Heteroplasmy and expression of mitochondrial genes in alloplasmic and euplasmic wheat

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The plant chondriome confers a complex nature. The \textit{atp4} gene (formerly called \textit{orf25}) of \textit{Aegilops crassa} (CR) harbors the promoter sequence of the \textit{rps7} gene from common wheat (\textit{Triticum aestivum} cv. Chinese Spring, CS). The \textit{rps7} gene of CR has the promoter sequence of CS \textit{atp6}. The \textit{atp6} gene of CR contains an unknown sequence inside of its coding region. Since repeat sequences have been found around the breaking points, these structural alterations are most likely generated through homologous recombination. In this study, PCR analysis was performed to detect structural alterations in each of three lines: euplasmic lines of \textit{Ae. crassa}, Chinese Spring, and alloplasmic Chinese Spring wheat with the cytoplasm of \textit{Ae. crassa ((cr)-CS)}. We found that each of these lines contained both genotypes, although mitochondrial genotypes of CR in Chinese Spring wheat and CS genotypes in \textit{Ae. crassa} were still retained as minor fractions (less than 10\%). On the other hand, CS mitochondrial gene frequencies in ((cr)-CS) were shown to be ca. 30\%. SNP analysis after DNA sequencing of these genes indicated that minor types of all three mitochondrial genes in alloplasmic wheat contained the mitochondrial gene types from pollens. Since the frequencies of paternal mitochondrial gene types in \textit{F\textsubscript{1}} were about 20\%, successive backcrossing increased the frequencies of paternal mitochondrial gene types to around 30\% in alloplasmic wheat. Expression profiles of these mitochondrial genes were quantitatively analyzed by RT-PCR. Transcripts of paternal mitochondrial gene types were scarcely found. This suggests that minor fractions including paternal mitochondrial gene types are maintained and silenced in the descendants.

Key words: wheat chondriome, heteroplasmy, paternal transmission, gene expression, interspecific hybrids

INTRODUCTION

DNA in plant cells is arranged into tripartite compartments: nucleus, plastids and mitochondria. Genes in the nuclear genome are transmitted biparentally in a Mendelian fashion, whereas plasmon is thought to show mostly maternal inheritance. This idea is mainly supported by the fact that a number of genetic traits in plants are inherited only through the maternal lineage in offspring after reciprocal crossings (Kihara, 1951; Grun, 1976). Phenotypic characters controlled by both plastome (plastid genome) and chondriome (mitochondrial genome) are stably inherited through generations, and rarely show segregation of traits among offspring, indicating the uniparental inheritance of the plasmon. While plastids contain multi-copies of identical circular DNA in most cases (Palmer, 1991), plant mitochondria harbor multi-forms of DNA such as highly branched circular and linear molecules (e.g., Woloszynska and Trojanowski, 2009). This heteroplasmy, defined as the coexistence of divergent mitochondrial DNA types, is well known in the plant chondriome. Although heteroplasmy is widely recognized in fungi and animals (Barr et al., 2005), plants characteristically maintain their traits over many generations (Arrrieta-Montiel et al., 2001). Sublimons of the plant chondriome are considered to generate through homologous recombination that is mediated by the tandem and/or inverted repeats in the master copy of mitochondrial DNA (Lonsdale et al., 1988; Ogihara et al., 2005). In addition to these sublimons, variants of mito-
chondrial DNA can be found in the chondriome. These variants are generated through small-scale mutation (Garicia-Diaz et al., 2003), paternal transmission of mitochondrial DNA (Hattori et al., 2002), and illegitimate recombination (Kmiec et al., 2006). Occasionally, minor sublimes in the heteroplasmic state of the plant chondriome are amplified and replaced to the main genome. This substoichiometric replacement takes place rapidly and sometimes reversibly (Jansa et al., 1998). Mechanism(s) underlying heteroplasy of plant chondriomes are still unclear.

In our study on the molecular basis of alloplasmic wheat showing photoperiod-sensitive cytoplasmic male sterility, we found that the mitochondrial gene apf4 (formerly named orf25) of Aegilops crassa (cytoplasm donor) confers a distinct promoter sequence and produces different transcripts from that of common wheat (nucleus donor) (Ogihara et al., 1999). In the Ae. crassa mitochondrial genome, the promoter region of the apf4 gene was replaced by that of rps7. Here, we report that the promoter of rps7 in the mitochondrial genome of Ae. crassa is replaced by that of atp6, and the atp6 gene of Ae. crassa contains an unknown sequence inside of the gene. In addition to these major mitochondrial gene types, chondromes of common wheat and Ae. crassa harbor their counterpart types as minor fractions. DNA sequence analysis shows that some of these minor types are transmitted through pollens. Analyses of their transcripts reveal that expression of minor fractions of mitochondrial genes including paternal types is silenced. These data shed more light on aspects of chondriome inheritance in interspecific hybrids of related plants.

