DCW11, Down-Regulated Gene 11 in CW-Type Cytoplasmic Male Sterile Rice, Encoding Mitochondrial Protein Phosphatase 2C is Related to Cytoplasmic Male Sterility

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Causes of cytoplasmic male sterility (CMS) in plants have been studied for two decades, and mitochondrial chimeric genes have been predicted to induce CMS. However, it is unclear what happens after CMS-associated proteins accumulate in mitochondria. In our previous study of microarray analysis, we found that 140 genes are aberrantly regulated in anthers of mitochondria. In our previous study of microarray analysis, we found that 140 genes are aberrantly regulated in anthers of *Oryza sativa* L. In the present study, we investigated DCW11, one of the down-regulated genes in CW-CMS encoding a protein phosphatase 2C (PP2C). DCW11 mRNA was preferentially expressed in anthers, with the highest expression in mature pollen. As predicted by the N-terminal sequence, DCW11 signal peptide–green fluorescent protein (GFP) fusion protein was localized in mitochondria. Knockdown of DCW11 in wild-type rice by RNA interference caused a major loss of seed-set fertility, without visible defect in pollen development. Since this knockdown phenotype resembled that of CW-CMS, we concluded that the down-regulation of DCW11 is correlated with CW-CMS. This idea was supported by the up-regulation of alternative oxidase 1a (*AOX1a*), which is known to be regulated by mitochondrial retrograde signaling, in DCW11 knockout lines. Down-regulation of DCW11 and up-regulation of *AOX1a* were also observed in two other types of rice CMS. Our result indicates that DCW11 could play a role as a mitochondrial signal transduction mediator in pollen germination.

**Keywords:** Cytoplasmic male sterility (CMS) — Mitochondrial retrograde signaling — *Oryza sativa* — Protein phosphatase 2C.

**Abbreviations:** AOX, alternative oxidase; CaMV, cauliflower mosaic virus; CMS, cytoplasmic male sterility; DCW, down-regulated in CW-CMS; GFP, green fluorescent protein; PP2C, protein phosphatase 2C; PPR, pentatricopeptide repeat; RNAi, RNA interference; RT-PCR, reverse transcription-PCR; UTR, untranslated region.

**Introduction**

Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the inability of plants to produce functional pollen, and it is widely utilized for F1 hybrid breeding. Beside their commercial use, CMS studies contribute to a better understanding of the plant nuclear–mitochondrial intracellular genomic barrier. For instance, many researchers have concluded that an aberrant chimeric gene in mitochondria possibly induces CMS in various plant species. On the contrary, nuclear fertility restorers have been genetically identified to ameliorate the ectopic protein derived from a chimeric gene (Hanson and Bentolila 2004). In addition to the above research, a recent review has pointed out that the cause of CMS is thought to involve the mitochondria to nucleus signaling pathway, the so-called mitochondrial retrograde signaling pathway (Zubko 2004). For example, wheat *APETALA3* homolog *WAP3* was considerably down-regulated in alloplasmic wheat expressing the pistillody phenotype (Murai et al. 2002). Linke et al. (2003) found that MADS box genes *DcMADS2* and *DcMADS3*, which are homologous to *Antirrhinum GLOBOSA* and *DEFICIENS*, are down-regulated in CMS carrot. The *APETALA3* gene was found to be expressed ectopically in CMS *Brassica napus* (Geddy et al. 2004). Teixeira et al. (2005) found evidence of a retrograde influence of the expression levels of various homeotic genes.

Retrograde responses derived from mitochondrial stress have been widely investigated in yeast and mammalian cells (Butow and Avadhani 2004). The RTG pathway is one of the most investigated systems of mitochondrial retrograde signaling. This pathway is characterized by two basic helix-loop-helix-leucine zipper (bHLH-Zip) transcription factors, Rtg1p and Rtg3p, that are thought to function in activation of the retrograde genes. In addition, the ATP-binding protein Rtg2g acts as the mediator of mitochondrial signals to Rtg1p and Rtg3p (Sekito et al. 2000). Thus, genes that possess the function of signaling and transcriptional regulation are likely to be involved in retrograde signaling. It is likely that pathways similar to the yeast or animal stress-derived mitochondrial retrograde response exist in plants. Mitochondrial-mediated signaling in wild-type plants is perhaps essential for normal male sporophyte or gametophyte development. Such factors related to retrograde signaling could be revealed as CMS.
downstream factors in a comprehensive transcriptomic study of CMS.

