A frameshift mutation of the chloroplast matK coding region is associated with chlorophyll deficiency in the Cryptomeria japonica virescent mutant Wogon-Sugi

Tomonori Hirao · Atsushi Watanabe · Manabu Kurita · Teiji Kondo · Katsuhiko Takata

Abstract Wogon-Sugi has been reported as a cytoplasmically inherited virescent mutant selected from a horticultural variety of Cryptomeria japonica. Although previous studies of plastid structure and inheritance indicated that at least some mutations are encoded by the chloroplast genome, the causative gene responsible for the primary chlorophyll deficiency in Wogon-Sugi, has not been identified. In this study, we identified this gene by genomic sequencing of chloroplast DNA and genetic analysis. Chloroplast DNA sequencing of 16 wild-type and 16 Wogon-Sugi plants showed a 19-bp insertional sequence in the matK coding region in the Wogon-Sugi. This insertion disrupted the matK reading frame. Although an indel mutation in the ycf1 and ycf2 coding region was detected in Wogon-Sugi, sequence variations similar to that of Wogon-Sugi were also detected in several wild-type lines, and they maintained the reading frame. Genetic analysis of the 19 bp insertional mutation in the matK coding region showed that it was found only in the chlorophyll-deficient sector of 125 full-sibling seedlings. Therefore, the 19-bp insertion in the matK coding region is the most likely candidate at present for a mutation underlying the Wogon-Sugi phenotype.

Keywords Cryptomeria japonica · Wogon-Sugi · Virescent mutant · matK gene · Frameshift mutation

Introduction

In higher plants, chlorophyll-deficient mutants such as albino, variegated and virescent mutants have been selected and utilized to elucidate the development and function of the chloroplast. The virescent forms of these mutants are phenotypically characterized as having a lag in chlorophyll accumulation in young leaves (Archer and Bonnett 1987; Archer et al. 1987) and have been isolated in a wide range of flowering plants including rice (Iba et al. 1991), barley (Jain 1966), maize (Hopkins and Elman 1984), cotton (Benedict and Kohel 1970), tobacco (Archer and Bonnett 1987; Archer et al. 1987), and peanut (Benedict and Ketting 1972).

The causative genes or alleles responsible for virescent mutations have recently been described in several plants. For example, virescent or delayed-greening mutants (cue3, cue6, cue8, and clpR1-1) of Arabidopsis are involved in the positive regulation of nuclear gene expression (López-Juez et al. 1998; Vinti et al. 2005; Koussevitzky et al. 2007). Plastid genome (plastome) mutants have been isolated in Hordeum (Rios et al. 2003; Landau et al. 2007), and the corresponding mutations have been characterized at the
DNA sequence level. In the case of plastome mutants, virescence is inherited in a non-Mendelian fashion, i.e., maternally in the majority of angiosperms and occasionally biparentally.

*Cryptomeria japonica* (Sugi) is a coniferous tree species that belongs to the group Cupressaceae sensu lato (Kusumi et al. 2000). In *C. japonica*, a number of mutant varieties show traits such as dwarfism, variegation, and morphological variation in needles and shoots. *Wogon-Sugi* is a virescent mutant, whose new shoots change color from yellowish-white in spring to normal green in late summer. Ohba et al. (1971) used reciprocal crosses between *Wogon-Sugi* and wild-type Sugi to demonstrate that the yellowish-white trait of *Wogon-Sugi* is inherited in a non-Mendelian fashion. In crosses using *Wogon-Sugi* as the male parent (pollen donor) and wild-type Sugi as the female parent, *Wogon-Sugi* occurred at a rate of 89.6%, green and white sectors at 7.5%, and the wild-type phenotype at 2.9%. In contrast, controlled crosses using *Wogon-Sugi* as the female and wild-type Sugi as the male parent resulted in 99% wild-type and 1% *Wogon-Sugi* and chimeric seedlings. This non-Mendelian transmission was maintained even in self-pollinated or backcrossed F1 hybrids derived from the reciprocal crosses. These data suggested paternal inheritance of the plastid genome in *C. japonica*, and provided the initial experimental evidence that at least some types of mutations are encoded by the plastid genome.

Genomic sequencing data provide detailed information on variations and mutations at the nucleotide level. We have already determined the complete nucleotide sequence of the *C. japonica* chloroplast genome using the shotgun sequencing method, and determined that 116 genes are encoded in the genome (Hirao et al. 2008, GenBank accession no. AP009377). Therefore, comparison with wild-type will determine whether there is a specific mutation in the *Wogon-Sugi* chloroplast genome that is responsible for its phenotype.

