An apple Atp9 pseudogene is maintained at high copy number in 'Golden Delicious'-type mitochondria but is present substoichiometrically in 'Delicious'-type mitochondria.
Short communication

An apple *atp9* pseudogene is maintained at high copy number in ‘Golden Delicious’-type mitochondria but is present substoichiometrically in ‘Delicious’-type mitochondria.

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Key words:

Apple, Cytoplasmic diversity, Mitochondrial *atp9* gene, Recombination, Substoichiometric sequences
Abstract

In this study, the mitochondrial \textit{atp9} gene sequence of an apple cultivar ‘Golden Delicious’ was found to exist in one intact version and two truncated versions (termed \textit{φatp9-1} and \textit{φatp9-2}). Interestingly, the \textit{φatp9-1} sequence is maintained at high copy number in the six ‘Golden Delicious’-cytotype cultivars examined but present substoichiometrically in eight ‘Delicious’-cytotype cultivars. Our data also suggest that \textit{φatp9-1} originated in a homologous recombination event mediated by the short repeat in a common ancestral mitochondrial genome of ‘Golden Delicious’ and ‘Delicious’, and was preferentially amplified in an evolutionary lineage that gave rise to the ‘Golden Delicious’-type genome. On the other hand, \textit{φatp9-2} was revealed to be present in high abundance in all 14 cultivars examined.
1. Introduction

A fundamental characteristic of higher plant mitochondrial DNA (mtDNA) is its propensity to recombine across dispersed repetitive sequences (Knoop, 2004; Mackenzie, 2007). Extensive recombination frequently generates considerable variation in genomic organization. Such recombination events are also likely to be involved in the creation of mitochondrial chimeric genes and pseudogenes (Conklin and Hanson, 1994; Fauron et al., 2004). In apples (Malus x domestica Borkh.), mtDNA has been used to characterize the cytoplasmic diversity of a wide range of cultivars and landraces (Ishikawa et al., 1992; Kato et al., 1993). The use of mitochondrial cox1 (cytochrome c oxidase subunit 1) and atp9 (ATP synthase F1 subunit 9) gene probes detected restriction fragment length polymorphisms (RFLPs), which enabled classification of a large number of apple genotypes into four cytoplasmic groups: ‘Golden Delicious’ type, ‘Delicious’ type, ‘McIntosh’ type, and ‘Dolgo Crab’ type.

In order to understand the molecular basis of the changes in the mitochondrial genome leading to these diverse cytoplasmic types, it is necessary to analyze the genomic regions in each of the four mtDNA types that can be used to distinguish apple germplasm. Wakatsuki et al. (2011) have recently shown that in two apple cultivars, ‘Golden Delicious’ and ‘Delicious,’ the cox1 reading frame exists as one full-length version (intact copy) and one truncated version (pseudocopy), and that the intact cox1 and pseudocopy have an 1115 bp segment in common. They also suggested that recombination events may have occurred within the 1115 bp repeats to create the two distinct mitochondrial genome organizations characteristic of the ‘Golden Delicious’ and ‘Delicious’ cytotypes. In this paper, we present an analysis of the rearrangements involving the atp9 loci of
the ‘Golden Delicious’ and ‘Delicious’ cytotype cultivars and rootstocks.

2. Materials and methods

2.1. Plant material and nucleic acid preparation

Leaf samples of apple cultivars and rootstocks (Table 1) were obtained from the collections at the National Institute of Fruit Tree Science, National Agriculture and Food Research Organization, Japan and the Field Center for Northern Biosphere, Hokkaido University, Japan. The preparation of total genomic DNA and total RNA from green leaves has been described previously (Wakatsuki et al., 2011).

2.2. Hybridization and sequence analysis

Restriction enzyme digestion, agarose gel electrophoresis, Southern and Northern blot analysis, DNA cloning and nucleotide sequencing were performed using standard protocols (Sambrook et al., 1989; Wakatsuki et al., 2011). The apple *atp9* gene copies were isolated from *HindIII*, *BamHI* or *EcoRI* libraries of total DNA by colony hybridization using the pea *atp9* sequence as the probe (Morikami and Nakamura, 1987).

2.3. PCR analysis

PCR amplifications were performed with 100-150 ng of template DNA, 5 pmol of each forward and reverse primer, and GoTaq (Promega, Madison, WI).
The following PCR program was used: initial denaturation at 94˚C for 5 min, then
35 cycles of incubation at 94˚C for 30 s, 54-60˚C for 30 s and 72˚C for 2-3.5 min,
and a final extension at 72˚C for 5 min. All PCR experiments were repeated at
least three times. The oligonucleotide primers used for PCR are listed in Table 2.

