Evidence of paternal transmission of mitochondrial DNA in a nucleus-cytoplasm hybrid of timopheevi wheat

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Structural heterogeneity depicted as heteroplasmy of the mitochondrial (mt) transcriptional unit of \textit{nad3-orf156} (\textit{atp8}) was studied in a nucleus-cytoplasm (NC) hybrid of \textit{Triticum timopheevi} with the D plasmon from the maternal \textit{Aegilops squarrosa} and compared with that of the parental lines. The tetraploid NC hybrid and the parental lines both showed varying degrees of heteroplasmy in this mtDNA region. The G plasmon of the paternal \textit{T. timopheevi} possessed five sequence types, while two sequence types were detected in the D plasmon of \textit{Ae. squarrosa}. The NC hybrid possessed all the five sequence types identical to those of the paternal parent in a 30% relative stochiometry. The remaining 70% comprised only one of the two maternal sequence types, suggestive of strong and selective NC interaction. No novel sequence types were detected and the relative stochiometries of the paternal sequence types were conserved in the NC hybrid. No paternal-identical or -related sequences were detected in the maternal D plasmon. These results provide evidence of the paternal transmission of the mtDNA and possibly account for the origin of the observed mtDNA heteroplasmy in the NC hybrid.

INTRODUCTION

Mitochondrial DNA (mtDNA) heteroplasmy is defined as conditions in which heterogeneous mtDNA molecules coexist in the organella and/or cells within individuals. MtDNA heteroplasmy is now widely recognized in a number of animal taxa. The origins of mtDNA heteroplasmy and the mechanisms of its proliferation, maintenance and transmission may differ in different systems and under different conditions (Chesser, 1998; Coller et al., 2001; Chinnery, 2002). Importantly, the phenomenon can provide a valuable experimental means of clarifying mtDNA inheritance, i.e. the molecular mechanisms of mtDNA proliferation, transmission and segregation during ontogeny. In humans, in particular, mtDNA heteroplasmy has recently attracted much attention because it is directly associated with many of the more than 100 pathogenic mutations occurring in the mtDNA (DiMauro and Andreu, 2000; McFarland et al., 2002; Manfredi et al., 2002). In plants, mtDNA heteroplasmy also has significant biological consequences, particularly in cases of cytoplasmic male sterility and fertility restoration, which involves a target mitochondrial gene(s) and a nuclear restorer gene(s) (He et al., 1995; Janska et al., 1998) and forms the basis for realizing hybrid heterosis in agricultural production.

The mitochondrial polycistronic transcriptional unit \textit{nad3-orf156} (\textit{atp8}) exhibits varying degrees of heteroplasmy in the nucleus-cytoplasm (NC) hybrids of tetraploid and hexaploid wheat possessing the D or D2 plasmon of \textit{Aegilops} species (Tsukamoto et al., 2000; Hattori et al., 2002). Similar heteroplasmic conditions occur in the same region in the respective parental pure lines (Hattori et al., 2002). Important issues to be addressed are concerned with the origin and the biological significance of the observed mtDNA heteroplasmy. The nuclear genome (AAGG) of timopheevi wheat (\textit{Triticum timopheevi} and \textit{T. araraticum}) is functionally different from that of durum wheat (AABB): The former is compatible and the latter is incompatible with the D plasmon of \textit{Ae. squarrosa} (Ohtsuka, 1991; Asakura et al., 1997). The G plasmon of timopheevi wheat also differs from the B plasmon of common wheat: It is incompatible with the nuclear genome of common wheat and causes male sterility (Tsunewaki, 1996). We observed in a previous study that a majority of the \textit{nad3-orf156} (\textit{atp8}) copies were the paternal types in the NC hybrids with the compatible nuclear genome of timopheevi wheat, whereas a majority were the maternal type in the NC hybrids with the incom-
patible nuclear genome of durum wheat (Tsukamoto et al., 2000). This observation prompted us to further examine the heteroplasmic status in timopheevi wheat and compare it with that in the NC hybrid. In the course of the study, we observed that timopheevi wheat was heteroplasmic, possessing five different copies of the region in its G plasmom. Furthermore, all of these copies were identical to the paternal copies and their relative stoichiometries were well conserved in the NC hybrid. These observations provided strong evidence that the mtDNA heteroplasmis is due to the paternal transmission of mtDNA molecules in the NC hybrid. Selective amplification of one maternal sequence type was also observed in the NC hybrid.

