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Effect of fertility restorer gene, *Rf2*, on mitochondrial RNA and proteins in Lead Rice-type cytoplasmic male sterile rice cells

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Summary

Cytoplasmic male sterility (CMS) is a maternally inherited trait in plants leading to dysfunctional pollen production and infertility. It is both useful in the breeding of hybrids and is an example of nucleus-mitochondrion cross talk in plants.
In Lead Rice-type (*Oryza sativa* L.) CMS is caused by an *atp6-orf79* gene, with an unusual, cytotoxic open reading frame, *orf79*, coupled to the mitochondrial *atp6* gene. Fertility is restored by a nuclear fertility restorer gene, *Rf2*. The mechanism of the protein product RF2 is however not fully understood. In this experiment I analyzed the protein and RNA content in *Rf2* over-expressing LD-CMS rice cells and observed that in lines with high expression of *Rf2*, *atp6-orf79* RNA was processed before any ORF79 was produced, indicating that RF2 acts by degrading the *atp6-orf79* RNA before translation.

Introduction

Cytoplasmic Male Sterility in rice

Genetic regulation between the nucleus and organelle genomes, so called anterograde regulation, is very important in eukaryotes. Likewise, retrograde signaling from organelles plays a big role in regulating nuclear gene expression. In plants, since they are prone to cross pollination, genome conflicts between the nucleus and a foreign cytoplasm can easily happen, leading to developmental disorders (Fujii & Toriyama 2008). Cytoplasmic Male Sterility (CMS) is such a trait in plants, caused by mitochondrion-nucleus incompatibility which leads to faulty gene regulation and finally a failure for the plant to produce functional pollen. Studies of the CMS system have contributed to revealing the importance of retrograde signaling since it was found that expression of a large number of nuclear genes differ between CMS and wild type lines (Carlsson et al. 2007). CMS is considered to have originally arisen in wild type plants through mutations, and the alleles have been conserved since it might acts as a population regulator. Restorer systems have thus co-evolved with the infertility-causing genes. (Hanson & Bentolila 2004). CMS can also be induced by crossing plants of different lines, so that the nucleus and mitochondrion regulation systems are mismatched. Because the CMS line has sterile pollen, so self-pollination can be avoided, this has commercial applications for example in the production of hybrid varieties (Mackenzie 2004). In many crops it is not necessary to restore the fertility of the hybrid line because seed production does not affect the harvested product, and it is more profitable for the seed companies if the farmers need to buy new seeds every season. In rice and other cereals however, it is necessary to restore fertility in the hybrid because a stable yield relies on the plants ability to produce seeds through self-pollination.

Understanding the fertility restorer systems of rice is especially of big importance in Asia, where the main cereal is rice, and the mechanisms in different rice varieties are currently under research. One of the most well studied fertility restorer systems in *Oryza sativa japonica* rice is the Chinsurah Boro II type BT-CMS/*Rf1* system. A fertility restorer gene has been identified and named *Rf1* (*restorer of fertility*) and the mechanism of action has been outlined. The restorer gene is a nuclear gene coding for a mitochondrion targeting protein involved in post-transcriptional regulation of the CMS causing gene (Kazama et al. 2008). A rice variety that has been shown to have a similar CMS causing gene and fertility restorer system as BT-CMS is the Lead Rice- type (LD)/*Rf2* type (Itabashi et al. 2011).

Infertility-causing open reading frames

In BT-CMS plants infertility is caused by the expression of a chimeric *atp6-orf79* gene in mitochondria. The open reading frame *orf79* is homologous in it's 5' region to *cox1*, the mitochondrial gene encoding cytochrome oxidase subunit 1 (Akagi et al. 1994). It's 3' region is of unknown origin and encodes a cytotoxic transmembrane protein which causes sterility by impeding pollen development (Wang et al. 2006). Infertility in LD-CMS plants is caused by a similar *atp6-orf79* gene to that of BT-CMS, differing only in a single nucleotide polymorphism in *orf79* and a 4bp insertion between *atp6* and *orf79*. Another difference between the two lines is that the BT-CMS mitochondria have two copies of the *atp6* gene, also possessing a normal copy of the *atp6* without the *orf79*. LD-CMS mitochondria however, only carry the *atp6-orf79* locus (Itabashi et al. 2009). A model comparing the infertility-causing genes in LD-CMS and BT-CMS lines can be seen in Figure 1.

