Transgenic Note

Cytoplasmic male sterility-associated ORF79 is toxic to plant regeneration when expressed with mitochondrial targeting sequence of ATPase γ subunit

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Abstract   Cytoplasmic male sterility (CMS) is a maternally inherited trait that results in the inability of plants to produce functional pollen. CMS is considered as a phenomenon caused by aberrant mitochondrial genomes. In BT-type CMS of rice (*Oryza sativa* L.), a CMS-associated mitochondrial gene has been reported to be an *orf79* gene. To confirm the effect of ORF79 on cell fate, we introduced the *orf79* gene with or without a mitochondrial targeting sequence into Taichung 65 carrying normal cytoplasm. It was revealed that ORF79 was toxic to plant regeneration when expressed as a fusion with mitochondrial targeting sequence of the ATPase γ subunit. This result implicates ORF79 as cytotoxic, and its toxicity depends on its combination with a mitochondrial targeting sequence.

Key words: Cytoplasmic male sterility, mitochondria, *orf79*, *Oryza sativa* L.

Cytoplasmic male sterility (CMS) is a widespread phenomenon observed in many higher plants that results in the inability of plants to produce functional pollen. CMS is a maternally inherited trait and is often associated with an aberrant mitochondrial gene. In some cases, nuclear-encoded fertility restorer genes (*Rf*s) have been reported to restore male fertility (Hanson and Bentolila 2004). To date, at least 14 CMS-associated open reading frames (ORFs) have been identified (Chase 2007), and these ORFs are considered to be newly arisen from unusual recombination events. In CMS plants, the products of such ORFs accumulate in mitochondria (Hanson and Bentolila 2004). Unfortunately, mitochondrial transformation is currently unavailable in higher plants. Hence, previous studies have attempted to introduce the CMS-associated genes into nuclear genome as a fusion to mitochondrial targeting sequence (MTS). Some people have reported that the introduction of the CMS-associated gene caused male sterility (He et al. 1996; Wang et al. 2006; Kim et al. 2007; Yamamoto et al. 2008), whereas others have failed to demonstrate the relation between the CMS-associated gene and male sterility (Wintz et al. 1995; Duroc et al. 2006; Chaumont et al. 1995). In the latter cases, they assumed that the precise form and localization of the gene products, which were essential to cause male sterility, was not achieved. Thus the mechanism in which the CMS-

associated protein causes male sterility is still unknown.

A BT-type CMS derived from the cytoplasm of Chinsurah Boro II and nucleus of Taichung 65 is the most widely studied CMS/*Rf* system in rice (Kazama et al. 2008). The mitochondrial genome of BT-type cytoplasm contains two duplicated copies of the *atp6* gene encoding a subunit of the ATPase complex (Kadowaki et al. 1990; Iwabuchi et al. 1993; Akagi et al. 1994). It has been reported that a unique sequence (*orf79*) located downstream from one of the *atp6* genes is responsible for pollen sterility (Wang et al. 2006). The *orf79* gene encodes a predicted transmembrane protein with a novel C-terminal region and an N-terminus showing similarity to the cytochrome oxidase subunit I (COX I; Iwabuchi et al. 1993; Akagi et al. 1994). Wang et al. (2006) have reported that introduction of *orf79* fused to mitochondrial targeting sequence into Taichung 65 (T65) caused pollen sterility. However whether accumulation of ORF79 protein was responsible for pollen sterility was not reported.

To reconfirm the effect of ORF79 on rice carrying normal cytoplasm, we introduced the *orf79* gene with or without a mitochondrial targeting sequence (MTS) into T65 via *Agrobacterium*-mediated gene transfer. Here we report submitochondrial localization and toxicity of ORF79 during plant regeneration.

Total cellular soluble protein was extracted from calli

in 10 mM Tris-HCl (pH 7.4). Mitochondria were purified from rice callus by the method of Tanaka et al. (2004). Total mitochondrial protein was dissolved in 10 mM Tris-HCl (pH 7.4) by sonication. To determine the submitochondrial localization of ORF79, the purified mitochondria were separated into membrane and soluble fractions (Nivison and Hanson, 1989). Proteins derived from each fraction were analyzed on SDS-PAGE immunoblot using antibody raised to recombinant ORF79 (Kazama et al. 2008). The recombinant ORF79 lacking the N-terminal region homologous to COX I was expressed in *Escherichia coli* and used as a positive control as described (Kazama et al. 2008). Anti-sera against cytochrome oxidase II (COX II) and isocitrate dehydrogenase (IDH) were obtained from Agrisera (Vännäs, Sweden).