MATERIALS AND METHODS

Plant Materials The alloplasmic line (cr-CS) of common wheat (Triticum aestivum cv. Chinese Spring; CS) with Aegilops crassa 6x (CR) cytoplasm and their parental lines were used in the present investigation. F1 hybrids of Ae. crassa 6x pollinated by CS were also used. Four F2 hybrids were confirmed by the two different PCR markers presented in Supplementary Table S1.

Isolation of DNA and RNA Total DNA was extracted from 14-day-old seedlings of the alloplasmic line and the CS and Ae. crassa euplastic lines, and from their F1 according to the CTAB method (Murray and Thompson, 1980). Total RNAs from CS, CR and (cr)-CS were extracted from 14-day-old seedlings by the phenol/SDS method (Ogihara et al., 1999). Mitochondrial DNAs and RNAs of the alloplasmic as well as the euplastic wheat (CS) were isolated as previously described (Ogihara et al., 1999).

Cloning and sequencing of mitochondrial genes The mitochondrial genomic clones containing CR-rps7 and CR-atp6 were screened against the mitochondrial genomic library of alloplasmic wheat (Ogihara et al., 1999) with the wheat respective genes (Ogihara et al., 2005). The PCR to amplify each type of three mitochondrial genes, i.e., atp4, rps7 and atp6, was carried out using the primers listed in Supplementary Table S2. The PCR products were cloned into the pGEM vector (Promega). The lambda phage clones and PCR clones containing these genes were sequenced as previously described (Ogihara et al., 1999). Sequenced data can be accessed to the DDBJ, continuous No. AB571683 to AB571714.

Quantitative analysis of different mitochondrial gene types in the mitochondrial genomes of the wheat lines To estimate the proportion of different mitochondrial gene types, total DNAs of the alloplasmic line (cr-CS) of common wheat (T. aestivum cv. Chinese Spring; CS) with Ae. crassa 6x (CR) cytoplasm, F1 hybrids of Ae. crassa 6x pollinated by CS, and their parental lines were isolated. Mitochondrial DNAs from two wheat lines of (cr)-CS and CS were isolated according to the method of Ogihara et al. (1999). The PCR primers were synthesized to distinguish the distinct mitochondrial gene types of atp4, rps7 and atp6. The primers for the coding regions of three mitochondrial genes were also synthesized. The primer sequences are listed in Supplementary Table S2, and their positions around the genes are shown in Fig. 1. Fifty ng of total DNAs and/or 5 ng of mitochondrial DNAs from CS and (cr)-CS were supplied for the PCR. The reactions were carried out in 20 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.25 mM dNTPs, 1.25 U Taq polymerase (Takara TaqTM), and 0.5 μM each combination of primers. The solutions were denatured at 94°C for 3 min, followed by 20, 23 or 26 cycles of reactions at 94°C for 30 sec, 59°C for 30 sec and 72°C for 60 sec. The PCR products were separated by agarose gel electrophoresis, and relative intensities of the corresponding PCR fragments were measured with the Digital Science EDAS290 (Kodak). Relative amounts of the PCR products for each mitochondrial gene type were adjusted with the amounts of those for the coding regions produced by the same PCR conditions.

Quantitative RT-PCR The purity and integrity of extracted RNAs were confirmed with RNA 6000 Nano Assay (Agilent). Total RNAs were treated with RNase-free DNase I to remove contaminated DNA. Five μg of DNase-treated RNAs were supplied for cDNA synthesis in 20 μl of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 1.25 μM Random primers (Promega), 10 mM dNTPs, 100 U ReverTra Ace (TOYOBO), and 8
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units RNasin Plus RNase Inhibitor (Promega) at 32°C for 10 min, followed by incubation at 42°C for 60 min. Reactions were stopped at 99°C for 5 min. The PCR primers for amplification of cDNAs from the distinct mitochondrial genes of atp4, rps7 and atp6 were also synthesized, as shown in Supplementary Table S2. Two µl of five-fold diluted RT products corresponding to ca. 100 ng of total RNAs were reacted in 20 µl of 4 mM MgCl₂, 0.5 µM primers, and 2 µl LightCycler-FastStart DNA Master SYBER Green I (Roche) in Light Cycler (Roche), with 50 cycles of 94°C for 10 sec, 59°C for 5 sec, and 72°C for 20 sec after 95°C for 10 min denaturation. The PCR products were calibrated using amplified products from a series of 1 ng, 10 pg, 100 fg, 1 fg, and 10 ag for the cloned PCR products with the same primers into the pGEM-T vector. Relative amounts of expressed genes were measured with the LightCycler Software ver. 3.5.3 by adjustment of the PCR products from each coding region.