MATERIALS AND METHODS

Plant materials. An NC hybrid and its parental lines, Triticum timopheevi var. typicum (KU107-1, 2n=4x=28) and Aegilops squarrosa var. typica (KU20-2, 2n=2x=14, DD), were used. The maternal Ae. squarrosa line used for producing the NC hybrid is a colchicine-doubled tetraploid derivative (KU29) of a diploid accession, KU20-2. The paternal nuclear genome of T. timopheevi is compatible with the D plasmom of Ae. squarrosa, due to its possession of two NC-compatibility genes, Ncc-tmp1A and Ncc-tmp1G, on the homoeologous chromosomes 1A and 1G, respectively (Asakura et al., 1997, 2000). The NC hybrid was produced by combining the D plasmom of Ae. squarrosa with the compatible nucleus of the recurrent T. timopheevi pollen parent (KU107-1) through repeated backcrosses (BC_{15}F_{x}) (Ohtsuka 1991). The diploid and tetraploid lines of Ae. squarrosa showed no differences in the constitution of the mitochondrial nad3-orf156 (atp8) region (Tsukamoto et al., 2000).

PCR amplification and identification of sequence types in the nad3-orf156 (atp8) region. Total DNA was extracted from 2- to 3-week-old plants from the NC hybrid and the parental lines. In each line, DNA from 10 plants was combined to equalize potentially varying degrees of heteroplasmy in individual plants. The nad3-orf156 (atp8) region (Fig. 1A) is a polycistronic mitochondrial transcriptional unit encompassing four open reading frames (ORFs; nad3, rps12, orf299 containing a duplicated part of the first exon of coxII, and orf156 (atp8)) (Gualberto et al., 1991). orf156 shows significant homology with the sugar beet atp8 gene encoding subunit 8 of ATP synthase (Kubo et al., 2000). The region was amplified by PCR using an oligonucleotide primer set: 01, 5'-GAGAGCCGAGAGACGGAATA-3' and 02: 5'-GAAAAAGTTGCTTGCTTTCTT-3'. Twenty-five cycles of PCR were performed using Taq DNA polymerase (Toyobo) and GeneAmp PCR System 9600 (Applied Biosystems) according to Hattori et al. (2002). The PCR-amplified fragments were cloned into pGEM-T vector (Promega) and used to transform Escherichia coli JM109. Sequences of the randomly cloned inserts were determined using the automated fluorescence dye deoxy terminator cycle sequencing system and an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The inserts were re-amplified by PCR using the same primer set and under the same conditions. Based on previously determined information on the restriction sites (Tsukamoto et al., 2000), the re-amplified inserts were digested with MspI and resolved by electrophoresis through 8% polyacrylamide gels. All MspI sites and polymorphic sites (nucleotide substitutions and insertions/deletions) were mapped in the region and PCR-RFLP patterns of the inserts were compared with the determined sequence types to identify individual clones.

Estimation of the relative stoichiometries of heteroplasmic sequence types in the nad3-orf156 (atp8) region. After determination of the identity of all sequence types, the random PCR clones from the NC hybrid and the parental lines were classified according to their RFLP patterns. More than 200 random clones were analyzed per line and their relative stoichiometries were determined based on the percentage frequency of each sequence type.

RESULTS

Detection and identification of heteroplasmic sequence types in the nad3-orf156 (atp8) region. Four different sequence types, designated g1, g2, g3 and g4, were recognized based on their MspI-RFLP patterns in the G plasmom of the paternal parent T. timopheevi, while two sequence types, d1 and d2, were recognized in the D plasmom of the maternal parent Ae. squarrosa (Fig. 1). Sequence analysis of the corresponding clones confirmed the positions of all single nucleotide polymorphisms (SNPs) including ones generating MspI sites (Fig. 1A) and all insertions/deletions (Fig. 2). The major copy of g1, i.e. g1a, was identical to the consensus b1 copy of the B plasmom of T. durum and T. aestivum within the overlapping region studied (Gualbert et al., 1991; Hattori et al., 2002). Another sequence type of g1 (g1b) was detected in the G plasmom based on the sequence comparison: This copy differed from the major g1a copy by five SNPs (Fig. 2). The major d1 copy of the D plasmom was identical to the previously identified major d1 copy in the D and D2 plasmoms of Aegilops species (Hattori et al., 2002). In total, there were 70 SNPs and five insertions/deletions of one to six nucleotides between the parental lines. Fifty-three of the SNPs were in the coding regions: six SNPs in nad3, three in rps12, six in orf156 (atp8) and 38 in orf299. Most of the SNPs were detected in orf299, but this ORF likely represents a pseudogene in
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the B plasmon of common wheat (Gualberto et al., 1991). Of the 15 SNPs found in the other three ORFs, four, two and three were synonymous substitutions in \textit{nad3}, \textit{rps12} and \textit{orf156} (\textit{atp8}), respectively. One insertion of three nucleotides (GGG) occurred in \textit{orf156} (\textit{atp8}) of the d2 copies. In the NC hybrid, six sequence types were recognized, and importantly five of them, \textit{g1a}, \textit{g1b}, \textit{g2}, \textit{g3} and \textit{g4}, were all identical to those detected in the