The objective of this study was to identify the gene that is mutated in the primary chlorophyll-deficient *C. japonica* virescent mutant, *Wogon-Sugi*. To identify candidate genes, we completed nucleotide sequencing of *Wogon-Sugi* and *Yaku-Sugi* by PCR-based genome walking. For wild-type we selected *Yaku-Sugi*, a local race of *C. japonica* in Japan. To identify the specific mutation of the *Wogon-Sugi* chloroplast genome, comparative analyses were conducted between *Wogon-Sugi* and wild-type chloroplast genomes and the mutations detected were examined in 16 *Wogon-Sugi* and 16 wild-type plants. Finally, we examined the relationship between phenotype and genotype using chloroplast DNA markers developed based on the specific mutation of the *Wogon-Sugi* chloroplast genome.

**Materials and methods**

Chloroplast DNA sequencing, sequence assembly, and gene annotation

The virescent mutant of *Cryptomeria japonica* (*Wogon-Sugi*) is preserved in 16 individuals in the Forest Products Research Institute, Forest Tree Breeding Center (FFPRI-FTBC) in Ibaraki, Japan. To determine the complete sequence of this mutant’s chloroplast genome, a *Wogon-Sugi* sample, W-77, was arbitrarily selected from among 16 individual plants. In addition, one wild-type *Yaku-Sugi* plant was employed as a comparative sample to detect mutations in the *Wogon-Sugi* chloroplast genome. The wild-type was collected from a natural *C. japonica* forest in Yaku Island (30°20′N and 130°30′E) and has been planted in FFPRI-FTBC.

Total cellular DNA of both plant types was prepared by the method of Shiraishi and Watanabe (1995). Approximately 100 mg of leaves was frozen in liquid nitrogen and ground in a homogenizer. The homogenized sample was mixed with 1 ml of CTAB buffer (100 mM Tris–HCl, pH 9.0, 20 mM EDTA, 2% CTAB (hexadecytrimethylammonium bromide)) with 0.1% beta-mercaptoethanol added just prior to use. The mixture was incubated at 65°C for 30 min and centrifuged for 10 min at 12,000×g, then 600 µl of the supernatant was transferred to a 1.5 ml microcentrifuge tube. The supernatant was mixed twice with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged for 10 min at 12,000×g. DNA was precipitated from the aqueous phase by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The precipitate was washed twice with 70% ethanol and dissolved in water. Extracted DNA was further purified using the DNeasy Plant Mini kit (Qiagen).

Complete nucleotide sequencing of the *Wogon-Sugi* and *Yaku-Sugi* chloroplast genomes was performed using 345 PCR genome walking primers (http://labglt.nftbc.affrc.go.jp/DNA_analysis_resource/sugi/sugi_cp_primer.html). DNA amplification reactions were carried out in a GeneAmp® PCR System 9700 (Applied Biosystems) programmed for touchdown PCR from 62 to 57°C for each primer. The PCR conditions were as follows: after initial melting at 94°C for 1 min, 30 s of denaturation at 94°C, 1 min of annealing at 62°C, and 1 min of extension at 72°C for 10 cycles of amplification. At each cycle, the annealing temperature was reduced 0.5°C, followed by 30 s of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C for 20 cycles, followed by a final extension at 72°C for 10 min. PCR was performed in a volume of 10 µl containing 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM each dNTP, 0.2 µM each primer, 100 ng genomic DNA and 0.1 U of Taq DNA polymerase.
Amplified PCR products were treated with exonuclease and shrimp alkaline phosphatase to remove excess dNTPs and primers. The exonuclease/alkaline phosphatase treatment was performed by mixing 5 μl PCR product with 0.2 μl exonuclease I (10 U/μl; TAKARA), 2.0 μl shrimp alkaline phosphatase (1 U/μl; Amersham), 1.0 μl SAP 10× buffer and 1.8 μl deionized water, and then incubating at 37°C for 30 min followed by 75°C for 15 min to inactivate the exonuclease and alkaline phosphatase. Cycle sequencing was performed according to the manufacturer’s instructions using BigDye® 2.0 Terminator Cycle Sequencing kit (Applied Biosystems). The sequencing primer (3.2 pmol, the same as the PCR primer), 1.0 μl ABI Dye Terminator Ready- Reaction sequencing premix and 1.5 μl 5× sequence buffer were added to the template. After a 2-min denaturation step at 96°C, dye-terminator reactions were incubated at 96°C for 15 s, 50°C for 1 s and 60°C for 4 min for 25 cycles. Excess dye terminators were removed by ethanol precipitation. The extension products were evaporated to dryness under vacuum, resuspended in Hi-Di formamide (Applied Biosystems), heated for 2 min at 94°C and loaded onto an ABI PRISM® model 3100 DNA sequencer (Applied Biosystems) according to the manufacturer’s directions. For sequence analysis and assembly, we used Sequencher® 3.1 software (Gene Codes Corporation). The determined sequence was annotated using DOGMA (Dural Organellar GenoMe Annotator) software (Wyman et al. 2004) after a FASTA-formatted file of the complete chloroplast genome was uploaded to the program’s server. The fully annotated chloroplast genome of Wogon-Sugi and one Yaku-Sugi plant was submitted to DDBJ GenBank with the following accession numbers: Wogon-Sugi chloroplast genome, AP010966; Yaku-Sugi chloroplast genome, AP010967. The complete nucleotide sequences of the Wogon-Sugi and Yaku-Sugi chloroplast genomes were aligned using GeneDoc software (Nicholas et al. 1997), and sequence variations between the two genomes were examined for single nucleotide polymorphisms (SNPs), simple sequence repeats (SSRs), and insertions or deletions (indels).