Transformation vectors used in this study are shown in Supplemental figure 1. *Orf79* was expressed under the control of the Maize *Ubiquitin* promoter, which is active in all tissue including pollen. N-terminal coding regions of *Rf1a*, restoration of fertility gene of BT-CMS (Kazama et al. 2003, Wang et al. 2006), or gene for the ATPase γ subunit ($\gamma ATPase$) of *Arabidopsis thaliana* (Niwa et al. 1999) were used as MTS. Transformation vectors in which *orf79* was replaced by *GFP* (Green Fluorescent Protein; Niwa et al. 1999) were also used as controls. These constructs were introduced into T65 via *Agrobacterium*-mediated gene transfer (Yokoi et al. 1997).

At first we examined the submitochondrial localization of ORF79 in the BT-CMS line (Figure 1). Separation of membrane fraction and soluble fractions was confirmed using antiserum against COX II and IDH, respectively. The immunoblot analysis detected ORF79 as a 7.9-kDa protein in mitochondrial membrane fraction but not in soluble fraction. ORF79 was not detected in T65. In the

BT-CMS line, ORF79 accumulated in mitochondrial membrane fraction.

In transgenic calli, ORF79 protein was detected in mitochondria when fused to *Rf1a* MTS or $\gamma ATPase$ MTS (Figure 2). Unexpectedly, ORF79 without MTS was also detected in mitochondria as well as in total cellular soluble fraction (Figure 2A). Next we investigated submitochondrial localization of ORF79 in the transgenic lines. While ORF79 was localized in both mitochondrial soluble and membrane fractions in all transgenic line, ORF79 was preferentially localized in mitochondrial membrane fraction when fused to $\gamma ATPase$ MTS or no MTS (Figure 2A). These results suggested that ORF79 might have mitochondrial targeting signal-like sequence, which might play a role in

Figure 2. Localization of ORF79 protein and predicted cleavage of MTS in transgenic lines. (A) Submitochondrial localization of ORF79 in transgenic lines detected by SDS-PAGE immunoblot analysis. Cellular total, total cellular soluble protein; Mt total, mitochondrial total protein; Mt soluble, mitochondrial soluble fraction; Mt membrane, mitochondrial membrane fraction. COX II and IDH were used for controls of mitochondrial membrane and soluble fractions, respectively. (B) Comparison of the size of ORF79 in BT-CMS and in transgenic lines with MTS, and schematic model of predicted cleavage site of MTS. Protein extracted from mitochondria membrane fraction was loaded. Lane 1, BT-CMS; lane 2, *Rf1a* MTS *orf79*; lane 3, $\gamma ATPase$ MTS *orf79*. Arrowhead in bottom panel indicates the cleavage site of MTS predicted by MITOPROT (http://ihg2.helmholtz-muenchen.de/ihg/mitoprot.html), a program of prediction of mitochondrial targeting sequence.
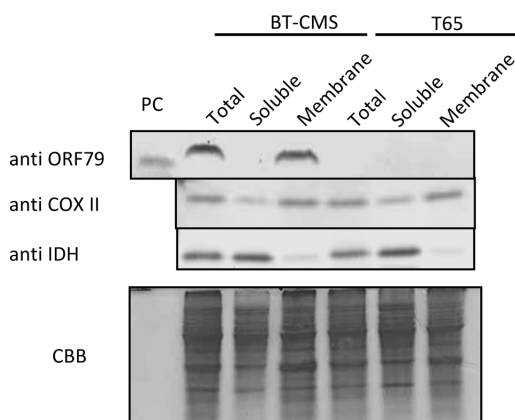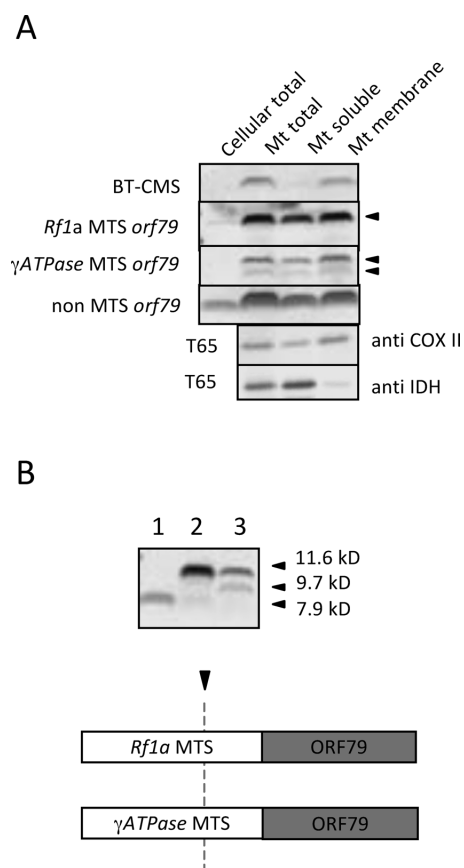
Figure 1. Localization of ORF79 protein in BT-CMS and T65 detected by SDS-PAGE immunoblot analysis. PC, Recombinant ORF79; Total, mitochondrial total fraction; Soluble, mitochondrial soluble fraction; Membrane, mitochondrial membrane fraction. The lower panel shows the Coomassie Blue (CBB) staining.
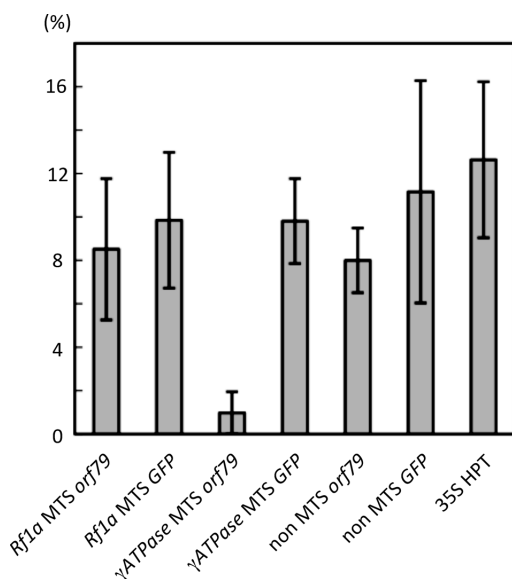
Figure 3. Plant regeneration rate from calli of each transgenic line. The rate of plant regeneration was calculated from the number of regenerated plants out of the number of hygromycin-resistant calli placed on the regeneration medium. Calli were grown on the medium for 90 days. Each value represents the mean±SE (n=5).