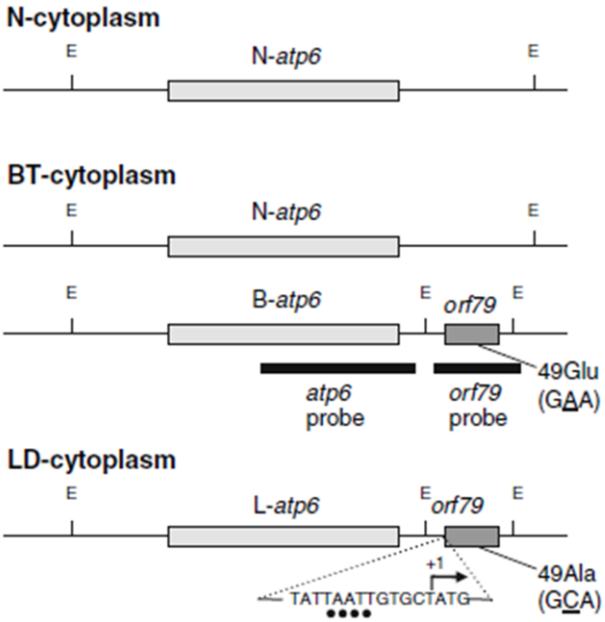

Figure 1. Schematic diagram of the *atp6-orf79* locus in normal *Oryza sativa Japonica* mitochondrial genome, compared with BT-CMS and LD-CMS varieties. N-*atp6* is the normal *atp6* gene and B-*atp6* is the duplicate with *orf79* present in BT-type cytoplasm. L-*atp6* is the locus present in LD-type cytoplasm. E, *Eco* RI site. (Adapted from Itabashi et al. 2009)

Fertility restorer genes

In CMS plants fertility can be restored either gametophytically or sporophytically. In most cases the restorer gene acts gametophytically, including BT and LD-CMS plants (Itabashi et al. 2009). Gametophytic restoration means that the individual pollen grain will determine their own fertility, depending on if they contain a functional restorer gene or not. Thus, in a restored line 50% of the T1 generation pollen will be infertile while fertility has been totally restored in the T2 generation (Fujii et al. 2008). The general principle of fertility restoration is that the nuclear restorer gene processes the RNA of infertility-causing ORF by regulation of mitochondrial gene expression. In BT-CMS the restorer of fertility, *Rf1*, encodes a pentatricopeptide repeat (PPR) containing protein, which locates to mitochondria and binds to RNA. Most fertility restorer systems consist of a PPR protein, exhibiting RNA-binding properties (Hu et al. 2012). It has been shown that RF1 binds to B-*atp6-orf79* RNA, in the region between *atp6* and *orf79*, and

processes it before translation. Wang et al. (2006), outlined the mechanism of two different fertility restorer genes, RF1A and RF1B. They showed that RF1A acts by endonucleolytic cleaving of the B-*atp6-orf79* transcript, and RF1B acts by degrading the B-*atp6-orf79* transcript. Kazama et al. (2008) showed that only the cleaving RF1 is required for fertility restoration, even though ORF79 accumulation was only reduced to 50%. These results show that reducing the amount of the infertility-causing ORF79 is the critical function of the fertility restorer protein.

In LD-CMS plants, restorer of fertility *Rf2* has been identified, but the mechanism of its protein product is not yet fully known. RF2 is a glycine-rich protein (GRP) containing a mitochondrial targeting sequence, but no known RNA-binding motif. It consists of 152 amino acids, with a glycine-rich region (Itabashi et al. 2011), which is hypothesized to be the functional region of the protein. GRPs are proteins containing more than 60% glycine, including plant cell wall proteins and RNA-binding proteins. Glycine-rich regions are often associated with other proteins in multi-protein complexes (Mousavi & Hotta 2005). It was recently discovered that a GRP, GRP162, in complex with a PPR-containing fertility restorer protein, RF5, restores fertility in Hong-Lian CMS rice as a fertility restoration complex (Hu et al. 2012). The GRP protein in that case contains the RNA-binding motif, but no mitochondrial targeting sequence. It is possible that RF2 functions in a similar way, but it is still not established at which step it prevents the production of the infertility-causing ORF79. Itabashi et al. (2009) could not detect ORF79 accumulation in the LD-CMS line, and concluded that the sterility induction and fertility restoration system in LD-CMS is different from that of BT-CMS. In later studies, with other anti-ORF79 antibodies, accumulation of the protein could be detected in LD-CMS. This result is still unpublished, but the same antibody is used in this project. Because of the similarity of the *atp6-orf79* transcripts of LD-CMS and BT-CMS lines it is likely that they are processed by a similar mechanism, even though the RF proteins differ.

Aims

The question I tried to answer in this project is: What is the function of RF2? Does it act by preventing transcription of *orf79*, by processing of the *atp6-orf79* transcript, by preventing translation of *orf79* or by destabilization of ORF79? If *atp6-orf79* RNA could be detected in samples where ORF79 was absent it would mean that RF2 acts by destabilizing the protein. I analyzed protein and RNA content in LD-CMS rice callus cells trough western and northern blotting and concluded that RF2 acts by RNA degradation.

Results

I used 5 lines of *Oryza sativa japonica* callus cells with LD-CMS type overexpressing *Rf2* under the control of ubiquitin promoter (Ubi::*Rf2*), including a sample of wildtype LD-CMS cells.