Verification of the mutation in the coding region by DNA sequencing

Mutations in the gene-coding regions, detected from sequence alignment, were examined in 16 wild-type plants and 16 Wogon-Sugi individuals by DNA sequencing. The 16 wild-type plants were 11 local races, Ajigasawa-Sugi, Ooshuku-Sugi, Toudou-Sugi, Makinosaki-Sugi, Honna-Sugi, Mura-Sugi, Kuma-Sugi, Tateyama-Sugi, Itoshiro-Sugi, Ashuu-Sugi, Hachirou-Sugi, plus three individuals from a natural population on Yaku Island and two plus-trees (Nagano 1, Iiyama 16) of Japan. Total cellular DNA of these 16 wild-type plants and 16 Wogon-Sugi individuals was prepared by the method of Shiraiishi and Watanabe (1995). Extracted DNA was further purified using the Mag-extractor (Toyobo). DNA sequencing was performed according to the above-described PCR genome walking methods. The sequences determined were aligned using GeneDoc software (Nicholas et al. 1997). Mutations in the nucleotide sequence of the coding region were translated to predict the amino acid sequence, and then the predicted primary structures were compared.

Genetic analysis of the chloroplast genome

A controlled artificial cross was made between a wild-type plant (Yoshiki 1) as the maternal parent and W-77 Wogon-Sugi as the paternal parent. Prior to pollination, the female cones of the wild-type plant were completely enclosed in crossing bags to eliminate foreign pollen contaminants. Mature pollen was collected from Wogon-Sugi male cones for pollination. After pollination, 131 mature F1 seeds were collected, germinated, and the seedlings grown for 1 year in a greenhouse. The phenotype of all 131 seedlings was evaluated for the 20th through the 25th leaves, which were sophomoric leaves and new shoots.

The chloroplast genomes of the 131 seedlings that were characterized for phenotype were examined using a chloroplast DNA marker developed based on a mutation in the Wogon-Sugi chloroplast genome. Total cellular DNA was prepared from each individual by the method of Shiraiishi and Watanabe (1995). Extracted DNA was further purified using the Mag-extractor (Toyobo). Fragment analysis was performed by sizing the PCR products. The forward primer used was 5’-AGGTTATTTTGGTCCCGGGGTTT-3’ and the reverse primer was 5’-ACTAAAATTTCTGGTTCGAGGTTT-3’; these were developed to detect the specific mutation in the marK coding region of the Wogon-Sugi chloroplast genome. The forward primer was labeled with HEX fluorescent dye. DNA amplification was carried out in a PTC-200 thermocycler (MJ Research). The PCR conditions were as follows: after initial melting at 94°C for 1 min, 30 s of denaturation at 94°C, 30 s of annealing at 60°C, and 1 min 30 s of extension at 72°C for 30 cycles, followed by a final extension at 72°C for 5 min. PCR was performed on samples of the same composition and volume described earlier for PCR genome walking. Amplified PCR products were separated using an ABI PRISM® model 3100 automated DNA sequencer. Labeled fragments were detected and sized using GeneScan® 350 ROXTM size standards (Applied Biosystems). All genotypes were determined using Genotypeer® fragment analysis software version 3.7 genotyping software (Applied Biosystems).
Table 1  Sequence variations between Wogon-Sugi (mutant) and Yaku-Sugi (wild-type plant) chloroplast genomes