its targeting cooperation with *Rf1a* or *γATPase* MTS. Indeed the amino acid sequence of ORF79 has a hydrophobic transmembrane domain and is predicted to be localized in mitochondrial membrane fraction based on PSORT (http://psort.ims.u-tokyo.ac.jp/form.html) programs. After transcribed in nucleus and translated in cytoplasm, ORF79 might be targeted to mitochondrial membrane. While *Rf1a* protein is targeted to mitochondrial matrix (unpublished data), the *ATPase γ* subunit has been reported to be superficial to the inner membrane on the side of matrix (Paga et al. 2000). This difference of each MTS might be responsible for preferential membrane localization of ORF79 in *γATPase* MTS transgenic plants.

The size of ORF79 in transgenic lines with MTS was larger than that of the BT-CMS line (Figure 2B). With *Rf1a* MTS, ORF79 was detected at 11.6 kD, indicating failure in cleavage of MTS. With *γATPase* MTS, ORF79 was detected at 11.6 kD and 9.7 kD, suggesting that incomplete cleavage of MTS or the presence of extra amino acids downstream of the expected cleavage site.

Plant regeneration from the transgenic calli was greatly inhibited in transgenic lines with *γATPase* MTS *orf79* (Figure 3). Although one plant was regenerated from callus introduced with *γATPase* MTS *orf79*, this callus did not accumulate ORF79. The rate of plant regeneration was calculated from the number of regenerated plants out of the number of hygromycin-resistant calli placed on the regeneration medium (Figure 3). When fused to *γATPase* MTS, introduction of *orf79* reduced the regeneration rate, but the rate in the transgenic line with *GFP* was identical to that with a

vector control, *35S HPT*, indicating that ORF79 acted as a cytotoxic protein during plant regeneration. Introduction of *orf79* with *Rf1a* MTS or no MTS, however, did not inhibit plant regeneration, suggesting that ORF79 toxicity might depend on its combination with MTS.

To examine the possible effect of ORF79 on pollen fertility, we observed pollen grains of transgenic T_1 plants carrying a single copy of hemizygous transgene with *Rf1a* MTS or no MTS. Accumulation of ORF79 was confirmed in calli induced from T_1 seeds and mature anther of T_1 plants (Supplemental figure 2). The expected pollen fertility was 50% or less if ORF79 caused pollen lethality. One plant with *Rf1a* MTS *orf79* showed pollen fertility of 50% (column 3, Supplemental figure 3). Other transgenic lines showed more than 70% pollen fertility, indicating that ORF79 did not affect pollen fertility. The fertility of pollen grains with the transgene was further confirmed in these transgenic lines, including the plant in column 3, by the segregation ratio of hygromycin resistant and sensitive plants, which fit to 3 : 1, in the self-crossed progeny (data not shown), whereas it was expected to be 1 : 1 assuming that the transgene was not transmitted from pollen.

