Chapter 4
Rice Organelle Genomics: Approaches to Genetic Engineering and Breeding

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Abstract Although organelle (mitochondria and plastid) genomes have less than 1% of the genes in the nucleus, they encode essential genes, such as those involved in energy production, respiration, and photosynthesis, and genes that control agronomically important characteristics such as cytoplasmic male sterility. Organelle genomes have high copy numbers in each cell (one to two orders of magnitude greater than in the nucleus) and are characterized by maternal inheritance. To know functions of genes encoded in the organelle genomes or to develop new plants adapted to various severe environments, genetic engineering of organelle genomes is one of the promising approaches. However, modifying the mitochondrial or plastid genomes in rice is presently impossible or difficult. Here, we discuss the characteristic features of these genomes and recent attempts at plastid transformation.

Keywords Mitochondria · Plastid · Chloroplast · Organelle genome

4.1 Mitochondrial Genomics in Rice

The common function of mitochondria in plants and animals is to act as an energy center to create ATP by oxidative phosphorylation. Despite this similarity, the genome sizes and structures of the mitochondrial genome in plants are quite different from those in animals. The size of the mitochondrial genome ranges

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from 200 to 11,300 kb in plants, while it is only about 17 kb in mammals, apparently as a result of the loss of many fragments. The large size of plant mitochondrial genome is mostly due to the presences of repeated sequences, noncoding regions, and introns rather than the increased number of genes.

4.1.1 Rice Mitochondrial Genome Sequencing

In flowering plants, the mitochondrial genome was first sequenced in *Arabidopsis thaliana* (Unseld et al. 1997) and subsequnetly in the rice *japonica* cultivar Nipponbare (Notsu et al. 2002). The genomes were sequenced by preparing phage clone libraries and restriction maps, followed by sequencing each clone. The rice mitochondrial genome was initially proposed to consist of five basic circular DNAs, with each circle sharing one or two fragments identical to those in other circles (Iwahashi et al. 1992). A master circle was hypothesized to form by homologous recombination between those identical fragments in the five circular DNAs (Iwahashi et al. 1992; Notsu et al. 2002).

So far, the mitochondrial genomes of 14 rice strains including wild rice have been reported (Table 4.1). The sizes of the genomes range from 402 kb (WA-type cytoplasmic male sterility, WA-type CMS) to 638 kb (IR 6888B, a maintainer line of WA-type CMS). The G + C contents of each genome are very similar. Except BT-type CMS, the genomes are hypothesized to have a single master-circle structure. Although the BT-type CMS mitochondrial genome is possible to have the same structure, a Southern blot analysis suggests that it consists of two separate circular molecules (Kazama and Toriyama 2016).

4.1.2 Gene Contents and Genes Associated with Cytoplasmic Male Sterility

The Nipponbare mitochondrial genome is reported to contain 35 genes for known proteins, 3 ribosomal RNAs, 2 pseudo-ribosomal protein genes, 17 tRNAs, and 5 pseudo-tRNAs (Notsu et al. 2002). Some genes consist of multiple exons that are dispersed throughout the mitochondrial genome and that are trans-spliced to form functional mRNAs (Table 4.2). Although 19 open reading frames (ORFs), which encode over 150 amino acids, have also been predicted, transcriptional products were detected in only 10 of those ORFs (Notsu et al. 2002). The existence of all genes except the predicted ORFs was confirmed in reported rice mitochondrial genome sequences (Tian et al. 2006; Fujii et al. 2010; Bentolila and Stefanov 2012; Zhang et al. 2012; Igarashi et al. 2013; Okazaki et al. 2013; Asaf et al. 2016;