3. Results and Discussion

3.1. ‘Golden Delicious’ atp9 locus

A previous study showed that hybridization of HindIII-digested apple
dNA with the pea atp9 probe produced a 4.8 kb fragment in gels of the ‘Golden
Delicious’ and ‘Delicious’ cytotype cultivars, and an additional 9.2 kb fragment in
subsequently showed that the 4.8 kb fragment from ‘Delicious’ contained an intact
atp9 gene and the first and second exons of the nad5 gene (nad5 ex1 and nad5
ex2).

Here, we isolated the 4.8 kb HindIII fragment from a ‘Golden Delicious’
DNA library. The restriction map of this clone was identical to that of the 4.8 kb
clone from ‘Delicious’ except for one EcoRI site (Fig. 1). Sequence analysis
indicated the presence of a 222 bp atp9 ORF, which shared 100% sequence
identity with the ‘Delicious’ atp9 (Figs. 1 and S1). The ‘Golden Delicious’ atp9 was
found to be expressed as a 0.5 kb mRNA (Fig. S2). Hybridization analysis of the
4.8 kb ‘Golden Delicious’ fragment also showed the existence of nad5 ex1 and nad5
ex2 homologous sequences in a 1.4 kb XhoI-EcoRI subfragment and a 1.4 kb
SacI-SalI subfragment, respectively (data not shown), confirming the
conservation of the atp9 nad5 ex1-nad5 ex2 linkage (Fig. 1).
3.2. *atp9* pseudocopies

Our results suggest the presence of a second *atp9* copy in the ‘Golden Delicious’ mitochondrial genome. The *atp9* probe was used to screen a BamHI library from ‘Golden Delicious’ and identified two clone families. Both families contained BamHI fragments (2.8 and 3.5 kb) corresponding to those detected previously by Southern blot experiments (see Table 1 of Kato et al., 1993). Restriction mapping of these clones indicated that the 4.8 kb *HindIII* fragment overlapped with the 3.5 kb BamHI fragment, but not with the 2.8 kb BamHI fragment. We therefore sequenced the 2.8 kb BamHI fragment.

As shown in Figs. 1, S1 and S3, we found a truncated *atp9* sequence. The truncated copy (termed *φatp9-1*) was virtually identical from nucleotide -116 to +91 to the intact ‘Golden Delicious’ *atp9*. Upstream of nucleotide -116 and downstream from nucleotide +91, the two sequences were completely different. The 3’ divergence resulted in a 62 codon extension of the *φatp9-1* ORF that was not homologous to any known sequence. The 5’ flanking region of *φatp9-1* included a 375 bp *rps12* locus (Gualberto et al., 1988), which in turn was preceded by a truncated *nad3* sequence (Gualberto et al., 1988).

We next asked whether the *φnad3·rps12·φatp9-1* gene cluster resulted from genomic recombination of the intact *atp9* locus with a distant genomic region. To investigate this question, an additional *nad3·rps12* cluster was isolated from a ‘Golden Delicious’ DNA library using the *φnad3* sequence as a probe. Sequence analysis of the isolated clone not only indicated the existence of an apparently intact *nad3·rps12* cluster (Figs. 1 and S1), but also revealed a very short sequence of homology (8/9 bp repeat, boxed in Fig. 1) across which recombination
has presumably taken place to generate the \( \phi_{atp9-1} \) arrangement. Over time, sequence divergence could have occurred in the repeat.

During the RFLP analysis, we became aware that the \( atp9 \) probe reproducibly gave an additional weak hybridization signal (6.0 kb) in EcoRI-digested DNAs of both ‘Golden Delicious’ and ‘Delicious’ (data not shown). In order to determine whether a third \( atp9 \) copy is present in the ‘Golden Delicious’ mitochondrial genome, the 6.0 kb EcoRI fragment was cloned from ‘Golden Delicious’. Restriction mapping and hybridization analysis of this clone indicated that an \( atp9 \) homologous sequence was located in a 0.45 kb \( BglII-BamHI \) subfragment (Fig. 1). Sequence analysis of the 0.45 kb subfragment revealed a segment homologous to the 3’ part (169 bp) of the \( atp9 \) ORF and its 3’ flanking sequence (3 bp) (Figs. 1, S1 and S4). Significant similarity between this truncated \( atp9 \) (termed \( \phi_{atp9-2} \)) and \( atp9 \) abruptly disappeared upstream of nucleotide + 54 and downstream from + 228 (Figs. 1 and S1).