We were unable to observe the pollen grains carrying *γATPase* MTS *orf79*, because transgenic plants were not regenerated. This observation would have been achieved if *orf79* was driven by pollen-specific promoter. Although we could not demonstrate toxic effects of ORF79 on pollen fertility, gametophytic pollen sterility has been reported in transgenic T65 transformed with MTS *orf79* under the control of CaMV 35S promoter (Wang et al. 2006). Their study also identified two restoration fertility genes, *Rf1a* and *Rf1b*, both of which restored male fertility. They used the N-terminal coding region of *Rf1b* as a MTS including an extra 83 amino acids downstream of the predicted cleavage site, whereas our study used the N-terminal coding region of *Rf1a* including an extra 19 amino acids. The difference of MTS might have caused the slight difference in ORF79 submitochondrial localization. In the study of Wang et al (2006), ORF79 was found to be possibly localized in a certain place of mitochondria where the protein specifically damages pollen development, although ORF79 accumulation in their transgenic plants has not been demonstrated. We concluded that precise localization of ORF79 within mitochondria might be an important factor causing pollen sterility. More detailed investigation of ORF79 localization would help with better understanding of the mechanism of how ORF79 causes male sterility.

Acknowledgements

References

Abe F, Sito K, Miura K, Toriyama K (2002) A single nucleotide polymorphism in the alternative oxidase gene among rice varieties differing in low temperature tolerance. *FEBS Lett* 527: 181–185

Akagi H, Sakamoto M, Shinjo C, Shimada H, Fujimura T (1994) A unique sequence located downstream from the rice mitochondrial *atp6* may cause male sterility. *Curr Genet* 25: 52–58

Chase CD (2007) Cytoplasmic male sterility; a window to the world of plant mitochondrial-nuclear interactions. *Trends Plant Sci* 23: 81–90

Chaumont F, Bernier B, Buxant R, Wiliams ME, Levings CS, Boutry M (1995) Targeting the maize T-*urf13* product into tobacco mitochondria confers methomyl sensitivity to mitochondrial respiration. *Proc Natl Acad Sci USA* 92: 1167–1171

Duroc Y, Gaillard C, Hiard S, Tinchant C, Berthome R, Pelletier G, Budar F (2006) Nuclear expression of a cytoplasmic male sterility gene modifies mitochondrial morphology in yeast and plant cells. *Plant Sci* 170: 755–767

Hanson MR, Bentolila S (2004) Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell* 16: S154–S169

He S, Abad AR, Gelvin SB, Mackenzie SA (1996) A cytoplasmic male sterility-associated mitochondrial protein causes pollen disruption in transgenic tobacco. *Proc Natl Acad Sci USA* 93: 11763–11768

Iwabuchi M, Kyozuka J, Shimamoto K (1993) Processing followed by complete editing of an altered mitochondrial *atp6* RNA restores fertility of cytoplasmic male sterile rice. *EMBO J* 12: 1437–1446

Kadowaki K, Suzuki T, Kazama S (1990) A chimeric gene containing the 5′ portion of *atp6* is associated with cytoplasmic male-sterility of rice. *Mol Gen Genet* 224: 10–16

Kazama T, Toriyama K (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. *FEBS Lett* 544: 99–102

Kazama T, Nakamura T, Watanabe M, Sugita M, Toriyama K (2008) Suppression mechanism of mitochondrial ORF79 accumulation by *Rf1* protein in BT-type cytoplasmic male sterile rice. *Plant J* 55: 619–628

Kim DH, Kang JG, Kim BD (2007) Isolation and characterization of the cytoplasmic male sterility-associated *orf456* gene of chili pepper (*Capsicum annuum* L.). *Plant Mol Biol* 63: 519–532

Nivison HT, Hanson MR (1989) Identification of a mitochondrial protein associated with cytoplasmic male sterility in petunia. *Plant Cell* 1: 1121–1130

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green fluorescent protein in living plants. *Plant J* 18: 455–463

Paga S, Zanotti F, Gaballo A (2000) The structural and function between the catalytic and proton translocating sectors of the mitochondrial F1F0-ATP synthase. *J Bioenerg Biomembr* 32: 401–411

Tanaka N, Fujita M, Handa H, Murayama S, Uemura M, et al. (2004) Proteomics of the rice cell: systematic identification of the protein populations in subcellular compartments. *Mol Genet Genomics* 271: 566–576

Wang Z, Zou Y, Li X, Zhang Q, Chen L, et al. (2006) Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. *Plant Cell* 18: 676–687

Wintz H, Chen HC, Sutton CA, Conley A, Cobb A, Ruth D, Hanson MR (1995) Expression of the CMS-associated *urfS* sequence in transgenic petunia and tobacco. *Plant Mol Biol* 28: 83–92

Yamamoto MP, Shinada H, Onodera Y, Komaki C, Mikami T, Kubo T (2008) A male sterility-associated mitochondrial protein in wild beets causes pollen disruption in transgenic plants. *Plant J* 54: 1027–1036

Yokoi S, Tsuchiya T, Toriyama K, Hinata K (1997) Tapetum-specific expression of the *Osg6B* promoter-*β*-glucronidase gene in transgenic rice. *Plant Cell Rep* 16: 363–367