exon of OsC2DPs formed a 2861 SNP set, the annotations were performed using website tools <http://bioinfo.sibs.ac.cn/>. PCA analysis and visualization were performed using R program by default functions using 2861 SNPs data. For haplotype analysis, SNPs located at OsC2DP17, OsC2DP29, and OsC2DP49 were collected following the above descriptions and the missing and heterozygote data were filtered. Phenotype data included an evaluation of the CT score following a previous study [74]. Haplotype grouping was carried out based on the SNP variations between each Hap. Significant tests of phenotype-haplotype and SNP-haplotype were carried out using SPSS software; different letters represent differences. Visualization of the haplotype network was performed using the software, PopArt [75]. LD analysis was performed using Haploview software [76].

Abbreviations

C2DP: C2 domain contain protein

MCTP: multiple C2 domains and transmembrane region protein

NTMC2: N-terminal-TM-C2 domain proteins

Ca: Calcium

miRNA: microRNA

MW: Molecular weight

NJ: Neighbor-joining

pI: Isoelectric point

HMM: Hidden Markov Model

Ka: Non-synonymous substitution rate

Ks: Synonymous substitution rate

ABA: Absciscic acid

IAA: Auxin

GA: Gibberellin

SA: Salicylic acid

MeJA: Methyl jasmonate

SNP: Single nucleotide polymorphism

Hap: Haplotype

LD: Linkage disequilibrium

CT: Cold tolerance

Trj: Tropical japonica

Tej: Temperate japonica

Ind: Indica

Adm: Admixture

qRT-PCR: Quantitative real-time polymerase chain reaction

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The genome, protein, and Generic Feature Format files of rice, *Arabidopsis thaliana*, Barley, Maize, Sorghum, Soybean, and Wheat were downloaded from the Phytozome database (<http://www.phytozome.net>) and Ensemble FTP (<http://plants.ensembl.org/info/data/ftp/index.html>). The RNA-seq data generated during the current study have been deposited and publicly available at the National Center of Biotechnology Information repository, GEO database (<https://www.ncbi.nlm.nih.gov/geo/>), accession numbers: GSE19024, GSE39429 and GSE37557. All datasets generated and analyzed during this study are included in this article and its additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (NRF-2021R1A4A2001968) provided by STK. The funders have no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

HZ and SWK conceived and designed research. HZ conducted experiments, HZ and YZ collected and analyzed the data, HZ wrote the preliminary manuscript. JS, YJK and STK gave critical revision of the manuscript. All authors have read and approved the manuscript.

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Acknowledgements

Not applicable.

Additional information

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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Figures

Figure 1

Phylogenetic tree of OsC2DPs in rice genome. Different colors represent different groups

Figure 2
Structural schematic diagram of the OsC2DP gene family. a Phylogenetic relationship of OsC2DPs. b Gene structure of OsC2DPs. c Putative domain structure of OsC2DPs

Figure 3

Syntenic analysis of OsC2DPs intra rice genome. Each rice chromosome is displayed in a different color. Duplicated gene pairs are displayed and linked using colored lines; red half lines represent tandem duplications

Figure 4

Expression profiles of OsC2DPs in diverse tissues across different stages. GS: germinating seed; PL: plumule; YR: radicle; YS: young seedling; SE: seedling at trefoil stage; TS: shoot under 2 tillers; TR: root under 2 tillers; BL: mature leaf blade under young panicle; BL2: mature leaf blade under mature panicle; BLS: mature leaf sheath under young panicle; BLS2: mature leaf sheath under mature panicle; BP1: developing panicle (length < 1 mm); BP2: developing panicle (3 mm < length < 5 mm); BP3: developing panicle (10 mm < length < 15 mm); BP4: developing panicle (40 mm < length < 50 mm); HL: flag leaf in heading date; HS: stem in heading stage; HP: panicle in heading stage; SP: spikelet; EN1: endosperm under 7 days after pollination; EN2: endosperm under 14 days after pollination; EN3: endosperm under 21 days after pollination; and ML: flag leaf under mature stage

Figure 5
Time-course expression analysis of OSc2DPs after exposure to abiotic stress. a Cold. b Heat shock. c Drought. d Salt. e Alkaline. Data represent the mean $\pm$ SE of three replicates

Figure 6
Putative regulation network for miRNA-targets of OSc2DPs. The predicted targets are connected by solid lines

Figure 7

Haplotype analysis of OsC2DP17. a Structural representation of OsC2DP17 and the upstream promoter region. b LD analysis of OsC2DP17 using SNPs in a; pure red squares represent complete linkage level between each SNP. c OsC2DP17 SNPs and haplotype groups in 137 rice accessions. SNP positions are given relative to the start of the 5' UTR. Hap: Haplotype. d Haplotype network variation of OsC2DP17. Circle size represents the number of accessions in each Hap, and the number of transverse lines between each Hap represents the number of nucleotide variations. Tej: Temperate Japonica; Trj: Tropical Japonica; Ind: Indica; and Adm: Admixture rice varieties. e Association of the CT score with haplotype. f SNP haplotype analysis for the CT score. Superscripts T and C in f indicate the T and C genotypes in the present accession panel. g SeqLogo plot for cold-related motif MYCCONSUSAT (CATCTG). Size of letters represent genotype frequency in the present accession panel. Different letters indicate significant differences among haplotypes (ANOVA, Duncan test), asterisks indicate significant differences in phenotype between genotypes (Student's t-test, ***p<0.001)

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