RESULTS

Euplasmic and alloplasmic wheat harbor two distinct mitochondrial gene types In this study, we compared mitochondrial gene structures between Chinese Spring wheat and Ae. crassa. We used entire sequencing data of master copy DNA of the mitochondrial genome obtained in Chinese Spring wheat (Ogihara et al., 2005). In our earlier work, we found that the leader
sequence of the *rps7* gene had recombined with that of *atp4* in the *Ae. crassa* chondriome (Ogihara et al., 1999). Therefore, we screened the *rps7* gene against the phage library of *Ae. crassa* mitochondrial DNA in the present study. When we sequenced the upstream region of the *rps7* gene of *Ae. crassa*, it showed homology with the leader sequence of one (*atp6-2*) of the two *atp6* genes (*atp6-1* and *atp6-2*) in the mitochondrial genome of common wheat. Then, we screened again the *atp6* gene against the phage library of *Ae. crassa* mitochondrial DNA. The *Ae. crassa* mitochondrial genome has one copy of the *atp6* gene whose structure is basically similar to that of common wheat (*atp6-1*), yet it contains unknown sequences at the 5' region of the coding sequences. Since the repeat sequences, named Box I and Box II, were identified in the leader sequences of these genes, alternative gene structures may have taken place by homologous recombination between these repetitive sequences (Bonen et al., 1993). These mitochondrial gene structures are shown in Fig. 1. Therefore, we questioned whether the alloplasmic line harbors both mitochondrial gene types. PCR primers specific for each mitochondrial gene type (Fig. 1) were synthesized (Supplementary Table S2). Surprisingly, we found that not only the alloplasmic line, but also two parental euplasmic lines harbor both types of mitochondrial genes (Fig. 1). Competitive PCRs showed that the proportions of the two types differed among the genes and lines. These results were confirmed by PCR using isolated mitochondrial DNA from the alloplasmic line of (cr)-CS and euplasmic CS (data not shown). The proportions of each mitochondrial gene type in the three lines were quantitatively estimated, as shown in Fig. 2. Euplasmic mitochondria contain about five to ten percent of distinct gene types as the minor fraction, although the proportions differed among the genes. Therefore, we concluded that minor mitochondrial DNA(s) are maintained even in euplasmic mitochondria. However, the alloplasmic line harbored about 30% of paternal gene types (Fig. 2). Then, we investigated the composition of both mitochondrial gene types in an F1 hybrid of *Ae. crassa* pollinated by CS. Interestingly, the contribution of the minor components to the mitochondrial gene types between the pure lines and alloplasmic line were found to be intermediate at 12% (*rps7*) to 25% (*atp4*), as presented in Fig. 2. Although alloplasmic wheat lines had been established by successive backcrossing and maintained for more than twenty years (Murai and Tsunewaki, 1993), the proportion of these minor mitochondrial gene types gradually increased with recurrent backcrossing, and eventually reached saturation after successive self-pollination.