Among several types of CMS in rice, we have been studying three types of gametophytic CMS: BT-CMS, LD-CMS and CW-CMS (for a review, see Fujii et al. 2008). BT-CMS originated from indica variety Chinsurah boro II (Shinjyo 1975). Although BT-CMS has been studied most extensively (Iwabuchi et al. 1993, Kazama and Toriyama 2003, Wang et al. 2006), we thought it was unsuitable for a comprehensive transcriptomic study because the pollen of the BT-CMS line shows apparent reduction of starch content and abnormal morphology. Pollen grains of the LD-CMS line, which originated from the Burnese cultivar Lead Rice, also show reduction of starch content and abnormal morphology. In contrast, pollen of the CW-CMS line, which is derived from Oryza rufipogon Griff., W1 strain, appeared to be normal but lacks pollen germination ability (Fujii and Toriyama 2005). Thus, we considered that CW-CMS should be the model for a rice CMS transcriptomic study using a microarray, so that the effects of morphological abnormality are minimally reflected. As a result, we have obtained 140 aberrantly regulated genes in a CW-CMS plant (Fujii et al. 2007). The genes differentially regulated in our CMS microarray were designated as up-regulated gene in CW-CMS (UCW) or down-regulated gene in CW-CMS (DCW). In order to identify the core factors that mediate retrograde signaling in CMS, or CMS downstream factors, we applied a reverse genetic approach in UCW and DCW genes.

In this study, we analyzed the expressional and functional characters of one of the DCW genes, DCW11, which encodes a protein phosphatase 2C (PP2C) gene. The possible role of DCW11 in CMS induction mechanism of rice is discussed.

**Results**

**DCW11 encodes a mitochondrial protein phosphatase 2C protein**

DCW11 (accession No. AK069289 in the rice full-length cDNA database, KOME, http://cdna01.dna.affrc.go.jp/cDNA/) was identified as a down-regulated gene under the CW-CMS background by our microarray analysis (Fujii et al. 2007). DCW11 is predicted to encode a PP2C family protein. The most similar protein in Arabidopsis is that encoded by At5g53140 with a similarity of 46.9% (Fig. 1). The PP2C region is fairly conserved between the two proteins.
In Arabidopsis, the PP2C family consists of 76 members (Schweighofer et al. 2004). It has been known that this gene family is involved in various biological processes such as the ABA response, the mitogen-activated protein kinase (MAPK) pathway, or even flower development (Schweighofer et al. 2004). Seventy-six Arabidopsis PP2Cs fall into 10 groups (A–J) (Kerk et al. 2002). At5g53140 is assigned to group F, but the functions of genes in group F remain unknown (Schweighofer et al. 2004). We searched for PP2C genes in rice, and found that 79 genes are predicted to contain a PP2C domain (data not shown).

The PSORT computer program (http://psort.nibb.ac.jp/) and the Mitoprot web site (http://ihg.gsf.de/ihg/mitoprot.html) predicted that DCW11 would be imported into mitochondria, and that the first 59 amino acids are possibly mitochondrial targeting signal peptides (Fig. 1, broken line). In order to verify that DCW11 localizes in mitochondria, we fused the fragment for putative signal peptides to a gene for green fluorescent protein (GFP) under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and introduced it into tobacco BY-2 cells by Agrobacterium-mediated transformation. As expected from the sequence, DCW11 signal peptide–GFP fusion protein was shown to co-localize with the Mito Tracker Red signal (Fig. 2). DCW11, therefore, was confirmed to localize in mitochondria.

DCW11 is preferentially expressed in anthers, with the highest accumulation in mature pollen

The expression specificity of DCW11 in wild-type rice was monitored in anthers at the tetrad stage, uninucleate microspore stage, bicalicular pollen stage and tricellular pollen stage, as well as in isolated tricellular pollen, stigmas, seeds, seedlings and roots using quantitative reverse transcription–PCR (RT–PCR) (Fig. 3). Expression of DCW11 increased during pollen development in anthers from the tetrad stage to the tricellular pollen stage. The highest accumulation of DCW11 mRNA was observed in tricellular pollen. The expression of DCW11 was not detected in stigmas, seeds, seedlings or roots. This expression profile indicates that DCW11 plays a role in pollen development.