| Location in Wogon-Sugi | Region                      | Mutation type | Wogon-Sugi | Yaku-Sugi |
|------------------------|-----------------------------|---------------|------------|-----------|
| 10876                  | rps 11-rp36 spacer          | SNP           | T          | G         |
| 16344                  | rpl2 intron                 | SSR           | (TA)<sub>h</sub> | (TA)<sub>h</sub> |
| 20693                  | psa1-trnP spacer            | SSR           | (T)<sub>h</sub> | (T)<sub>h</sub> |
| 21018                  | trnP-trnW spacer            | SNP           | A          | G         |
| 23754                  | psb1-ψ clpP spacer          | SSR           | (T)<sub>h</sub> | (T)<sub>h</sub> |
| 23912                  | psb1-ψ clpP spacer          | SNP           | G          | T         |
| 24189                  | psb1-ψ clpP spacer          | SSR           | (A)<sub>h</sub> | (A)<sub>h</sub> |
| 39584                  | chL-trnH spacer             | SSR           | (T)<sub>h</sub> | (T)<sub>h</sub> |
| 43948                  | matK coding region          | Indel         | 19 bp insertion | –        |
| 78669                  | atpl-atpH spacer            | SSR           | (TA)<sub>h</sub> | (TA)<sub>h</sub> |
| 82805                  | trnG intron                 | SSR           | (A)<sub>h</sub> | (A)<sub>h</sub> |
| 86642                  | trnL-trnF spacer            | SSR           | (TA)<sub>h</sub> | (TA)<sub>h</sub> |
| 92241                  | ndhA intron                 | SSR           | (T)<sub>h</sub> | (T)<sub>h</sub> |
| 105857                 | ψycf68-rps12 spacer         | SNP           | T          | G         |
| 105858                 | ψycf68-rps12 spacer         | SNP           | C          | A         |
| 115914                 | ycf2 coding region          | Indel         | 33 bp insertion | –        |
| 121743                 | ycf2-ycf1 spacer            | SNP           | A          | G         |
| 121981                 | ycf2-ycf1 spacer            | SNP           | A          | G         |
| 128800                 | ycf1 coding region          | Indel         | 66 bp deletion | –        |
| 130055                 | trnL-ccsA spacer            | Indel         | 19 bp deletion | –        |
| 131579                 | ccsA-petA spacer            | SNP           | G          | T         |

The locations and regions of polymorphic sites between Wogon-Sugi and Yaku-Sugi were documented according to the nucleotide and gene order of the annotated Wogon-Sugi chloroplast genome sequence. Mutations were classified into three types: single nucleotide polymorphism (SNP), simple sequence repeat (SSR), and insertion or deletion (indel). SSR mutations of each chloroplast genome are indicated by repeat motif and repeat number.

Results

General characteristics of Wogon-Sugi and wild-type chloroplast genomes

The size of the Wogon-Sugi chloroplast genome was determined to be 131,804 bp, which is slightly larger than the 131,781 bp of wild-type Yaku-Sugi. Both chloroplast genomes encoded a total of 116 genes, 112 of which were single copy and two (trnI-CAU and trnQ-UUG) were duplicated as inverted repeat sequences. Of the 116 genes, there were four ribosomal RNA genes (3.5%), 30 individual transfer RNA genes (25.9%), 21 genes encoding large and small ribosomal subunits (18.1%), four genes encoding DNA-dependent RNA polymerases (3.5%), 48 genes encoding photosynthesis-related proteins (41.4%), and nine genes encoding other proteins, including those with unknown functions (7.8%). Of the 112 single copy genes, 17 contained introns, and three (clpP, trnT-GGU, and ycf68) were identified as pseudogenes. In addition, stop codons were found in the matK (maturase) coding region of the Wogon-Sugi chloroplast genome.

Sequence variations between the two chloroplast genomes

A total of 21 sequence variations (eight SNPs, nine SSRs, and four indels) were identified from the sequence alignment of the Wogon-Sugi and Yaku-Sugi chloroplast genomes (Table 1). The eight SNPs, five of the SSRs, and one indel were identified in intergenic spacer regions. The remaining four SSRs were identified in intron regions. The remaining three indels were identified in three gene coding regions: matK (maturase), ycf2 (hypothetical protein RF2) and ycf1 (hypothetical protein RF1).

An insertional mutation in the matK gene of the Wogon-Sugi chloroplast genome causes a frameshift mutation