**Sequence analysis of both types of three mitochondrial genes in alloplasmic and euplasmic lines**

The PCR products of both types of three mitochondrial genes (*atp4, rps7* and *atp6*) in three lines were individually cloned into the plasmid vector. The cloned mitochondrial genes were sequenced to compare the mitochondrial gene types among the lines. DNA sequences of 14 clones for each gene type (AE, mitochondrial gene type of *T. aestivum*, CR; mitochondrial gene type of *Ae. crassa*, see Fig. 1) found in three lines (CS, (cr)-CS and *Ae. crassa*) were determined. Both of the AE and CR types were found in the mitochondrial genomes of the three lines, i.e., CS, (cr)-CS and *Ae. crassa* (Supplementary Figs. S1–S3). In CS and *Ae. crassa*, one AE and one CR type were found. Each type of AE and CR were distinguishable from each other, due to their SNPs. Sequence homologies were more than 95% between two of three genes. On the other hand, we found it striking that three AE(s) for three genes, i.e., *atp4, rps7* and *atp6*, were found in (cr)-CS lines, one of which (AE (cr)-CS) was identical to that of CS. The other two (AE (cr)-CS-1 and AE (cr)-CS-2) were grouped into the AE type of CS rather than that of CR, based on their SNPs (Supplementary Figs. S1–S3). While the CR type in (cr)-CS was identical to that of *Ae. crassa*, it differed from that of CS as to three genes (Supplementary Figs. S4–S6). In order to assess the inheritance mode of the mitochondrial genome in interspecific hybrids, mitochondrial genome types of four F1 hybrid plants were examined. Sequence analysis of three mitochondrial genes in F1 hybrids showed that major mitochondrial gene types were maternally inherited, but minor portions of mitochondrial genes homologous to the genotypes in pollen were also found. The original AE type in the *Ae. crassa* mitochondrial genome
was still retained, and that corresponding to the pollen type was also found in the F1 hybrids (Supplementary Figs. S7–S9), although only maternal CR type was found in F1 plants (Supplementary Figs. S10–S12). These lines of evidence clearly show that both of the AE and CR types are maintained in wheat and goat grass, and mitochondrial DNA can be paternally transmitted through pollen in interspecific hybridization. The original minor AE type maintained in the Ae. crassa mitochondrial genome was gradually decreased through successive backcrossing, replacing the paternal AE type of CS as a minor fraction in the mitochondrial genome of alloplasmic wheat.

Mitochondrial genes are preferentially expressed in maternally transmitted DNA of mitochondrial genomes The promoters of three mitochondrial genes were mapped in common wheat (Bonen et al., 1993; Ogihara et al., 1999). Taking this information into account, primers were synthesized (Supplementary Table S2) to amplify the respective transcripts by reverse transcription (RT)-PCR (Fig. 3). Competitive RT-PCR revealed that the transcripts were expressed from the maternally transmitted major gene types in the respective mitochondrial genomes (Fig. 3). Further amplification to detect minor components in the mitochondrial genomes was performed, and real-time RT-PCR was later carried out to estimate the contents of the minor fractions in each mitochondrial genome. The results show that minor transcripts contain less than 0.1%, compared to the PCR products of the major components (Table 1). The PCR products for coding sequences (CDSs) of three genes were cloned into the plasmid vector. The eight cloned products were sequenced. Sequence analysis revealed that all products were from the major types of the respective mitochondrial genes (Supplementary Figs. S13–S18). This clearly shows that expression of the minor genes and/or the paternally transmitted genes were mostly suppressed regardless of their content frequencies in the mitochondria.

**DISCUSSION**

**Heteroplasmy of the mitochondrial genome in euplasmic lines of wheat species** In the process of investigating the structure of mitochondrial gene types in *Ae. crassa* (Ogihara et al., 1999), three mitochondrial genes were identified, i.e., *atp4*, *rps7* and *atp6*, which had distinct gene types from those of *T. aestivum* cv. Chinese Spring (Fig. 1). Since the shared repeat sequences named Box I and Box II were found upstream of the start codon of all of the three genes, distinct types are predicted to take place through homologous recombination. Strikingly, competitive PCR analysis showed that both types of mitochondrial genes were contained even in the parental euplasmic lines with different stoichiometry. Frequencies of the minor components were less than 10%, whose stoichiometries were different from gene to gene in each species (Fig. 2). It is well known that plant mitochondrial genomes are complex, consisting of sublimons (e.g., Woloszyńska and Trojanowski, 2009), in which circular molecules as well as linear ones can be observed with electron microscopy. Restriction fragment analysis supports this observation (Bonen, 1994). To explain this complexity, the master copy of mitochondrial DNA is assumed (Lonsdale et al., 1988), and a number of variant sublimons for mitochondrial DNAs are generated through homologous recombination mediated by repeat sequences. In fact, we completed the sequencing of the master copy DNA in mitochondria of Chinese Spring wheat as well as all major variants that had been cloned into a cosmid, and were able to trace the recombination events (Ogihara et al., 2005). In the master copy of CS wheat, no mitochondrial gene types of *Ae. crassa* (CR type) were found. Furthermore, the CS types of mitochondrial genes were not found in the major DNAs of *Ae. crassa* mitochondria (Ogihara et al., 1999). These lines of evidence suggest that minor mitochondrial gene types were maintained through successive generations in each mitochondrial genome.