Knockdown of DCW11 in Taichung 65 results in reduced seed sets and AOX1a up-regulation

To gain insight into the nature of DCW11 involvement in CMS, we produced DCW11 knockdown plants by the RNA interference (RNAi) method. The DCW11 cDNA-specific region in the 3′ untranslated region (UTR) of the gene was cloned for utilization as the trigger for RNAi. We obtained seven independent RNAi transgenic lines. In five transgenic plants, expression of DCW11 was shown to be
knocked down to 4–38% in comparison with that of the wild-type in the T₀ generation (Fig. 4A, KDDCW11-1, KDDCW11-3, KDDCW11-4, KDDCW11-5, KDDCW11-7). Seed sets of knockdown lines were reduced to 4–56%, whereas the control plants with β-glucuronidase (gus) genes were reduced to 75%. Since reduced seed fertility is often observed in the transgenic T₀ generation, we planted eight plants per line for the T₁ generation, of which DCW11 mRNA was successfully down-regulated in the T₀ generation. The seed sets in the T₁ generation were reduced to 15–49%, which was almost the same as that in the T₀ generation (Fig. 4B). T₁ plants that lost the RNAi transgenes showed normal seed-set fertility and normal DCW11 expression, which proved that reduction of seed set in knockdown plants was caused by DCW11 mRNA down-regulation. Since DCW11 mRNA was barely detected in stigmas or seeds (Fig. 2), we considered that down-regulation of DCW11 caused male sterility but did not cause dysfunction in female organs or seed maturation. This was supported by the fact that we obtained F₁ seeds by crossing wild-type pollen onto DCW11 knockdown plants (data not shown). We could not find any morphological defects in the pollen of DCW11 knockdown plants under light microscopy (data not shown). The values of pollen stainability were ≥85% in the T₁ generation of DCW11 knockdown plants, which was the same as that observed in the CW-CMS line (Fig. 4C). We suspected that DCW11 functions in pollen germination ability. This phenotype resembled CW-CMS, in which the pollen looks normal until it fails to germinate on the stigma.

Fig. 4 Characterization of DCW11 knockdown plants and three types of CMS. (A) Relative DCW11 expression level in anthers at the tricellular pollen stage monitored as described in Fig. 3. (B) Seed set and (C) pollen stainability. (D) Relative AOX1α expression level investigated using the same method as that for DCW11. Scores in each plant are shown as percentages for (A–C) (untransformed Taichung 65 = 100%), and relative expression values in (D) (untransformed Taichung 65 = 1).
These results led us to believe that DCW11 could be related to CW-CMS.

In order to obtain further evidence, we investigate the expression levels of \textit{AOX1a} mRNA in \textit{DCW11} knockout lines. \textit{AOX1a} is known to be up-regulated in the mitochondrial complex I mutant of tobacco (Sabar et al. 2000). Our previous study detected ectopic overdrive of \textit{AOX1a} in CW-CMS mature anthers (Fujii et al. 2007). To the best of our knowledge, \textit{AOX1a} is the most common nucleus-encoded marker gene for CMS. As expected, the \textit{AOX1a} mRNA level was highly overexpressed in anthers at the tricellular pollen stage in \textit{T\_0} and \textit{T\_1} knockdown lines (Fig. 4D). These data indicate that repression of \textit{DCW11} causes a CW-CMS-like effect, and DCW11 could be involved in a pathway stimulating \textit{AOX1a} expression in rice CMS.

**DCW11 is down-regulated in other CMS mature anthers**

To determine if \textit{DCW11} is involved in other CMS systems in rice, we checked the expression level of \textit{DCW11} in mature anthers of BT-CMS and LD-CMS lines, both of which exhibit the pollen abortion phenotype (Fig. 4A). We also isolated total RNA from uninucleate microspores, bicellular pollen and tricellular pollen of BT-CMS and LD-CMS lines, as well as CW-CMS and Taichung 65. The resulting total RNA was subjected to quantitative real-time RT-PCR. As a result, down-regulation of \textit{DCW11} was evident in pollen at the tricellular stage in all three CMS cytoplasms, but not in uninucleate microspores or bicellular pollen (Fig. 5). From these results, we expect that transcriptional regulation of \textit{DCW11} is common in all these CMS systems.

**Discussion**

\textit{DCW11} is involved in pollen germination

\textit{DCW11} encodes PP2C targeted to mitochondria. The function of \textit{DCW11} or an ortholog of \textit{Arabidopsis} has not yet been elucidated. We have analyzed the expression and functional characters of \textit{DCW11} in wild-type rice. Knockdown of \textit{DCW11} resulted in reduced seed set although pollen grains of the knockdown lines were visibly normal.