Detection of the transferred *Rf2* gene and *Rf2* expression

To confirm the presence of the *Rf2* transgene in the callus cells I extracted total DNA and conducted PCR with *Rf2* primers (Fig. 2). The DNA analysis detected the Rf2 gene in all lines except for line 4. PCR with actin primers confirmed the quality of the DNA and similar loading size of the samples.

I extracted total RNA and carried out RT-PCR analysis to check the expression of *Rf2* in each sample (Fig. 3). *Rf2* expression was detected in all samples except for 2 and 4. Based on the intensity of the bands, expression level is higher in lines 1 and 3 and weak in line 5.

An RT-PCR analysis with Hygromycin resistance (HM) primers was also carried out, because the vectors used to induce *Rf2* expression contain the HM resistance gene as a selection marker. The analysis with HM resistance primers shows transgene expression in all samples except for line 4 (Fig. 3).

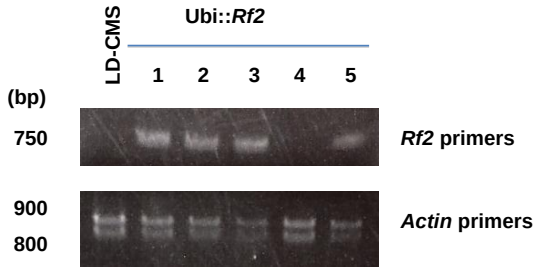

Figure 2. PCR analysis of total DNA extracted from Rf2 overexpressing LD-CMS rice cells. Rf2 primers were used to check transgene expression and actin primers as sample control. The samples were separated in 1% agarose gel and stained with EtBr.

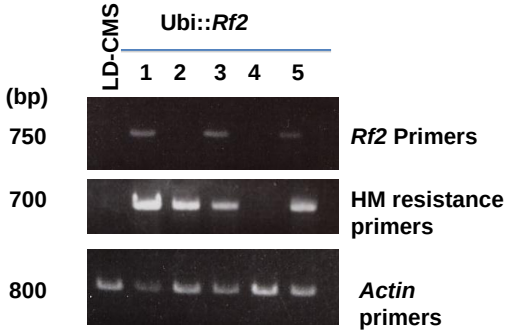

Figure 3. RT-PCR analysis of total RNA extracted from Rf2 overexpressing LD-CMS rice cells using Rf2 primers and Hygromycin resistance primers to visualize transgene presence and Actin primers as sample control. The samples were separated in 1% agarose gel and stained with EtBr.

Detection of ORF79 protein

A western blot analysis with anti-ORF79 antibodies was carried out in order to detect ORF79 protein (Fig. 4). ORF79 was detected in lines 2, 3 and 5 as well as in the wild LD-CMS wild type sample, at size 7.9 kD. The amount of ORF79 in lines 2 and 3 was less than that of line 5. In line 1, ORF79 could not be detected, and is assumed to have been broken down in the presence of RF2. In line 4, ORF79 could also not be detected, even though RF2 is absent in this line.

Anti-isocitrate dehydrogenase (IDH), a cellular compartment marker for mitochondrial matrix with a molecular weight of 45 kD, was detected equal amounts. This indicates equal loading of protein in each sample (Fig. 4).