The insertion or deletion mutations in coding regions matK, ycf2, and ycf1 were examined in 16 wild-type and 16 Wogon-Sugi plants by DNA sequencing. All three indels of the coding region consisted of duplicated repetitive sequences. The 19-bp insertion in the matK coding region was found only in the 16 Wogon-Sugi individuals. This insertion resulted in a reading frameshift after the amino acid residue phenylalanine (F) at the 24th amino acid residue, introducing a stop codon just after the serine (S) residue at the 39th amino acid residue (Fig. 1). The ycf2 coding region of all 32 plants was classified into four types based on the 33-bp repetitive sequence unit, which is equivalent to an insertion of 11 amino acids (Fig. 2). Four indel variants were identified among the 16 wild-type plants, which are referred to as Types I–IV in Fig. 2. Two wild-type
plants had the DNA sequence depicted as Type I, with five copies of the 33-bp sequence repeat. The Type II variants had four copies (two wild-type plants), Type III had three copies (10 plants), and Type IV had two copies (two plants). All 16 *Wogon*-Sugi plants had the Type I indel. Despite the length variation in the *ycf2* coding region, none of the variants caused a change in the reading frame. Similarly, the *ycf1* coding region was classified into four types based on the 66-bp repetitive sequence unit found in all 32 plants investigated (Fig. 3): Six plants were classified as Type I, which contained five copies of the 66-bp repeat; six plants were Type II with four copies; three were Type III with three copies, and one plant was Type IV with two copies of the repeat. All *Wogon*-Sugi plants belonged to Type II. The indel mutations of the *ycf2* and *ycf1* coding regions do not shift the reading frames, but each additional repeat in the *ycf2* gene is equivalent to an insertion of 11 amino acids and each additional repeat in the *ycf1* gene is equivalent to an insertion of 22 amino acids.

The framenshift mutation of the *matK* coding region of *Wogon*-Sugi is associated with the primary chlorophyll deficiency of offspring

Of the 131 *Wogon*-Sugi seedlings produced through controlled crossing, 114 (87.0%) showed yellowish-white leaves (Fig. 4a), six (4.6%) showed normal green leaves (Fig. 4b), and 11 (8.4%) showed chimeric leaves (Fig. 4c). The chimeric seedlings exhibited yellowish-white sectors (Fig. 4c-i), normal green sectors (Fig. 4c-ii), and variegated sectors (Fig. 4c-iii).

The results of genetic analysis using chloroplast DNA markers are presented in Table 2. The 114 seedlings that showed yellowish-white leaves had the genotype of the *Wogon*-Sugi chloroplast genome, namely the 196-bp PCR product (Fig. 5a). The six seedlings that showed normal green leaves had the genotype of the maternal wild-type chloroplast genome, namely the 177-bp PCR product (Fig. 5b). The 11 seedlings that showed chimeric leaves had the genotypes of both the paternal *Wogon*-Sugi and the maternal wild-type: both the 196- and 177-bp PCR products were present (Fig. 5c-i, ii, iii). For details, in each sector of chimeric seedlings, the yellowish-white sector showed the 196 bp PCR product (Fig. 5c-i), the green sector showed 177 bp PCR product (Fig. 5c-ii), and the variegated sectors showed both 196 and 177 bp PCR products (Fig. 5c-iii). That is, yellowish-white leaves that has the 196-bp PCR product is paternal inheritance of chloroplast genomes, normal green leaves that has the 177-bp PCR product is maternal inheritance, and the variegated sectors that has both 196 and 177 bp PCR products is biparental inheritance.

Discussion

Previous study of the virescent mutation in *Wogon*-Sugi was restricted to observation of chloroplast structure and evaluation of the mode of inheritance of the phenotype (Ohba et al. 1971). Although these data provided the initial experimental evidence that at least some types of mutations are encoded by the chloroplast genome, the causative gene
responsible for the yellowish-white (chlorophyll-deficient) leaves of Wogon-Sugi was not identified. The present results, which used the chloroplast genomic sequence and genetic analysis using chloroplast DNA markers, indicated that a frameshift mutation in the matK coding region is associated with the primary chlorophyll-deficiency traits of Wogon-Sugi.

Although comparative analysis of Wogon-Sugi and Yaku-Sugi identified 21 sequence polymorphisms (including eight SNPs, nine SSRs, and four indels), only three of the polymorphisms were located in the coding region; these three polymorphisms were indels. Therefore, these variations in the matK, ycf2, and ycf1 coding regions were considered candidates for the mutation responsible for the Wogon-Sugi phenotype. In Wogon-Sugi individuals, a 19-bp insertional mutation was found in the matK coding region. This insertion disrupts the matK reading frame. On the other hand, the indels of ycf2 and ycf1, which were respectively 33 and 66 bp repetitive sequence units, were found in both Wogon-Sugi and wild-type chloroplast genomes. Therefore, the insertional sequence in the matK coding region must be the mutation specific to the Wogon-Sugi chloroplast genome and the one responsible for the developmental chlorophyll deficiency, because the 19 bp insertion in the matK gene leads to a reading frameshift after the 24th residue, phenylalanine, and results in a stop codon after the 39th residue in the Wogon-Sugi mutant.