Table 4.1 A summary of reported rice mitochondrial genome sequence

Accession no.	Strain	Organism	Length (bp)	% GC	References
BA000029	Nipponbare	*Oryza sativa japonica* group	490,520	43.9	Notsu et al. (2002)
DQ167399	93-11	*Oryza sativa indica* group	491,515	43.8	Tian et al. (2006)
DQ167400	Nipponbare S	*Oryza sativa japonica* group	490,669	43.8	Tian et al. (2006)
DQ167807	PA64S	*Oryza sativa japonica* group	490,673	43.8	Tian et al. (2006)
AP011076	CW-CMS	*Oryza rufipogon*	559,045	44.0	Fujii et al. (2010)
AP011077	LD-CMS	*Oryza sativa indica* group	434,735	43.9	Fujii et al. (2010)
JF281153	IR 6888B	*Oryza sativa indica* group	637,692	43.9	Bentolila and Stefanov (2012)
JF281154	WA-CMS	*Oryza sativa indica* group	401,567	43.9	Bentolila and Stefanov (2012)
JN861111	Hassawi	*Oryza sativa indica* group	454,820	43.8	Zhang et al. (2012)
JN861112	IR 1112	*Oryza sativa indica* group	454,894	43.8	Zhang et al. (2012)
AP012527	RT98-CMS	*Oryza rufipogon*	525,913	44.2	Igarashi et al. (2013)
AP012528	RT102-CMS	*Oryza rufipogon*	502,250	44.0	Okazaki et al. (2013)
KU176938	W1340	*Oryza minuta*	515,022	44.0	Asaf et al. (2016)
AP017385	BT-CMS	*Oryza sativa indica* group	95,643[a]	44.0	Kazama and Toriyama (2016)
AP017386	BT-CMS	*Oryza sativa indica* group	440,134[b]	43.9	Kazama and Toriyama (2016)

[a]Subgenome 1
[b]Subgenome 2

Kazama and Toriyama 2016). The sequence complexity of plant mitochondrial genomes sometimes makes new sequences and ORFs via illegitimate homologous recombination. Expression of such new ORFs sometimes leads to male sterility, called cytoplasmic male sterility (CMS). Several studies have identified CMS-associated genes (or CMS-causative genes) (Iwabuchi et al. 1993; Akagi et al. 1994; Fujii et al. 2010; Bentolila and Stefanov 2012; Okazaki et al. 2013; Igarashi et al. 2013). Previously, CMS-associated genes have been identified by comparisons of gene structures and their expression profiles between CMS-causing and normal mitochondria. In rice, CMS lines are bred by cytoplasmic substitution via repeated backcrossing. In this case, the cytoplasmic donor cultivar carries *restorer of fertility* (*Rf*) genes in its nuclear genome. This indicates that the

Table 4.2 Location of homologous fragments of the mitochondrial genes in the nuclear genome

Gene	Function	Location in the nuclear genome				
		Exon1	Exon2	Exon3	Exon4	Exon5
nad1	Complex I	N.D.	chr. 1	chr. 8	chr. 9	chr. 12
nad2	Complex I	chr. 9	chr. 9	chr. 1	chr. 1	chr. 1
nad3	Complex I	chr. 12	–			
nad4	Complex I	chr. 12	chr. 12	chr. 12	chr. 12	–
nad4L	Complex I	N.D.	–			
nad5	Complex I	chr. 12	chr. 12	chr. 12	N.D.	N.D.
nad6	Complex I	chr. 12	–			
nad7	Complex I	chr. 12	chr. 12	chr. 12	chr. 12	chr. 12
nad9	Complex I	chr. 1	–			
cob	Complex III	N.D.	–			
cox 1	Complex IV	chr. 12	–			
cox 2	Complex IV	N.D.	chr. 12	–		
cox 3	Complex IV	N.D.	–			
atp1	Complex V	chr. 9	–			
atp4	Complex V	chr. 12	–			
atp6	Complex V	chr. 1	–			
atp8	Complex V	chr. 12	–			
atp9	Complex V	chr. 12	–			
ccmB	Cytochrome c	chr. 12	–			
ccmC	Cytochrome c	chr. 12	–			
ccmFc	Cytochrome c	chr. 12	N.D.	–		
ccmFn	Cytochrome c	N.D.	–			
mat-r	Transcription	N.D.	–			
rps1	Translation	N.D.	–			
rps2	Translation	chr. 12	–			
rps3	Translation	chr. 12	chr. 12	–		
rps4	Translation	N.D.	–			
rps7	Translation	chr. 12	–			
rps11	Translation	N.D.	–			
rps12	Translation	chr. 12	–			
rps13	Translation	chr. 12	–			
rps14	Translation	chr. 9	–			
rps19	Translation	N.D.	–			
rpl2	Translation	N.D.	N.D.	–		
rpl5	Translation	chr. 9	–			
rpl16	Translation	chr. 12	–			
orfX	Transporter	chr. 12	–			

Sequences existed in nuclear genome entirely are listed
N.D. means full-length sequences are not detected in the nuclear genome

cytoplasmic donor cultivar has both CMS-associated and *Rf* genes; RF suppresses the expression of the CMS-associated gene, and male sterility does not occur. The most common approach to identify CMS-associated genes has mostly relied on Northern blot screening between CMS and fertility restored lines (Hanson and Bentolila 2004).