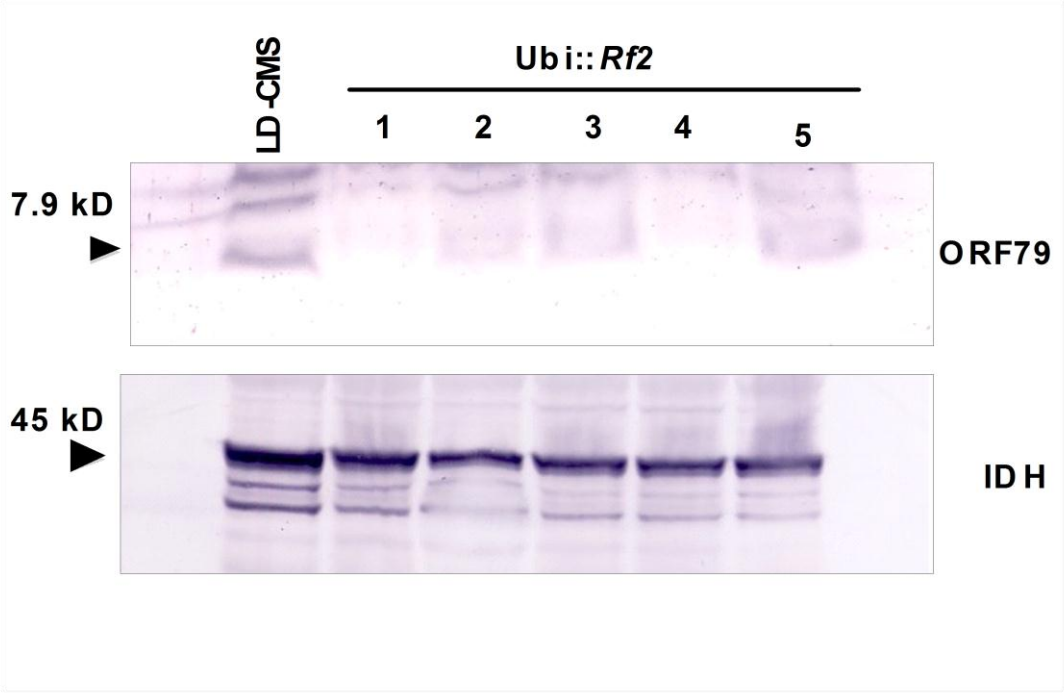

Figure 4. Western analysis of mitochondrial proteins extracted from Rf2 overexpressing LD-CMS rice cells using anti-ORF79 antibodies. The control using anti-IDH confirms equal loading of protein in each sample (size 45 kD).

Detection of *atp6* and *orf79* transcripts

Northern hybridization was carried out in order to detect the state of the *atp6-orf79* transcript in each line (Fig. 5)

The *atp6* probe detected an intense band at 1.5 kb in all the lines, which corresponds to the size of *atp6* RNA. A weaker band was detected at 2.0 kb in line 2, 3, 5 and the LD-CMS, corresponding to co-transcribed *atp6-orf79*. This band was also detected by the *orf79* probe in the same samples, confirming that it is the co-transcribed transcript.

Orf79 RNA was only detected in the same samples in which ORF79 was detected in the western analysis.

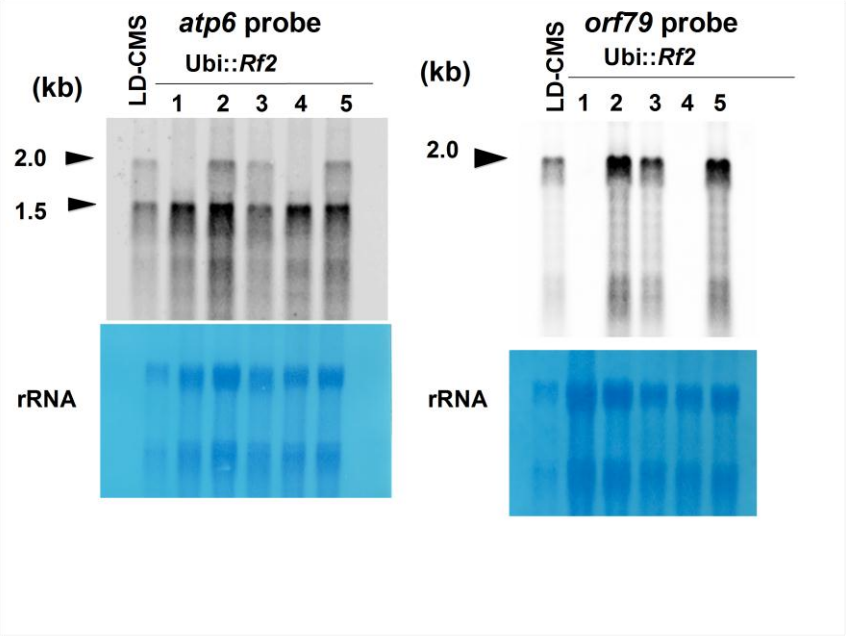

Figure 5. Northern hybridization results with atp6 and orf79 probes used on RNA extracted from Rf2 overexpressing LD-CMS rice cells. The arrows show 2.0 kb, which is the length of atp6-orf79 and 1.5 kb which is the length of atp6.

Discussion and conclusion

The question that I wanted to answer with this study was how RF2 restores fertility in LD-CMS lines. The *Rf2* expression levels and amount of ORF79 protein and *atp6-orf79* transcript was very varied between the lines. This is because the lines used in this study might contain different copy number of the *Rf2* transgene. If the T1 line carries a single transgene, then 50% of the the T2 lines might carry *Rf2* homozygously (*Rf2*, *Rf2*) and 50% might carry the gene heterogously (*Rf2*, -). Because *Rf2* gametophytically restores fertility, 100% of the T2 generation will be fertile even though the expression level of *Rf2* might be different between the lines. In line 2, *Rf2* expression was not detected by RT-PCR although the *Rf2* transgene was present. In this line the *orf79* transcript does not seem to have been processed. It is possible that the transgene lost its function over time during the callus development. In line 4, the *atp6-orf79* transcript and ORF79 were not detected even though the transgene was not detected. Since genomic PCR and RT-PCR was carried out with a time delay after the western and northern analysis it is possible that the transgene could have fallen away over time, as usually happens in transgenic cell cultures. In the other samples varying levels of *Rf2* expression was observed. I assume that the *atp6-orf79* transcript was totally degraded in line 1, under high expression of *Rf2*. In line 3 the *Rf2* expression was weaker, and the *atp6-orf79* transcript is weakly degraded. Expression of *Rf2* in line 5 is likely too weak to degrade the *atp6-orf79* transcript. Tables 1 summarizes the results of my study. Line 4 is not included, because of the absence of the *Rf2* transgene.