The region surrounding the 0.45 kb \( BglII-BamHI \) subfragment was further sequenced. For this purpose, a 6.5 kb \( BamHI \) fragment overlapping the 6.0 kb EcoRI fragment was also cloned and partially sequenced. The third exon of \( nad5 \) (\( nad5 \) ex3) was shown to lie between 1245 and 1224 bp upstream of \( \phi_{atp9-2} \) (Figs. 1 and S4; Knoop et al., 1991). Furthermore, located downstream from the \( \phi_{atp9-2} \) stop codon were two ORFs with high similarity to the sequences of \( rpl5 \) and \( rps14 \) genes in other higher plant mitochondria (Figs. 1 and S4; Brandt et al., 1993; Ye et al., 1993).

### 3.3. Distribution of \( atp9 \) pseudocopies in apple cultivars

Next, we sought to determine whether the \( atp9 \) pseudocopies are present
in diverse apple genotypes. Total genomic DNAs were prepared from six ‘Golden Delicious’ cytotype cultivars and eight ‘Delicious’ cytotype cultivars and then used for PCR amplification. PCR experiments were performed using the oligonucleotide primers P1 and P2 that are specific to \( \phi \text{atp9-1} \). The DNAs from all 14 genotypes generated a single product of the expected size (ca. 180 bp) (Fig. S5). Some of the PCR products were sequenced to verify their identity. We did note, however, that the amounts of amplification product differed among the cytotypes: the \( \phi \text{atp9-1} \) sequence from six ‘Golden Delicious’ cytotype cultivars was always more intensely amplified (Fig. S5). This indicates that \( \phi \text{atp9-1} \) exists at a higher copy number within the ‘Golden Delicious’ type mitochondrial genome but is maintained substoichiometrically within the ‘Delicious’ type mitochondrial genome (Table 1, see Mackenzie (2007) for substoichiometric molecules). Taken together, our observations suggest that \( \phi \text{atp9-1} \) originated in a homologous recombination event mediated by the 8/9 bp repeat in the common ancestral mitochondrial genome of ‘Golden Delicious’ and ‘Delicious’, and was preferentially amplified in the lineage that led to the ‘Golden Delicious’ type mitochondrial genome.

We carried our further PCR assays with two pairs of primers (P3/P4 and P3/P5), which allow the detection of the \( \phi \text{atp9-2} \) sequence, using the DNAs from the 14 apple genotypes as templates. All the plants examined yielded an intense fragment of the predicted size (ca. 100 bp for P3/P4 and ca. 110 bp for P3/P5), thereby revealing the presence of \( \phi \text{atp9-2} \) in high abundance irrespective of the cytotype (Table 1, Fig. S5).

Acknowledgements

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References


Fig. 1. Genomic organization of an intact *atp9* gene and two pseudocopies (φ*atp9*-1 and φ*atp9*-2) from ‘Golden Delicious’. The locations of *nad3*, φ*nad3*, *rps12*, *nad5* ex1, *nad5* ex2, *nad5* ex3, *rpl15* and φ*rps14* are also indicated. The homologous regions are shaded. The sequences of the homologous portions of 9.0-kb *BamHI* clone, 2.8-kb *BamHI* clone and 4.8-kb *HindIII* clone were aligned to reveal the 8/9-bp repeat boxed in the nucleotide sequences shown enlarged above for each of the three clones. Restriction sites are given for *BamHI*(B), *BglII*(Bg), *EcoRI*(E), *HindIII*(H), *NcoI*(N), *PstI*(P), *PvuII*(Pv), *SacI*(Sc), *SalI*(Sl) and *XhoI*(X). An *EcoRI* site lost in the ‘Golden Delicious’ *atp9* locus relative to the ‘Delicious’ *atp9* locus is circled (see Fig. S1).
Supplementary Figure Captions

Fig. S1. Nucleotide sequence comparison of ‘Delicious’ atp9 (1) (Kato et al. 1995), ‘Golden Delicious’ atp9 (2), and two ‘Golden Delicious’ pseudocopies, φatp9·1 (3) and φatp9·2 (4). Numbering begins with the first nucleotide of the presumed start codon (+1). Start and stop codons are boxed. Identical bases are interconnected by asterisks. The 38-bp sequence shared by the four loci is shaded. Nucleotide substitutions in the ‘Golden Delicious’ atp9 locus at positions 12 and 11 result in the loss of an EcoRI site (see text and Fig. 1). ‘Delicious’ atp9 sequence is referred from accession number D37958 (nucleotide position 121-556). ‘Golden Delicious’ atp9 sequence contains four nucleotide changes relative to ‘Delicious’ atp9: T to A, T to A, T to G and C to A at nucleotide positions 80, 81, 170 and 171, which are located in the 5’ non-coding region of atp9. Nucleotide sequence data for (3) and (4) are included in the sequences deposited as accession numbers AB674548 and AB674549, respectively.