Recently, it has become more common to use next-generation sequencing to identify CMS-associated genes. Whole mitochondrial genomic sequences of CMS rice were obtained and compared between those of standard cultivars, such as Nipponbare, to screen for new ORFs that are absent from the reference genome (Bentolila and Stefanov 2012; Kazama and Toriyama 2016). Then, CMS-associated gene candidates were selected based on the criteria that they are chimeric in structure with known mitochondrial genes or encode peptide containing a transmembrane domain, because all reported CMS-associated genes have such characteristics. Subsequently, the expressions of candidate genes were checked to determine whether they exhibit different patterns in the presence or absence of *Rf* genes. Expression pattern differences that depend on the presence or absence of *Rf* genes indicate that the expression of CMS-associated genes is the cause of CMS. However, it is presently impossible to modify a plant mitochondrial genome by DNA fragment knock-in and specific ORF knock-out. Thus, there is not yet an absolute proof that a CMS-associated gene is a CMS-causative gene that has not been obtained.

4.1.3 Mitochondrial DNA Fragments in the Nuclear Genome

Many DNA fragments identical to mitochondrial DNA have been found in the nuclear genome (Notsu et al. 2002; Bentolila and Stefanov 2012). This kind of nuclear fragment is called "promiscuous" DNA and thought to be translocated from mitochondria through evolutionary processes and/or organelle establishment. In *Arabidopsis*, a 620-kb insertion of a mitochondrial genome sequence was reported on chromosome 2 (Stupar et al. 2001). In rice, fragments representing 60% of the mitochondrial genome were found in the nuclear genome (Bentolila and Stefanov 2012). Interestingly, such promiscuous DNA fragments are unequally distributed among the chromosomes.

To identify which mitochondrial genes exist in the nuclear genome, BLAST search was performed (Table 4.2). In this analysis, genes except tRNAs and the predicted ORFs in the Nipponbare mitochondrial genome were used as queries. Homologous sequences were found for all but 14 mitochondrial genes (Table 4.2) (International Rice Genome Sequencing Project 2005). The similarity between the mitochondrial and nuclear gene sequences was more than 88%. This indicates that almost all of the mitochondrial genes in rice have been translocated into the nuclear genome and that the translocation events of mitochondrial genome could have occurred in relatively recent times.

4.2 Chloroplast Genomics in Rice

4.2.1 Use of Rice Chloroplast Genomes for Comparative Genomics

Chloroplasts are organelles that are representative of plants; they are the most prominent plastid present in green tissues, and play a crucial role in plants as a metabolic center involving various biochemical processes, including photosynthesis. Chloroplasts possess their own genome, which is generally circular and consists of a large single copy (LSC) region and a small single copy (SSC) region, respectively, that separate two inverted repeat regions (IRs). The chloroplast genome contains three major groups of genes that encode (i) subunits of the photosynthetic complex, (ii) components of the gene expression machinery, and (iii) a few proteins that may function in metabolic pathways and unknown proteins (Shimada and Sugiura 1991). Compared with the mitochondrial genome, the chloroplast genome of land plants has a well-conserved structure, although the gene content, gene order, and fine structural details depend on the species (Jansen et al. 2007; Xu et al. 2015). In the grass family (Poaceae), the chloroplast genome shares some structural arrangements that are different from those of other plant families (Doyle et al. 1992; Katayama and Ogihara 1996; Saski et al. 2007). These include three inversions in the LSC; deletions of *accD*, *ycf1*, and *ycf2*; and loss of introns in *clpP* and *rpoC1* (Katayama and Ogihara 1996; Saski et al. 2007; Daniell et al. 2016).