Table 1. Summary of Rf2 expression, degradation of atp6-orf79 transcripts and accumulation of ORF79 protein.

Line	**LD-CMS**	**1**	**2**	**3**	**5**
Rf2 expression level	-	+++	-	++	+
Degradation of *atp-orf79*	-	+++	-	++	+
ORF79 accumulation	++++	-	+	++	+++

My results indicate that two types of RNAs are transcribed from the single *atp6-orf79* locus in LD type cytoplasm. One is the 2.0 kb transcript of *atp6-orf79* and the second one is the 1.5 kb transcript of *atp6*. Because *atp6* was detected in all samples, RF2 does not affect the *atp6* transcript. Selective transcription of *atp6* already takes place, so it is most likely that the added effect of RF2 happens after RNA transcription. I conclude that RF2 play a role in degrading the *atp6-orf79* transcripts before translation.

I constructed a simple model of the different transcriptions at the CMS-causing locus (Fig. 6). In both fertility restored and CMS plants *atp6* is transcribed from the *atp6-orf79* locus. In CMS plants the whole *atp6-orf79* is also transcribed and both are translated. In fertility restored cells with *Rf2* whole *atp6-orf79* transcripts are degraded before translation of *orf79* RNA.

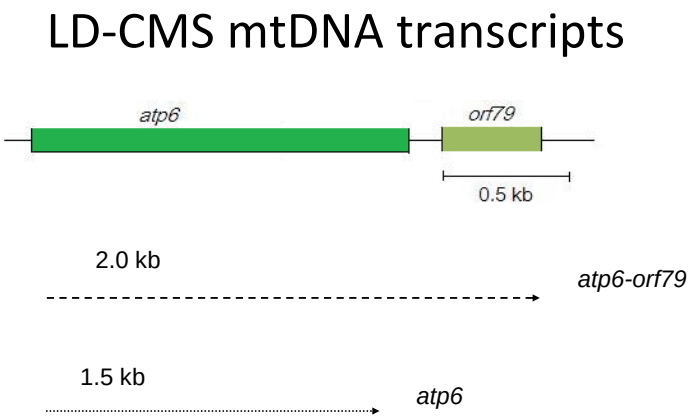

Figure 6. Model of the transcription ways in LD-CMS at the CMS-causing locus

The function of RF2 is not yet determined. RF2 has no RNA-binding motif (Itabashi et al. 2011), so its direct interaction with *atp6-orf79* has not been confirmed, but according to these results its function could be to process *atp6-orf79* RNA by inhibiting translation, leading to degradation of the *atp6-orf79* transcript. In order to confirm the interaction and to determine if RF2 acts on the RNA transcript an *in vitro* binding assay between RF2 and *atp6-orf79* RNA and DNA should be conducted. If RF2 binds to *atp6-orf79* RNA *in vitro* it confirms that it acts post-transcriptional by facilitating the degradation of *atp6-orf79*. If it binds to the DNA it confirms that RF2 acts by inhibiting transcription of *orf79*. If it binds to neither some unknown co-factors might be involved. Hu et al (2012) proved that fertility restoration is regulated by a fertility restoration complex in the HL-CMS line, consisting of a PPR containing RF5 and an RNA-binding GRP. This might also be the case in LD-CMS. The samples in which RF2 could not degrade *atp6-orf79*, might not express the right cofactors, which samples 1 and 4 might be doing. The gene regulation mechanism of *atp6* is also still unknown, with parallel

transcription of *atp6* and *atp6-orf79*. Further studies should therefore focus on answering the question of how *atp6* transcription is regulated and to confirm if RF2 acts after transcription.

Materials and methods

Samples

The *Rf2* overexpressiong LD-CMS *Oryza sativa japonica* callus cells that I received were produced as follows:

Rf2 cDNA including 5' untranslated region was PCR-cloned by using cDNA template synthesized from anthers at the tricellular stage of F3 plants between LD-Akihikari and CSSL204 carrying *Rf2* homozygously. The primer pair used to amplify *Rf2* was

5'-GGATCCGCCCTCAGCAGCAGGATCCAC-3' and

5'-GGATCCTTATTGTTGGAACATATCAT-3'. *Bam* HI sites are underlined. The Bam HI-digested fragment was cloned into the *Bam* HI site downstream the maize ubiquitin promoter in the DHA6His binary vector (Kagaya et al. 2002). This construct was introduced into LDA by Agrobacterium-mediated method (Itabashi et al. 2011). Callus from T2 seeds was induced and the cells were grown on solid N6CI medium and kept in a growth chamber under light (Kazama et al. 2008).