To date, there have been large numbers of \textit{Arabidopsis} male gametophyte mutants identified, with morphologically unaffected pollen but deficient in male transmission of the mutant allele. A calmodulin-binding protein mutant no pollen germination1 exhibited complete loss of T-DNA transmission through male gametophytes, although no obvious defects were observed in pollen development (Golovkin and Reddy 2003). Johnson et al. (2004) screened a large number of \textit{Arabidopsis} T-DNA tag-lines and identified in total 32 hapless mutants, which displayed distorted genotype segregation. Twenty-nine of them were deficient in steps later than pollen germination rather than pollen development. \textit{SETH1} and \textit{SETH2} are genes encoding glycosylphosphatidylinositol-anchoring proteins, and essential for maintenance of polarized pollen tube growth (Lalanne et al. 2004). To the best of our knowledge, there have been no reports demonstrating that a PP2C-encoding gene is required for the pollen germination process.

Although we have not yet determined whether DCW11 possesses PP2C activity or not, our results demonstrated that \textit{DCW11} mRNA is accumulated in mature pollen and functions in pollen germination. Additional information about the pollen tube growth mechanism could be obtained by searching the signaling transduction components that \textit{DCW11} is involved in.

Repression of \textit{DCW11} results in \textit{AOX1a} up-regulation

Expression of \textit{AOX1a} is well known to be regulated by mitochondrial retrograde signaling (Djajanegara et al. 2002). Zarkovic et al. (2005) designed a forward genetic screening to isolate the mitochondrial retrograde regulation factor using mutagenized \textit{Arabidopsis} carrying the firefly luciferase gene fused to the \textit{AOX1a} promoter, resulting in the \textit{Arabidopsis} plants with mutations in the genes related to mitochondrial retrograde regulation failing to activate the \textit{AOX1a} promoter under antimycin A treatment. In our case, such a forward genetic approach using a reporter gene was difficult due to the nature of CMS. We therefore applied a reverse genetic approach in \textit{DCW} genes. Since knockdown of \textit{DCW11} resulted in \textit{AOX1a} up-regulation in this study (Fig. 4D), DCW11 could be participating in suppression of \textit{AOX1a} mRNA expression under normal conditions, or the knockdown of \textit{DCW11} was causative of the
CMS-like effect. This is also supported by the fact that AOX1a is up-regulated in BT-CMS and LD-CMS, in which DCW11 was down-regulated (Fig. 4D). To the best of our knowledge, there have been no reports on AOX1a negative regulators, although Zarkovic (2005) has indicated the existence of a candidate positive regulator. Understanding the molecular functions of DCW11 would help us interpret the regulatory network of AOX1a and retrograde signaling in plants.

In mammals, a recent study on novel mitochondrial matrix-localized PP2C, PP2Cm, revealed that knockdown resulted in cell death associated with loss of mitochondrial membrane potential (Lu et al. 2007). Although DCW11 had no significant similarities to human PP2Cm (data not shown), we are not able to rule out the possibility of DCW11 participating in a part of the cell death program. Considering that DCW11 homologous At5g53140 in Arabidopsis belongs to group F of PP2C division and its function was previously unidentified, some of the members of group F could be related in mitochondrial stress signaling.

DCW11 could be the downstream factor of CMS

Although CMS is caused by genetic incompatibility between nuclei and mitochondria within male reproductive organs, only a few hints are provided for the essentiality of mitochondria in pollen development from mutant studies. Recently, the Arabidopsis mitochondrial complex II mutant sdh1-1 was isolated and was shown to have deficiency in both male and female gametophyte development (Leon et al. 2007). Pollen of the mutants develops normally until the vacuolated microspore stage, but fails to undergo mitosis I. The result indicates that mitochondrial complex II activity may be indispensable for generating a vegetative nucleus. One of the hapless mutants, hap11, was shown to encode a mitochondrial ATP synthase subunit (Johnson et al. 2004). In the case of hap11, the pollen tube growth path appears normal, but the tube fails to enter the micropyle. Since mitochondria are ubiquitous organelles and provide energy to all kinds of cells, mitochondrial mutants should exhibit embryo lethality or early developmental dysfunctions as they do in the case of the maize empty pericarp4 (EMP4) mutant (Gutierrez-Marcos et al. 2007). These authors showed that EMP4 encodes a mitochondrial pentatricopeptide repeat (PPR) protein. Many mitochondrial PPR mutants are embryonic lethal in Arabidopsis, indicating that proper mitochondrial function is critical for early plant development (Lurin et al. 2004).