Fig. 1. (A) A schematic illustration of the \textit{nad3-orf156} (\textit{atp8}) region and \textit{MspI} restriction sites in the six \textit{nad3-orf156} (\textit{atp8}) clones. (B) PCR-RFLP patterns of the various \textit{nad3-orf156} (\textit{atp8}) clones. The PCR products were digested with \textit{MspI}. The relative mobility of the fragments in the non-denaturing polyacrylamide gel does not necessarily correspond to their molecular size. \textit{(squarrosa)-T. timopheevi} represents the NC hybrid of \textit{T. timopheevi} with the D plasmon of \textit{Ae. squarrosa}.
G plasmon of the paternal *T. timopheevi*. Only the major d1 copy but no d2 copy of *Ae. squarrosa* was detected in the NC hybrid, suggesting complete selection of the d1 copy. A similar selective amplification of the d1 copy was shown in a previous study of hexaploid NC hybrids of cv. Chinese Spring with the D or D2 plasmom from var-

Fig. 2. Polymorphic sites detected among seven sequence types of the nad3-orf156 (*atp8*) region in the NC hybrid and the parental lines. The g1a and d1 copies had sequences identical to the consensus sequence of the B and D plasmons, respectively (Gualberto et al., 1991; Tsukamoto et al., 2000; Hattori et al., 2002). Dashed lines indicate identical nucleotides and blanks deleted nucleotides. Asterisks indicate nonsynonymous substitutions; they are omitted in orf299 because it is likely a pseudogene.
Estimation of the relative stoichiometry of the heteroplasmic sequence types in the \textit{nad3-orf156} (\textit{atp8}) region. The g1 (g1a + g1b), g2, g3 and g4 copies were found in 66.8%, 7.7%, 14.9% and 10.6% of the 208 random PCR clones from the G plasmon of \textit{T. timopheevi}, respectively (Fig. 3). The stoichiometries of the g1a and g1b copies were not studied separately because they could only be identified by direct sequencing. The d1 and d2 copies occurred in 90.6% and 9.4% of the 203 clones from the D plasmon of \textit{Ae. squarrosa}, respectively. No copies identical or paralogous to the copies found in the G plasmon were detected from the D plasmon. Seventy percent of the 200 clones from the NC hybrid possessed the d1 copy and the remaining 30% had the four copies found in the G plasmon. It was notable that these four G-plasmon copies occurred with similar frequencies to those observed in the paternal G plasm of \textit{T. timopheevi}.

**DISCUSSION**

We observed a high level of heteroplasmy in the \textit{nad3-orf156} (\textit{atp8}) region in the NC hybrid of timopheevi wheat with the D plasmon of \textit{Ae. squarrosa} and its parental lines. No polymorphisms were detected in six chloroplast DNA regions studied between the NC hybrid and the parents (data not shown). In this study we addressed the question “what is the origin of the observed
mtDNA heteroplasmy in the nad3-orf156 (atp8) region in wheat, Aegilops and NC hybrids?". We presented a line of evidence, albeit still circumstantial, that the observed mtDNA heteroplasmy in the NC hybrid can be ascribed to the paternal transmission of the mtDNA molecules. There are two reasons for drawing this conclusion. First, five paternal sequence types detected in the NC hybrid of T. timopheevi with the D plasmon were all identical to the G-plasmon copies of T. timopheevi. This argues against the possibility that random modifications (such as nucleotide substitutions and recombinations) of the D-plasmon copies (either d1 or d2) could result in the accidental generation of these G-plasmon-identical sequences. It is well known that intensive and extensive modifications of nuclear genomes occur in synthetic allopolyploids of wheat that are produced through interspecific hybridization followed by chromosome doubling (Comai, 2000; Ozkan et al., 2001; Shaked et al., 2001; Kashkush et al., 2002). With respect to plasmons, however, no relevant information on such so-called McClintock's genome shock effect has been available so far. We observed only the paternal-identical copies in addition to the maternal-identical copies in the NC hybrid (Figs. 2 and 3). This result suggests that the plasmon has been rather stable at the molecular level in the NC hybrid during hybridization, amphidiploidization and subsequent propagation through backcrossing and self-fertilization over the last ca. 30 years. Second, we could not detect even a single copy of the G-plasmon-identical or -related sequences in the 203 random PCR clones from the maternal parent. We previously studied more than 115 clones from the D and D2 plasmons of Aegilops species and detected no paternal type copies (Hattori et al., 2002). Laser et al. (1997) observed that a paternal rye-homologous sequence of the mitochondrial orf25 detected in the cytoplasm of the wheat-rye hybrid crop triticale was present in a small sub-stoichiometric amount (0.1%) in the cytoplasm of the maternal wheat parent. They suggested that the observed mtDNA heteroplasmy was due to preferential amplification of the hidden rye homologue in the wheat mitochondria. The origin and significance of this rye-homologous sequence in the wheat mtDNA remains unknown. Although proof of absence is difficult, it is improbable that all the five identical copies of paternal sequences were present in the D plasmon of the maternal parent. In plants, paternal transmission of mtDNA has been reported in several cases (Erickson et al., 1989; Kuroiwa et al., 1992; Faure et al., 1994). In Pelargonium, paternal mtDNA is present in pollen grains at least until just after pollen mitosis (Kuroiwa et al., 1992; Nagata et al., 1999). In wheat and related species, however, cytoplasmic organelles (mitochondria and chloroplasts) have long been believed to obey strict maternal inheritance. Further detailed studies must be conducted to solve this issue.