Fig. S2. Transcription analysis of the ‘Golden Delicious’ atp9 gene. The atp9-specific probe was generated by PCR and was allowed to hybridize to a Northern blot containing total RNA from ‘Golden Delicious’. The 2.9-kb band probably corresponds to bicistronic transcripts covering atp9, nad5 ex1 and nad5 ex2, whereas the 0.5-kb band most likely represents the processed atp9 mRNA.
Fig. S3. Nucleotide sequence comparison of the *nad3-rps12* gene cluster (1) and *φnad3-rps12-φatp9-1* gene cluster (2) in the ‘Golden Delicious’ mitochondrial genome. Amino-acid translations are given for *nad3* and *rps12*. Positions of the primers used are underlined. Nucleotide sequence data for (1) and (2) have been deposited as accession numbers AB674547 and AB674548, respectively.

Fig. S4. Nucleotide sequence of *φatp9-2* and flanking regions in the ‘Golden Delicious’ mitochondrial genome. Exon 3 of the *nad5* gene is located upstream of *φatp9-2*, whereas *rpl5* and *φrps14* are located downstream from *φatp9-2*. Amino-acid translations are given for *nad5 ex3* and *rpl5*. Positions of the primers used are underlined. Nucleotide sequence data have been deposited under accession number AB674549.

Fig. S5. PCR amplification of the *φatp9-1* and *φatp9-2* sequences. The PCR reaction mixture consisted of 100ng template DNA, 5pmol of each forward and reverse primer, and GoTaq (Promega, Madison, WI). The experiments were carried out using primers P1/P2 for the detection of *φatp9-1*, and P3/P4 and P3/P5 for *φatp9-2*. Total genomic DNA was prepared from ‘Golden Delicious’ (1), ‘Delicious’ (2), ‘Cellini’ (3), ‘Geneva’ (4), ‘Hopa Crab’ (5), ‘Red Astrachan’ (6), ‘Yellow Transparent’ (7), ‘M9’ (8), *M. prunifolia* (acc. 6109011-0001) (9), ‘Fuji’ (10), ‘Jonathan’ (11), ‘Jonagold’ (12), ‘Tsugaru’ (13) and ‘Natsunobeni’ (14).
Table 1

Apple cultivars and rootstocks analyzed for the presence of *atp9* pseudogene copies (see Fig. S5)

<table>
<thead>
<tr>
<th>Cultivar/rootstock</th>
<th>Parentage</th>
<th>Relative abundance of <em>φ</em>&lt;sub&gt;atp9&lt;/sub&gt;-1</th>
<th><em>φ</em>&lt;sub&gt;atp9&lt;/sub&gt;-2</th>
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<tbody>
<tr>
<td><strong>‘Golden Delicious’-type cytoplasm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Golden Delicious</td>
<td>Possibly Grimes Golden × Golden Reinette</td>
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<td>High</td>
</tr>
<tr>
<td>Fuji</td>
<td>Ralls Janet × Delicious</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Jonathan</td>
<td>Esopus Spitzenburg × Unknown</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Jonagold</td>
<td>Golden Delicious × Jonathan</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Tsugaru</td>
<td>Golden Delicious × Jonathan</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Natsunobeni</td>
<td>Vista Bella × Unknown</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td><strong>‘Delicious’-type cytoplasm</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delicious</td>
<td>Possibly Yellow Bellflower × Unknown</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Cellini</td>
<td>U. K. cultivar</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Geneva</td>
<td>Canadian cultivar</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Hopa Crab</td>
<td>U. S. A. cultivar</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Red Astrachan</td>
<td>Russian cultivar</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Yellow Transparent</td>
<td>Russian cultivar</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>M9</td>
<td>Jaune de Metz Paradise</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>6109011-0001</td>
<td><em>M. prunifolia</em></td>
<td>Low</td>
<td>High</td>
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</table>
List of primers used (see Figs. S3 and S4 for the positions of primers)

<table>
<thead>
<tr>
<th>Primer</th>
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<td>P1</td>
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</tr>
<tr>
<td>P2</td>
<td>5'-GCACCTTCTAACATCTCGAGTTGATC-3'</td>
</tr>
<tr>
<td>P3</td>
<td>5'-TCACGAAGAGGATTTCCGGACTG-3'</td>
</tr>
<tr>
<td>P4</td>
<td>5'-GATTTCGGGCCACGGAATGGATCA-3'</td>
</tr>
<tr>
<td>P5</td>
<td>5'-TGATTGGTTAGCCAATGATGGATTCGGGC-3'</td>
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</table>
Fig. 1
Fig. S3
Fig. S5