The rice chloroplast genome was first sequenced in the *Oryza sativa* L. cv. Nipponbare (Hiratsuka et al. 1989); its length was 134,525 bp with 38.99% GC content. The present annotation data (https://www.ncbi.nlm.nih.gov/nuccore/NC_001320.1) show that the Nipponbare chloroplast genome contains 159 genes, including 108 protein-coding genes, 8 ribosomal RNA genes, 38 transfer RNA genes, and 5 pseudogenes. To date, more than 100 rice chloroplast (plastid) genome sequences, including that of wild rice, are available in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/nucleotide/) because of advances in sequencing technologies.

Oryza, which includes 23 species, is divided into 10 nuclear genome types, which include 6 diploids (AA, BB, CC, EE, EF, and GG) and 4 allotetraploids (BBCC, CCDD, HHJJ, and HHKK) (Ge et al. 1999). The AA genome type includes three cultivated rice species (*Oryza sativa* ssp. *japonica* and *O. sativa* ssp. *indica* in Asia and *O. glaberrima* in Africa) and six wild relatives: *O. barthii*, *O. glumaepatula*, *O. longistaminata*, *O. meridionalis*, *O. nivara*, and *O. rufipogon*. The Asian cultivated rice, *O. sativa*, is generally thought to have originated from *O. rufipogon* (Khush 1997; Vaughan et al. 2008; Huang et al. 2012). However, despite numerous studies (Ma and Bennetzen 2004; Vitte et al. 2004; Zhu and Ge 2005; Londo et al. 2006; Molina et al. 2011; Huang et al. 2012; Yang et al. 2012; Civáň et al. 2015; Huang and Han 2015), the domestication history remains under debate.

Recent studies of the evolutionary and phylogenetic relationships among the AA genome species based on whole chloroplast genome sequences showed that two subspecies of *O. sativa*, *japonica* and *indica*, had distinct maternal origins (Wambugu et al. 2015; Kim et al. 2015; Tong et al. 2016). It is indicated that the maternal genome of *japonica* has been derived from *O. rufipogon*, whereas the *indica* maternal genome has been originated from *O. nivara*, although the nuclear genomes seem to be of complex origin (Kim et al. 2015). Nuclear genome analyses suggest that the African cultivated rice *O. glaberrima* was domesticated from *O. barthii* (Li et al. 2011b; Wang et al. 2014). Phylogenetic relationships of several chloroplast genomes also indicated that the maternal origin of *O. glaberrima* was *O. barthii* (Wambugu et al. 2015; Kim et al. 2015; Tong et al. 2016). Moreover, chloroplast genome analyses of worldwide rice accessions revealed that South American wild rice, *O. glumaepatula*, has a distinct chloroplast genome (Kim et al. 2015), and *O. rufipogon* contains cytoplasm derived from different geographic regions (Kim et al. 2015; Wambugu et al. 2015). Chloroplast-based phylogenies usually reflect geographic distributions better than nuclear genomes and should be useful for resolving the evolutionary and domestication history of rice. Wild species have disease-resistance and stress-tolerance genes, which make them valuable genetic resources for rice improvement and breeding (Brar and Khush 1997). In recent years, wild species with the EE genome (*O. australiensis*) and BBCC genome (*O. minuta*) chloroplast sequences were also reported (Nock et al. 2011; Waters et al. 2012; Asaf et al. 2017). These chloroplast genome studies will elucidate the detailed genetic relationships among rice species and will be helpful in developing future breeding strategies using new genetic resources. In addition, the accumulated rice chloroplast sequences also provide insight for chloroplast genome engineering to improve chloroplast function such as photosynthetic capacity.

4.2.2 Perspectives on Rice Chloroplast Genome Engineering

In higher plants, the first successful chloroplast transformation was achieved in tobacco by particle bombardment about 30 years ago (Svab et al. 1990). Chloroplast transformation has many advantages over nuclear transformation, including a high level of transgene expression, multigene transformation, site-directed gene integration, absence of gene silencing, and maternal inheritance that prevents transgene flow into the environment. Each plant cell has dozens of chloroplasts, each of which contains multiple genome copies. Thus, gene expression can be ten to a hundred times greater in chloroplast genomes than in nuclear genomes. With this property, plants can be used to make large amounts of foreign proteins, such as pharmaceutical proteins (e.g., vaccine antigens and antibiotics) and industrial enzymes (e.g., hydrolases, redox enzymes, and transferases). Many functional proteins have been expressed in tobacco and lettuce chloroplasts (reviewed in Jin and Daniell 2015;

Daniell et al. 2016), and expression can be as high as 70% of total leaf protein (Ruhlman et al. 2010). Chloroplasts have great potential as bioreactors for commercial production. Now, many therapeutic proteins are in clinical development (Zhang et al. 2017).