The type of frameshift mutation in the matK coding region has not been reported in other plant species. On the other hand, a similar indel mutation within ycf1 and ycf2 has been reported in intraspecies comparisons of Oenothera hookeri (Blasko et al. 1988) and between plants from Fig. 2 Repetitive indel mutations in the ycf2 coding region of Wogon-Sugi and four wild-type chloroplast genomes. The nucleotide and deduced amino acid sequences of part of the ycf2 gene of Types I–IV, and of Wogon-Sugi, showing variation in the number of 66 bp insertions. Colored nucleotides show the 33-bp repetitive units.
Fig. 3  Repetitive indel mutations in the ycf1 coding region of Wogon-Sugi and four wild-type chloroplast genomes. The nucleotide and deduced amino acid sequences of part of the ycf1 gene of Types I–IV, and of Wogon-Sugi, showing variation in the number of 198 bp insertions. Colored nucleotides show the 66-bp repetitive units.

| Type   | Sequence of ycf1 Coding Region (198 bp) | Sequence of Deduced Amino Acid (446 bp) |
|--------|----------------------------------------|----------------------------------------|
| Type I | TTTAGGGAAGA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA | REK L R K F L T E S R D S L P K L I S S F |
| Type II| TTTAGGGAAGA ATCCTAGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA | F R E K L R K F L T E S R D S L P K L I S S F |
| Wogon-Sugi | TTTAGGGAAGA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA | F R E K L R K F L T E S R D S L P K L I S S F |
| Type III| TTTAGGGAAGA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA | F R E K L R K F L T E S R D S L P K L I S S F |
| Type IV | TTTAGGGAAGA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA | F R E K L R K F L T E S R D S L P K L I S S F |

| Type I   | GGGGAAACTA TAGAAATT   | CTAAACGGAAA GTAGAGTCTC TCTCTCCAAA TTGATAAGTT CTTTATTGGA |
|---------|----------------------|--------------------------|
| Type II  | GGGGAAACTA TAGAAATT   | CTAAACGGAAA GTAGAGTCTC TCTCTCCAAA TTGATAAGTT CTTTATTGGA |
| Wogon-Sugi | GGGGAAACTA TAGAAATT   | CTAAACGGAAA GTAGAGTCTC TCTCTCCAAA TTGATAAGTT CTTTATTGGA |
| Type III | GGGGAAACTA TAGAAATT   | CTAAACGGAAA GTAGAGTCTC TCTCTCCAAA TTGATAAGTT CTTTATTGGA |
| Type IV  | GGGGAAACTA TAGAAATT   | CTAAACGGAAA GTAGAGTCTC TCTCTCCAAA TTGATAAGTT CTTTATTGGA |

| Type I   | GAACTTGAA AAGCTCAA   | CCGAAACTGAG ATCAAGCTCT CCTCTAGGG AAAATTGATA AGTTCTTTTA |
|---------|----------------------|--------------------------|
| Type II  | GAACTTGAA AAGCTCAA   | CCGAAACTGAG ATCAAGCTCT CCTCTAGGG AAAATTGATA AGTTCTTTTA |
| Wogon-Sugi | GAACTTGAA AAGCTCAA   | CCGAAACTGAG ATCAAGCTCT CCTCTAGGG AAAATTGATA AGTTCTTTTA |
| Type III | GAACTTGAA AAGCTCAA   | CCGAAACTGAG ATCAAGCTCT CCTCTAGGG AAAATTGATA AGTTCTTTTA |
| Type IV  | GAACTTGAA AAGCTCAA   | CCGAAACTGAG ATCAAGCTCT CCTCTAGGG AAAATTGATA AGTTCTTTTA |

| Type I   | CTAGAAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
|---------|----------------------|--------------------------|
| Type II  | CTAGAAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Wogon-Sugi | CTAGAAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Type III | CTAGAAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Type IV  | CTAGAAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |

| Type I   | GAAATTTCT AGACGGAAAG AGATTTCTCCT CTCTCAAAAAT TTGATAATTC TCTTATTGGA GAAACTAAA |
|---------|----------------------|--------------------------|
| Type II  | GAAATTTCT AGACGGAAAG AGATTTCTCCT CTCTCAAAAAT TTGATAATTC TCTTATTGGA GAAACTAAA |
| Wogon-Sugi | GAAATTTCT AGACGGAAAG AGATTTCTCCT CTCTCAAAAAT TTGATAATTC TCTTATTGGA GAAACTAAA |
| Type III | GAAATTTCT AGACGGAAAG AGATTTCTCCT CTCTCAAAAAT TTGATAATTC TCTTATTGGA GAAACTAAA |
| Type IV  | GAAATTTCT AGACGGAAAG AGATTTCTCCT CTCTCAAAAAT TTGATAATTC TCTTATTGGA GAAACTAAA |