Thus, why male-specific defects are observed in CMS is quite interesting. There might be a special function of mitochondria in male organ development rather than energy production, such as regulation of nuclear genes required for pollen development (for a review, see Hanson and Bentolila 2004). Since this knockdown phenotype of a defect in pollen germination ability resembled that of CW-CMS, we have concluded that the down-regulation of DCW11 is strongly correlated with CW-CMS. DCW11 could be involved in mitochondrial signal transduction in pollen development, especially in acquisition of germination ability.

Materials and Methods

Plant materials

Oryza sativa L. japonica cultivar Taichung 65 was used as the wild type throughout the study. A BT-CMS line was obtained from a successive backcross of Chinsurah Boro II and Taichung 65 (Shinjyo 1975). A CW-CMS line was derived from four backcrosses of japonica Reimei into O. rufipogon Griff., W1 strain (Toriyama and Hinata 1987), and five subsequent backcrosses of Taichung 65. An LD-CMS line was derived from nine backcrosses of japonica Akihikari into Lead Rice, followed by three successive crosses of Taichung 65. All of the CMS lines were confirmed to be occupied mainly by the Taichung 65 nuclear genome by marker-assisted selection.

Plasmid construction and plant transformation

DCW11 RNAi constructs driven by the maize ubiquitin promoter were produced using the pANDA vector provided by Dr. Miki and Dr. Shimamoto, following their protocols (Miki and Shimamoto 2004). A gene-specific region of the 400 bp 3' UTR of DCW11 (accession No. AK069289 in the rice full-length cDNA database, KOME http://cdna01.dna.affrc.go.jp/cDNA/) was PCR cloned by the primer set 5'–CACCCCCCTTGCGAAAACA GAAGAC–3' and 5'–CCGTAATGGCAGGTAATTAG–3'. Nucleotides CACC were added to the original DCW11 sequence for cloning into TOPO vector (Invitrogen, Carlsbad, CA, USA). The resulting RNAi construct was introduced into Agrobacterium tumefaciens EHA105, and further introduced into plants by the method described by Yokoi et al. (1997). We also developed rice lines with the gus gene driven by the maize ubiquitin promoter as a negative control.

Pollen stainability

Spikelets 1 d before anthesis were harvested, and pollen grains were stained with 1% (w/v) iodine-potassium iodide solution. The numbers of darkly stained pollen grains were counted for >200 pollen grains per spikelet. Pollen stainability was calculated as the average of the values of six spikelets. The seed set percentage was calculated as the average of that of the five panicles for each plant.

Quantitative RT–PCR analysis

Total RNA from various stages of anthers, pollen, stigmas, seeds, seedlings and roots was extracted using RNeasy (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. DNA contamination was eliminated by treatment of RNase-free DNase I (TAKARA-BIO INC., Ohtsu, Japan). cDNA synthesis was accomplished using the ReverTraAce (TOYOBO, Osaka, Japan). PCR was performed for three biological replicates, using SYBR Premix Ex Taq (TAKARA BIO INC.) and Thermal Cycler Dice Real Time System TP800 (TAKARA BIO INC.).
RT-PCR was performed using the specific set of primers for each amplification reaction as follows: 5'-ATGAAAGACTTGGAATGCCCTC-3' and 5'-CGTATATGCCCAGGTATAGAATT-3' for DCW11; 5'-GTCCTATGCCCAGATGTTGCATCACC-3' and 5'-CGGTATACGTTAAAAAGCTCTTCTC-3' for AOX1a (accession No. AK064040 in the rice full-length cDNA database, KOME, http://cdna01.dna.affrc.go.jp/cDNA/); 5'-TACAACGCGTGGCTCGCCAC-3' and 5'-ACATTGCACAGGAGGTCAG-3' for tubulin alpha-1 chain (accession No. AK069140 in the rice full-length cDNA database, KOME, http://cdna01.dna.affrc.go.jp/cDNA/).

Transformation of tobacco BY-2 cells

To analyze the DCW11 cellular localization, a 198 bp fragment encoding predicted signal peptides was PCR cloned by primers FORWARD: GGATCCATGTGATGCTGAGC AGCT and REVERSE: GGATCCGGTACGATCACAT CCTGG. A BamHI site was added to the original DCW11 sequence for cloning into the CaMV 35S- and GFP-containing binary vector. Transformation of BY-2 cells was performed as described by Shaul et al. (1996). Transformed cells were selected by kanamyacin treatment, and counterstained by Mito Tracker Red (Invitrogen). Cells were observed under fluorescent microscopy.

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