**Paternal mitochondrial gene types are contained in the interspecific hybrid of wheat** To address the inheritance manner of heteroplasmic mitochondrial genomes, we generated the F1 hybrid of *Ae. crassa* pollinated by Chinese Spring wheat, and analyzed the mitochondrial genome type of the F1 plants. The major type of mitochondrial genome was maternally transmitted, as expected (CR type; Fig. 2). The AE type was found as
the minor fraction, indicating heteroplasmy of the F₁ plants. The PCR products corresponding to the CR and AE types were sequenced. Even though the major CR type of the F₁ plants was identical to that of the maternal Ae. crassa line, the AE type of the F₁ plants as the minor fraction contained both types derived from the biparental lines (Supplementary Figs. S7–S9). This indicates that the mitochondrial genome harbors the genes type identical to those paternally transmitted through pollen in interspecific hybrids. The paternal transmission of organellar genomes is not exceptional: it has been reported not only in plants (Hattori et al., 2002) but also in animal mitochondrial genomes (Kondo et al., 1990; Gyllensten et al., 1991; Shitara et al., 1998). It should be pointed out herein that the original AE type maintained in the mitochondrial genome of Ae. crassa coexisted as a minor fraction with the additional AE type from CS wheat after pollination. Hence, both the maternal and paternal minor mitochondrial genome types were maintained as heteroplasmy in the F₁ seedling after a series of successive cell divisions.

**Inheritance of the mitochondrial genome in alloplasmic wheat through backcrossing and self-pollination** Alloplasmic wheat was generated from an interspecific hybrid between Ae. crassa and T. aestivum cv. Chinese Spring by successive backcrossing of Chinese Spring wheat more than ten times, and maintained for more than 40 years by self-pollination (Tsunewaki, 1993). In alloplasmic wheat, the major mitochondrial genome type showed maternal inheritance, so that the mitochondrial gene types of alloplasmic wheat were identical to those of the maternal parent, Ae. crassa in terms of gene structure and DNA sequences (Fig. 1 and Supplementary Figs. S1–S6). In fact, a number of sublimons have been produced by homologous recombination (Ogihara et al., 2005; Woloszynska and Trojanowski, 2009). Since the plant mitochondrial genomes showed low rate of nucleotide substitutions, the DNA sequences per se have been relatively stable during the evolutionary course (Wolfe et al., 1987) compared to chloroplast DNA, although frequent rearrangements of the mitochondrial genomes took place within and between wheat mitochondrial DNA molecules (Ogihara et al., 2005; Liu et al., 2011). In these heteroplasmy of mitochondrial gene types, paternal mitochondrial gene types were found in the descendants of wheat interspecific hybrids. The proportion of minor mitochondrial gene types distinct from the major types in the euplasmic lines is gradually increased by successive backcrossing and reaches a plateau of about 30% in the mitochondrial genome of alloplasmic wheat (Fig. 2). Recently, major type of the mtDNA of alloplasmic wheat with the cytoplasm of Ae. kotschyi which belongs to the Polyides section (Tsunewaki, 1993), has been sequenced (Liu et al., 2011). That contains the CR type of atp4, rps7 and atp6 genes, although they did not report the minor type (AE type) of those genes. Therefore, this indicates that drastic change(s) of sublimon’s constituents took place after differentiation of wheat-related group in the Sitopsis section. The mechanism(s) for maintenance of heteroplasmy of mitochondrial DNAs are still remained to be clarified.

**Expression of mitochondrial genes in heteroplasmic conditions** Mitochondrial genes are predominately expressed from the maternally transmitted major type of the mitochondrial genome even in alloplasmic wheat (Fig. 3 and Table 1). This strongly suggests that silencing system(s) play a role on the minor fraction(s) of the mitochondrial genome. Silenced mitochondrial genes seem not to be expressed through the maternal lineage. Data presented in Fig. 3 and Table 1 indicate that silencing of the minor fraction genes is consistent through the generations. Another possibility is that the maternal lineage can only transmit the major type of mitochondrial genes. This situation is unlikely, because this case should lead to the drastic decrease of proportions of minor fractions in the mitochondrial genome through the generations. But, the proportion of the minor mitochondrial genes are maintained at about 30% in the alloplasmic wheat (Fig. 2). Nuclear genes in alloplasmic wheat seem not to influence to expression of mitochondrial genes in the minor fractions (Fig. 3 and Table 1). Methylation of mitochondrial genes in the pollen through hybridization process and/or that of paternal genes in zygote is plausible. Thus, future experiments are required to further understand mitochondrial gene expression from the heteroplasmy.

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