Total RNA and DNA analysis

Total RNA extraction from callus cells

In order to check the expression levels of *Rf2* in the samples callus cells from five lines samples of *Oryza sativa* with LD-CMS (Ubi::Rf2) were used, as well as a control LD-CMS wildtype sample. RNA extraction was carried out with the RNeasy Mini Kit (Qiagen), following the protocol for "Purification of Total RNA from Plant Cells and Tissues and Filamentous Fungi". The protocol was used as follows:

1. 100 mg of each callus sample was picked to a microtube in a clean bench and weighed.
2. Because callus cells have a high water content, liquid nitrogen freezing was not used. Instead, the callus cells were put in a mortar cooled on ice, 400 µl RLT buffer and 4.5 µl β-merceptoethanol was added, and the sample grinded to a fine paste.
3. The paste was transferred to a microtube by pipetting, vortexed and centrifuged at max speed (20,352 *g*) for 5 min.

Steps 4-8 followed the protocol.

9. Step 8 was repeated and the optional step 10 was carried out.
11. After placing the RNeasy spin column in a new microtube 57 µl of RNase free water was added directly to the membrane and the samples were incubated at room temperature for 5 min. Finally the samples were centrifuged for 2 minutes at max speed

and the flow through containing the extracted RNA was kept in freezer.
To check the quality of extracted RNA a 1% agarose gel electrophoresis was carried out and RNA detected with EtBr staining. Contaminating DNA was removed by incubating the samples with DNase I (50 µl RNA sample, 10 µl 10xDNase I buffer, 5 µl DNase I (TaKaRa), 35 µl RNase free DDW) at 37°C for 2 hours. Then 100 µl phenol-chloroform was added to remove the DNase, the samples centrifuged for 5 min at 20,352 *g* and the upper layer transferred to new microtubes. Finally the samples were concentrated by ethanol precipitation. The extracted RNA was used for RT-PCR to confirm expression of *Rf2*.

RT-PCR

Reverse Transcription was carried out with the kit SuperScript III (Invitrogen). 1 µg RNA sample was mixed with 5 µl dT primer, 1 µl dNTP and 7 µl DDW in a PCR tube. The samples were incubated at 65°C for 5 min then chilled on ice for 1 min. In a mictrotube the RT reaction mix was made with 4 µl Buffer, 0.1 M DTT, 1 µl RNase inhibitor (Invitrogen) and 1 µl SuperScript III Reverse Transcriptase (Invitrogen). 7 µl was added to each PCR tube, and the tubes were incubated at 50°C for 60 min for the reaction, 70°C for 10 min to stop the reaction and finally incubated at 12°C for cool down.
The samples were then amplified through normal PCR with 1 µl of the sample 2 µl 10x rTaq buffer (TaKaRa), 2 µl dNTP, 1 µl each of forward and reverse primers, 0.1 µl of rTaq (TaKaRa) and 12.9 µl DDW(H_2O). The PCR program started with 94°C for 1 min, then 30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec, finished by 72°C for 2 min. One PCR was carried out with *actin* primers [RAc1; AACTGGGATGATATGGAGAA/ RAc2; CCTCCAATCCAGACACTGTA] as control, one with the transgenic primers for *Rf2* [K7-genome9; GGTTCACAATTTCAGACATCT/NOSterR.2: AAGACCGGCAACAGGATTCA] and one with transgenic primers for Hygromycin resistance [HPTf; GAGAGCCTGACCTATTGCAT/ HPTr; TCGGCGAGTACTTCTACACA].

Total DNA extraction from callus cells

In order to confirm the presence or absence of the transgene in the samples a total DNA extraction was carried out. Callus from each sample was picked in a clean bench into a microtube. 400 µl DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added and each sample grinded with a plastic pestle. The samples were centrifuged at 13,025 *g* for 5 min, then 300 µl was transferred to a new tube and 300 µl of phenol chloroform was added. The tubes were vortexed and

centrifuged again under the same conditions. 150 µl was transferred from the upper layer into a new mictrotube and to concentrate the samples 150 µl of 2-propanol was added. The samples were mixed by inverting the tube a few times and centrifuged again. The liquid was discarded, then the samples were washed by adding 500 µl of 70% ethanol and gently inverting the tube. The ethanol was discarded and the samples flashed at 0.8 *g* for a few seconds, then the remaining ethanol was discarded by pipetting and the tubes left open for 5 min to air dry the samples. Finally 100 µl of DDW was added and the samples mixed by tapping and flashing.

The presence of the transgene was controlled by conducting a PCR with the K7/Nos primer pair and Actin primers as control. The PCR product was electrophoresed in 1% agar gel and stained with EtBr.