| Type I   | GAAACCAATGAA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA |
|---------|----------------------|--------------------------|
| Type II  | GAAACCAATGAA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA |
| Wogon-Sugi | GAAACCAATGAA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA |
| Type III | GAAACCAATGAA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA |
| Type IV  | GAAACCAATGAA ACCCTGGAAA ATTCTTACG GAAATGGAG ATTTCTCCTCC AAAATTGATA AGTTCTTTTA |

| Type I   | CTTAGAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
|---------|----------------------|--------------------------|
| Type II  | CTTAGAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Wogon-Sugi | CTTAGAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Type III | CTTAGAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |
| Type IV  | CTTAGAAAAT TCTGAAAGGA AAGTGGAGAT TCTCTCCAAA AATTGATAAGTT CTTTATTGGA GAAACTAA |

| Type I   | R K F L T E S R D S L P K L I S S F R E K L |
|---------|----------------------|--------------------------|
| Type II  | R K F L T E S R D S L P K L I S S F R E K L |
| Wogon-Sugi | R K F L T E S R D S L P K L I S S F R E K L |
| Type III | R K F L T E S R D S L P K L I S S F R E K L |
| Type IV  | R K F L T E S R D S L P K L I S S F R E K L |

| Type I   | R K F L T E S R D S L P K L I S S F R E K L |
|---------|----------------------|--------------------------|
| Type II  | R K F L T E S R D S L P K L I S S F R E K L |
| Wogon-Sugi | R K F L T E S R D S L P K L I S S F R E K L |
| Type III | R K F L T E S R D S L P K L I S S F R E K L |
| Type IV  | R K F L T E S R D S L P K L I S S F R E K L |

| Type I   | R K F L T E S R D S L P K L I S S F R E K L |
|---------|----------------------|--------------------------|
| Type II  | R K F L T E S R D S L P K L I S S F R E K L |
| Wogon-Sugi | R K F L T E S R D S L P K L I S S F R E K L |
| Type III | R K F L T E S R D S L P K L I S S F R E K L |
| Type IV  | R K F L T E S R D S L P K L I S S F R E K L |
subsection *Oenothera* (Nimzyk et al. 1993; Greiner et al. 2008), which were also changes in a repetitive sequence without a reading frameshift. One point to consider is the possibility that the *Wogon*-Sugi chloroplast genome has point mutations that prevent proper annealing of primers in a PCR reaction, and a nuclear paralog with a better amplification instead. Consequently, we conducted the PCR-RFLP analysis as a supplementary analysis, namely detection of longer chloroplast regions, including *matK* from *Wogon*-Sugi total DNA with two different primer combinations. The results showed a clear size differentiation between *Wogon*-Sugi and wild-type (supplementary data S1); the longer regions including *matK* (with the insertional mutation) were only observed in *Wogon*-Sugi, and not for the nuclear paralog.

The *matK* gene, which is encoded in the *trnK* intron of the chloroplast genome, is utilized in systematic studies because of its high mutation rate and resolution (Shaw et al. 2005). Furthermore, indels are frequent in *matK*, though they primarily occur in multiples of three, maintaining the reading frame (Barthet and Hilu 2007). We conducted an additional comparative analysis with other five plant species (three angiosperm species and two gymnosperm species) to examine the conservation of the *matK* amino-acid sequence in *C. japonica*, especially in the location of the insertional mutation of *Wogon*-Sugi. The results indicated that the mutation point of *Wogon*-Sugi after the amino acid residue phenylalanine (F) at the 24th amino acid residue is highly conserved between *C. japonica* generally and other five plants (supplementary data S2). Thus, the location of

![Table 2](image)

Table 2  Results of genetic analysis in 131 offspring using a chloroplast DNA marker

| Inheritance patterna | Wogon-Sugi type (Paternal) | Wild-type type (Maternal) | Chimeric type (Biparental) | Total |
|---------------------|---------------------------|--------------------------|---------------------------|-------|
| Number of observed seedlingsb | 114                       | 6                        | 11                        | 131   |
| Rate (%)            | 87.0                      | 4.6                      | 8.4                       | 100   |

| The 131 offspring were obtained by an artificial cross between wild-type (Yoshiki 1; maternal parent) and *Wogon*-Sugi (paternal parent) |
| The chloroplast genomes of each of the 131 offspring were genotyped based on the length variation of PCR product (Fig. 5) 

![Fig. 4](image)

The phenotypes obtained from an artificial cross between *Wogon*-Sugi (paternal parent) and wild-type (maternal parent). a *Wogon*-Sugi type with yellowish-white leaves. b Wild-type with normal green leaves. c Chimeric type with yellowish-white sectors and normal green sectors. In the chimeric type, the yellowish-white sector, the green sector, and the variegated sectors are shown as c-i, c-ii, and c-iii, respectively. Red arrows show the sample used for genetic analysis.
the insertional mutation of Wogon-Sugi is likely to affect some kind of functional domain in MatK, though it is not clear whether it is an important functional domain such as a reverse-transcriptase (RT) domain, domain X (the proposed maturase functional domain), and a zinc-finger-like domain (Mohr et al. 1993).