Chloroplast transformation has also been used to control agronomic traits, including resistance to insects, diseases, and herbicides, and abiotic stress tolerance of plants (reviewed in Jin and Daniell 2015; Daniell et al. 2016). For example, double-stranded (ds) RNA expressed in chloroplasts was shown to induce RNA interference (RNAi) of target genes in insect hosts (Jin et al. 2015; Zhang et al. 2015), demonstrating a new approach to protecting crops without chemicals.

Chloroplast engineering of metabolic pathways or photosynthetic ability could be used for crop improvement (Maliga and Bock 2011; Hanson et al. 2013; Wani et al. 2015). To manipulate the isoprenoid pathway, seven genes were simultaneously inserted into the chloroplast genome, resulting in the accumulation of high levels of target metabolites (Kumar et al. 2012). Photosynthetic efficiency was also enhanced by replacing the tobacco chloroplast *rbcL* gene with three genes from the cyanobacterium *Synechococcus* (*rbcL*, *rbcS*, and an assembly gene) (Lin et al. 2014).

Despite the remarkable progress in chloroplast transformation technologies, there are still substantial limitations to their use. Successful examples of chloroplast genome engineering have been restricted to only tobacco and a few dicots (Jin and Daniell 2015). Development of fully chloroplast-engineered monocots has not yet been achieved because of the lack of transformation protocols (Bock 2007; Clarke and Daniell 2011; Khan 2012; Rigano et al. 2012). Major obstacles to chloroplast transformation in monocots are thought to be (i) difficulty of tissue culture and regeneration from green tissue and (ii) no effective selection systems for retaining the transformed chloroplasts (plastids) and facilitating the transition from heteroplasmic to homoplasmic state (that is, all chloroplast genomes are replaced with identical transformed chloroplast genomes). Indeed, efficient protocols for tobacco chloroplast transformation were established based on tissue culture systems of green leaf materials using spectinomycin selection with the aminoglycoside 3″-adenyltransferase gene, *aadA*, as a selection maker (Lutz et al. 2007; Verma and Daniell 2007; Scotti and Cardi 2012). Unfortunately, most monocotyledonous plants are endogenously resistant to spectinomycin, because of the point mutations in their targeted *16S rRNA* gene (Fromm et al. 1987). Additionally, regenerable tissue culture materials in monocots are generally nongreen tissues, such as dark-grown embryogenic calli or suspension cells. In nongreen tissues, a small number of plastids exist as undifferentiated plastids called proplastids. Proplastids are about fivefold smaller in size than chloroplasts, and the gene expression levels are lower than those of chloroplasts (Vera and Sugiura 1995; Sakai et al. 1998; Silhavy and Maliga 1998; Daniell et al. 2002; Pyke 2007; Liebers et al. 2017).

Even with these disadvantages, several advancements have been reported in monocotyledonous cereal crops, particularly in rice. Khan and Maliga (1999) were the first to transform rice plastids. They used a fluorescent antibiotic resistance gene, *aadA11gfp*, a fusion gene of the *Aequorea victoria* green fluorescence protein

gene (*gfp*) and *aadA*. They introduced the fusion gene to rice suspension culture cells and selected the transplastomic lines with streptomycin: *aadA* confers resistance to both spectinomycin and streptomycin, and rice cells are sensitive to streptomycin. Twelve shoots regenerated from surviving cells on streptomycin-containing medium from 25 bombarded plates. The plastid transformation was proven by PCR detection of the transgene, but the GFP fluorescence was only observed in a few populations of highly heteroplasmic cells, including wild-type and transgenic chloroplasts. Despite this success, the authors did not indicate inheritance of the transgene or the fertility of the transplastomic plants. Subsequently, Lee et al. (2006) again attempted to introduce *gfp* and *aadA* by using streptomycin selection with the conventional rice cell culture with mature seed-derived calli. They successfully produced fertile transplastomic rice plants and demonstrated transmission of both transgenes to the progeny. However, the transformation efficiency was quite low, with two transgenic lines out of approximately 4000 calli on 100–120 bombarded plates; and all transplastomic lines still have been in heteroplasmic state.