Mitochondrial protein analysis through western blotting analysis

Extraction of mitochondria

I extracted the mitochondria according to standard procedures with A. buffer and G. buffer mixed as follows. MillQ is ultrapure water (Millipore corporation).

A. Buffer	G.Buffer (Lysis buffer)
0.35 M sorbitol	0.3 M sucrose
50 mM Tris-HCl (pH 8.0)	0.05 M Tris
5 mM EDTA (pH 8.0)	0.001 M EDTA
0.1% BSA	Adjust pH to 7.5 with HCl
1.25 ml/L β -merceptoethanol	0.05% BSA
MillQ H_2O	0.001 M β -merceptoethanol
	MillQ H_2O

Each sample was weighed and put in a mortar with 4.0 ml/g A. buffer and grinded to a fine paste. The paste was squeezed through a filter made up of 4 layers of gauze and 1 layer Miracloth, thereafter centrifuged at 565 *g* (at 4°C) for 10 min. The supernatant was transferred to a new plastic tube and centrifuged again at 5789 *g*, for 10 min. The supernatant was discarded and the remaining pellet dissolved in 1 ml G. buffer by vortexing and tapping, thereafter centrifuged at 565 *g* for 10 min. The supernatant was transferred to a new microtube and centrifuged at 5789 *g* for 20 min. Finally the supernatant was discarded with an aspirator to completely remove all liquid. The resulting pellets containing the mitochondria were stored in the freezer and used for either protein or RNA analysis.

Protein separation trough SDS-PAGE electrophoresis

I conducted three SDS-PAGE electrophoreses, as follows.

I dissolved the mitochondrial pellets in 100 µl H.S dilution buffer (2.5 ml HEPES-KOH pH 8.0, sorbitol 3.0 g, DDW 47.5 ml) and measured protein concentration in the samples with NanoDrop. They were diluted to 100 µg/ml in H.S dilution buffer. 12 µl samples plus 3 µl SDS sample buffer (SB) was used, and they were denaturated at 95°C for 5 minutes. The 15 µl samples were loaded to a 10% Tris-glycine SDS-PAGE gel and electrophoresed for at 20 mA, constant current. Western analysis with an anti-ORF79 antibody was then conducted. Because of weak signals it was necessary to concentrate the samples through acetone precipitation to a concentration of 600ng/µl. I transferred a volume containing 600 ng of protein to new microtubes and filled up to 50 µl with H. S. buffer. 150 µl of chilled acetone was added and the samples were incubated at -20°C for 10 min, thereafter centrifuged at 12,000 *g* for 5 min. The supernatant was discarded and the samples left to air dry. Finally I dissolved the concentrated samples in 20 µl SB and separated them through 10% Tris-Tricine SDS-PAGE, with electrophoresis at 30 mA, constant current, and again analyzed them with western analysis.
The former procedure was repeated with anti-IDH antibody to visualize functional protein concentration in each sample and to confirm equal loading size.

Western blotting

The samples separated in SDS-PAGE gel were blotted to an Immobiolon-P membrane, which was first soaked in methanol for 3 minutes and then in transfer buffer. The SDS-PAGE gel was placed on the membrane sandwiched between 12 filter papers and electroblotted for 2 hour at 72 mA, constant current. Then I soaked the membrane for at least one hour in hybridization buffer (1% BSA/1xTBS-T). In the first western analysis primary antibody α-ORF79 was used (1/3000 α-ORF79 /1% BSA/1x TBS-T) as primary antibody and α-rabbit IgG (1/5000 α-rabbit IgG /1% BSA/1x TBS-T) as secondary antibody. In the second western blot analysis primary antibody α-IDH (1/5000 α-IDH/ 1% BSA/ 1x TBS-T) and secondary antibody α-rabbit IgG (1/5000 α-rabbit IgG/ 1% BSA/1xTBS-T) was used. Each antibody was incubated with the membrane for at least 1 hour in a hybridization bag under constant shaking. I washed the membrane with hybridization buffer between hybridization of the primary and secondary antibody and in TBS-T after the secondary antibody hybridization. A substrate of Alkaline Phosphatase (AP) was used to stain the membrane (45 µl NBT /35 µl BCIP/ 10 ml AP 9.5). In the α-ORF79 analysis the AP dye was incubated with the membrane over night in order to give a stronger signal.