A crossing test carried out to identify segregation confirmed that the yellowish-white trait of Wogon-Sugi is inherited in a non-Mendelian fashion (Table 2). Of the 131 F1 full-sibling offspring, 87% were Wogon-Sugi type, 8.4% were chimeric, and 4.6% were wild-type. These results were very similar to those reported by Ohba et al. (1971), suggesting that segregation of the phenotype is significantly different from the theoretical ratio of 3:1 for a Mendelian trait, and therefore one recessive nuclear gene is not responsible for this trait.

The trait of yellowish-white sectors was tightly linked with the genotype of the 19-bp frameshift mutation of the matK gene in the Wogon-Sugi chloroplast genome. A plastid DNA marker targeting the 19-bp insertion of the Wogon-Sugi matK region was useful to objectively evaluate the genotype of the Wogon-Sugi plastid genome. The yellowish-white sectors were dissected from 125 virescent offspring, including 11 chimeric offspring, and all had the genotype of the Wogon-Sugi chloroplast genome, as shown in Fig. 5. Green sectors were harvested from six green offspring and 11 chimeric offspring, and all lacked the matK insertion in the maternal chloroplast genome. These results indicate that the 19-bp insertion of the matK coding region is the most likely candidate for a polymorphism underlying the Wogon-Sugi phenotype.

The matK gene is assumed to be the splicing factor for group II introns in the chloroplast genome (Neuhaus and Link 1987). Although the maturase function of MatK is not clearly understood, in white barley its maturase-like function is indirectly associated with the mutant albostrians, which has a chloroplast ribosome deficiency that results in the loss of all chloroplast-encoded proteins including MatK (for a review, see Hess et al. 1994a; Schmitz-Linneweber and Barkan 2007). The group II intron-containing precursor transcripts of trnK, trnA, trnI, rps12, rpl2, and atpF remain unspliced in albostrians plastids (Hess et al. 1994b; Hübschmann et al. 1996; Vogel et al. 1997, 1999). Barthet and Hilu (2007) suggested that MatK has an essential function as a posttranscriptional splicing factor at a particular developmental stage, and thus its function indirectly contributes to photosynthetic competency of the chloroplast.

In the primary yellowish-white sector of new shoots in Wogon-Sugi plants, there is the possibility that matK of the mutated version lacks or has insufficient function as a result of the frameshift mutation, and that genes containing group II introns might not be spliced. However, there are several unclear aspects about the frameshift mutation of matK in the Wogon-Sugi plastid genome. For example, it is not a lethal mutation, and the yellowish-white traits of Wogon-Sugi often change to normal green in late summer (supplementary data S3). Furthermore, once the yellowish-white leaves change to green, their color is stable. C. japonica is a
perennial plant and the virecence of new shoots in Wogon-Sugi is observed only early in development, even though later the shoot plastids still have the Wogon-Sugi genotype.

Regarding the behavior of the virecent mutation in Wogon-Sugi, one possibility is that a nuclear gene might be associated with the splicing of genes having the group II intron in the plastid genome. Leon et al. (1998) reviewed the range of mutants known to have altered chloroplast development and concluded that almost every step of plastid development depends on the direct action of nuclear-encoded genes. In fact, in maize (Zea mays) chloroplasts, genetic analyses have shown that nuclear-encoded proteins are associated with the splicing of at least 10 of the 17 group II introns (Jenkins et al. 1997; Jenkins and Barkan 2001; Till et al. 2001; Ostheimer et al. 2003; Ostesetzer et al. 2005). Therefore, we hypothesize that a nuclear-encoded splicing factor is recruited as an alternative splicing factor for the chloroplast during the greening of virecent Wogon-Sugi shoots. To test this hypothesis, more detailed analysis is required at the transcriptional and post-transcriptional levels to establish whether the Wogon-Sugi behavior originates with the frameshift mutation of the matK gene, and to determine its association with a nuclear-encoded splicing factor.

Acknowledgments We thank Dr. Shohab Youssefian (Akitia Prefectural University) for helpful discussions, comments and advice, and Dr. Barry Jaquish (BC Ministry of Forests and Range, Victoria) for helpful comments and advice.

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