A different selection approach using PPT (L-phosphinotricin), the active ingredient of the herbicide Basta, and the resistance gene *bar* was also attempted for rice plastid transformation (Li et al. 2009). Although the transformation efficiency was not clear, six transplastomic plants were obtained with various heteroplasmic levels, and maternal transmission of *bar* was suggested based on crossing experiments (Li et al. 2009). More recently, Li et al. (2016a, b) tried other selection strategy using a rice-specific chloroplast transformation vector with hygromycin-resistance cassette that contains the hygromycin phosphotransferase gene *hpt*. The antibiotic hygromycin B blocks protein biosynthesis in both prokaryotic and eukaryotic cells (Gonzalez et al. 1978), and hygromycin selection was widely used for nuclear transformation in rice (Hiei and Komari 2008). Fertile transplastomic plants were obtained, and transgene inheritance in progeny was demonstrated. However, only a few transformation events occurred in these experiments (24 surviving plants with approximately 20,000 total calli), and the number of transformed plastids was very small, probably because of weak selection pressure (Li et al. 2016b).

A possible reason for the lower transformation efficiencies observed in nontobacco species is reduced activity of plastid homologous recombination (Sikdar et al. 1998). Assuming this was the case, the application of transcription activator-like effector nucleases (TALENs) was tested in rice plastid transformation (Li et al. 2016a). TALENs are a powerful tool for targeted genome modification (Bogdanove and Voytas 2011). They induce genomic double-strand breaks (DSBs) for gene insertion and thus greatly stimulate the homologous recombination DNA-repair pathway (Wyman and Kanaar 2006). To improve the insertion efficiency of exogenous DNA fragments into the rice plastid genome by homologous recombination, Li et al. (2016a) conducted co-transformation of the TALEN vector and plastid transformation vector into rice calli by particle bombardment. As a result of preliminarily PCR analyses of T_0 plants, the transformation efficiency

(detection frequency of transgene fragments) of co-transformation (0.35%) was much higher than that of transformation with a single vector (0.11%).

In summary, despite the interest in chloroplast transformation of rice, it has not yet been successful. Issues to be solved include (i) choice of target tissues for gene introduction, (ii) activation of homologous recombination, and (iii) development of efficient selection methods. The candidate target tissues (plastids) include nongreen tissue (proplastids) and green tissue (chloroplasts). As mentioned above, calli, a nongreen tissue used in conventional tissue culture systems, contain small proplastids with low introduction efficiency and low levels of gene expression. In contrast, green tissue contains large chloroplasts that are thought to have high efficiency and high levels of gene expression. However, no culture system capable of regenerating green tissues has yet been established in rice. In maize, a leaf-based culture system has been constructed (Ahmadabadi et al. 2007). We attempted a similar culture method using rice seedlings, and confirmed that it can be used to culture (unpublished data). We are now testing this green tissue to see if it can be used for chloroplast transformation of rice.

The selection methods, for transplastomic rice plastids, streptomycin-*aadA*, PPT-*bar*, and hygromycin-*hpt*, all failed to achieve homoplasmy, which indicates that these selection methods are not suitable for rice plastid transformation. In dicots, a few alternative selection systems have recently been developed for plastid transformation (Barone et al. 2009; Li et al. 2011a; Bellucci et al. 2015; Dunne et al. 2014; Yu et al. 2017). The selection methods for dicots and other novel selection methods need to be tested for transforming rice plastids.

Furthermore, because of their low plastid transformation efficiency, monocots, including rice, seem refractory to integration of foreign genes into the plastid genome. Perhaps monocots have specific biological mechanisms to prevent it. At present, many aspects of monocot plastid biology remain unclear. Elucidation of the molecular mechanisms of plastid genome maintenance will be indispensable for establishing methods for plastid transformation in rice.

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