mtDNA heteroplasmy is not restricted to the NC hybrids but also occurs in the parental pure lines (Hattori et al., 2002; and present results). At least two possibilities can be envisaged for the origin of mtDNA heteroplasmy in these pure lines. One explanation simply assumes mutations in the mtDNA that occurred and accumulated in their plasmons. The mtDNA heteroplasmy observed in the D plasmon of Aegilops squarrosa is likely to be ascribed to this mutation mechanism: the d1 and d2 copies detected in the D plasmon differed in 18 SNPs and two insertions/deletions between them (Fig. 2). The other scenario is that the paternal mtDNA was transmitted to zygotes during interspecific hybridization and maintained throughout chromosome doubling and subsequent propagation. This can be applied to species of allopolyploid origin in Triticum and Aegilops. In fact, we detected a D-plasmon-identical copy of the nad3-orf156 (atp8) region (d1 copy) in all hexaploid wheat species and synthetic hexaploid wheat lines so far studied (Hattori et al., 2002; and our unpublished results). These allopolyploids originated through either natural or artificial hybridization between various sources of maternal tetraploid wheat and paternal Ae. squarrosa.

Mutations of mtDNA are the most important cause of mitochondrial genetic diseases in humans (Chinnery et al., 2000). MtDNA mutations associated with nuclear mutations are also likely related to male sterility and fertility restoration in common bean (He et al., 1995; Janska et al., 1998) and also to some other peculiar plant phenotypes such as non-chromosomal stripe in maize (Newton and Coe, 1986; Newton et al., 1990) and chloroplast mutator in Arabidopsis (Martinez-Zapater et al., 1992; Sakamoto et al., 1996). In both human and plant mtDNAs, the relative stoichiometries of variants to normal mtDNA molecules, i.e. the mutation load, seem to be the primary determinant of the phenotypes in heteroplasmic individuals. We detected only the d1 copy in all the NC hybrids so far studied, in spite of the heteroplasmic conditions of the maternal parents (Tsukamoto et al., 2000; Hattori et al., 2002; and Fig. 3). This suggests that the selective proliferation of particular heteroplasmic copies is under the control of strong NC interaction. Heteroplasmic states occur in at least five other mtDNA regions (coxI, coxIII, atpA, atpA-atp9, rps13-atp6) in the tetraploid NC hybrids (Tsukamoto et al., 2000). It remains to be seen if stoichiometries of these heteroplasmic regions are also under control of NC interaction.

An important question that remains to be addressed is the biological significance of the observed mtDNA heteroplasmy. The mtDNA heteroplasmy so far studied in wheat, Aegilops and the NC hybrids seems not directly related to male sterility/fertility- restoration and/or to any other particular phenotypes. To obtain clues about the physiological significance, biochemical and phenotypic variations between the NC hybrids and parental lines
have to be carefully studied at different developmental stages and under different environmental conditions. Such biological data must then be compared with transcriptional, post-transcriptional and translational characteristics of the heteroplasmic copies in the NC hybrids. Several wheat NC hybrids with alien plasmons from distantly related species, e.g. *Agropyron* and rye (*Secale cereale*), are known to show distinct differences in phenotypes including overall plant vigor, variegation and male sterility/fertility-restoration, depending on the nuclear genotypes, i.e. the presence and absence of at least a single dose of specific maternal chromosomes carrying NC-compatibility genes (Nakata et al., 1986; Nakamura et al. 1991, Suzuki et al., 1994, 1995). These materials should be useful in studying this novel phenomenon in wheat and related species.

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