Mitochondrial RNA analysis trough Northern hybridization analysis

Mitochondrial RNA extraction

Mitochondrial RNA was extracted from the same LD-CMS/Ubi::Rf2 rice callus cells as above, using the same extraction procedures. RNA extraction was carried out with RNAiso Plus (TaKaRa). I dissolved the mitochondrial pellets in 1 ml RNAiso Plus, by vortexing and incubated them for 5 min at room temperature. This was followed by centrifugation at 12,000 *g* for 5 min. I transferred the supernatant to a new tube and added 0.2 volumes of chloroform, vortexed the samples and incubated them at room temperature for 10 min. The samples were centrifuged again at 12,000 *g* for 5 min, and 500-600 µl of the supernatant in the upper layer was transferred to a new microtube. An equal volume of 2-propanol was added, and mixed by inversion. This was followed by 10 min incubation at room temperature and centrifugation for 10 min at 12,000 *g*. I discarded the supernatant and washed the samples with 500 µl 70% EtOH by gently inverting the tubes. The EtOH was completely discarded, and the tubes left open to air dry for 5 min. Finally I eluted the RNA in 50 µl RNase-free water and mixed them by vortexing for 10 min. I checked the RNA quality by separating 2 µl of each sample in a 1% agarose gel, staining it with EtBr and measured the RNA concentration of each sample with NanoDrop.

The samples were concentrated trough ethanol precipitation. I transferred volumes containing 3 µg RNA to new microtubes and filled up to 100 µl with RNase free water. I added 10 µl 3M NaOAc, 1 µl glycogen and 300 µl 100% EtOH. The samples were incubated for at least 15 min at -30°C, thereafter centrifuged for 15 min at 20,352 *g*. I removed the supernatant and washed the samples with 70% EtOH, by gently inverting the tubes. The EtOH was completely discarded and the samples left to air dry for 5 min. Finally I eluted the samples in 3 µl RNase free water.

Probe construction

Orf79 probe was received, constructed with primers
[B-GSP6; ATGGCAAATCTGGTCCGATG/
B-GSP1; AGGGGTGGGATATTTGCCTGGTCCACC].
I constructed the probe for *atp6* with primers
[primer-i; TCTCCCTTTCTAGGAGCAGAGC/
primer-g; CCTCGTTTTTATTCAATT]
and DIG-labeled dUTP (Roche PCR DIG Labeling Mix). First I amplified the probe template using 1 µl of template (BTR genome), 2 µl 10x ExTaq buffer (TaKaRa), 2 µl dNTP, 1 µl each of forward and reverse primers, 0.1 µl of ExTaq (TaKaRa) and 12.9 µl DDW (Deonized Distilled water). The PCR program started with 94°C for 1 min, then

30 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec, finished by 72°C for 2 min.

Then I separated the PCR product trough 1% agarose gel electrophoresis and detected the DNA by soaking the gel in EtBr for 10 min and then viewing it with ATTO Gel Picture Printgraph. I cut out the band containing the template and extracted the DNA with UltraClean^{TM}15 DNA Purification Kit (MO BIO), using the kit's ULTRABIND protocol.

The extracted PCR template was diluted 1/1000 and I used 1 µl as template for Dig-labeling. In each PCR tube I added 5 µl 10x ExTaq buffer (TaKaRa), 5 µl dNTP, 2 µl each of forward and reverse primers, 0.2 µl of ExTaq (TaKaRa) and 34.8 µl DDW. Otherwise the same protocol was used as above: PCR amplification followed by separation in agarose gel, EtBr staining and DNA purification. Before use the probes were diluted 25 µl probe/20 ml hybridization buffer and denaturated through boiling for 10 min.

RNA separation and Northern blotting

I diluted 3 µl of the samples in 10.5µl premix (20x MOPS 100 µl, formaldehyde 350 µl, formamide 1 ml, DDW 100µl), denaturated them for 5 min at 75°C and thereafter added 2.7 µl loading dye. The samples were separated on a 1.2% agarose- 18% formaldehyde gel at 100 *V*, constant voltage. This was followed by northern blotting to a Nytran-N membrane, afterwards the membrane was put in a UV-cross-linker and dried for 1 hour at 55°C. The membrane was soaked in DEPC'd hybridization buffer at 65°C for 3 hours, then Dig-labeled DNA probes for *atp6* and *orf79* were hybridized to the membrane over night at 65°C. The unspecifically hybridized probes were washed away by putting the membrane on a shaker with 2xSSC buffer for 2x5 min, then with 0.1xSSC buffer for 2x20 min, then soaked in TBS for 1 min. Then the membrane was transferred to a new hybribag with 10 ml blocking buffer and shaked for 1 hour. The solution was then switched to anti-Dig antibody solution (10 ml blocking buffer, 2 µl anti-Dig) and incubated for 1 hour. The membrane was washed in TBS for 3x10 min and finally stained with CSPD mix (10 ml AP 9.5m 20 µl CSPD), after soaking the membrane in AP 9.5 for 3 min it was put in CSPD mix for 4 min at 37°C in darkness. CSPD fluorescence was detected in a LAS-400 machine for 1 hour.

Ribozomal staining

After northern hybridization the membrane was put in 5% acetic acid on shaker for 15 minutes. The solution was switched to methylene blue solution, and soaked for 30 minutes on shaker, finally washed in MillQ for 1 hour on shaker.

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