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RESEARCH ARTICLE

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

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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 *mat*K coding region in the *Wogon*-Sugi. This insertion disrupted the *mat*K reading frame. Although an indel mutation in the *ycf*1 and *ycf*2 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 *mat*K 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 *mat*K coding region is the most likely candidate at present for a mutation underlying the *Wogon*-Sugi phenotype.

Keywords *Cryptomeria japonica* · *Wogon*-Sugi · Virescent mutant · *mat*K gene · Frameshift mutation

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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 Elfman 1984), cotton (Benedict and Kohel 1970), tobacco (Archer and Bonnett 1987; Archer et al. 1987), and peanut (Benedict and Ketring 1972).

The causative genes or alleles responsible for virescent mutations have recently been described in several plants. For example, virescent or delayed-greening mutants (*cue*3, *cue*6, *cue*8, 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

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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 ion 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 (hexadecyltrimethylammonium bromide)) with 0.1% beta-mercaptoethanol added just prior to use. The mixture was incubated at 65°C for 60 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 ten 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 $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, 100 ng genomic DNA and 0.1 U of *Taq* DNA polymerase

(Invitrogen). 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 (Dual 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 Shiraishi 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 Shiraishi 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′-AGGTTATTCTTGGGTCCGGGGGTTT-3′ and the reverse primer was 5′-ACTAAAATTTCCGTTGG TTCGGAG-3′; these were developed to detect the specific mutation in the *mat*K 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 ROX™ size standards (Applied Biosystems). All genotypes were determined using Genotyper® 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-*rpl*36 spacer	SNP	T	G
16344	*rpl*2 intron	SSR	$(TA)_9$	$(TA)_8$
20693	*psa*J-*trn*P spacer	SSR	$(T)_{19}$	$(T)_{20}$
21018	*trn*P-*trn*W spacer	SNP	A	G
23754	*psb*J-*ψ clp*P spacer	SSR	$(T)_{10}$	$(T)_{11}$
23912	*psb*J-*ψ clp*P spacer	SNP	G	T
24189	*psb*J-*ψ clp*P spacer	SSR	$(A)_{11}$	$(A)_{12}$
39584	*chl*L-*trn*H spacer	SSR	$(T)_{11}$	$(T)_{10}$
43948	*mat*K coding region	Indel	19 bp insertion	–
78669	*atp*I-*atp*H spacer	SSR	$(TA)_7$	$(TA)_6$
82805	*trn*G intron	SSR	$(A)_{12}$	$(A)_{11}$
86642	*trn*L-*trn*F spacer	SSR	$(TA)_{22}$	$(TA)_{12}$
92241	*ndh*A intron	SSR	$(T)_{13}$	$(T)_{14}$
105857	*ψycf*68-*rps*12 spacer	SNP	T	G
105858	*ψycf*68-*rps*12 spacer	SNP	C	A
115914	*ycf*2 coding region	Indel	33 bp insertion	–
121743	*ycf*2-*ycf*1 spacer	SNP	A	G
121981	*ycf*2-*ycf*1 spacer	SNP	A	G
128800	*ycf*1 coding region	Indel	66 bp deletion	–
130055	*trn*L-*ccs*A spacer	Indel	19 bp deletion	–
131579	*ccs*A-*pet*A 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 (*trn*I-CAU and *trn*Q-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 (*clp*P, *trn*T-GGU, and *ycf*68) were identified as pseudogenes. In addition, stop codons were found in the *mat*K (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: *mat*K (maturase), *ycf*2 (hypothetical protein RF2) and *ycf*1 (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 *mat*K, *ycf*2, and *ycf*1 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 *mat*K 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 *ycf*2 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

Fig. 1 Reading frameshift mutation in the *mat*K coding region of the *Wogon*-Sugi chloroplast genome. The nucleotide and deduced amino acid sequences of part of the *mat*K gene of *Wogon*-Sugi and wild-type chloroplast genomes are shown. *Blue nucleotides* show the insertion sequence in *Wogon*-Sugi, and *red nucleotides* show the introduced stop codon

```
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     5         15         25         35         45         55
Wogon-Sugi   ATGGGTGAAT TCCAAAGAAA TGAAAACAAA CATAAATCTT GGCAACAATT CTTTTTATAT
               M  G  E   F  Q  R   N  E  N  K  H  K  S   W  Q  Q   F  F  L  Y
Wild type    ATGGGTGAAT TCCAAAGAAA TGAAAACAAA CATAAATCTT GGCAACAATT CTTTTTATAT
               M  G  E   F  Q  R   N  E  N  K  H  K  S   W  Q  Q  F   F  L  Y

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    65         75         85         95        105        115
Wogon-Sugi   CCGCTTTTTT TTTTTATATC CGCTTTTTTT TCGGGAAGAT CTTTACGCAA TTGCTCATGA
               P  L  F   F  F  I   S  A  F  F   S  G  R   S  L  R   N  C  S  *
Wild type    CCGCTTTTTT TT-------- ---------- -CGGGAAGAT CTTTACGCAA TTGCTCATGA
               P  L  F   F                          R  E  D   L  Y  A   I  A  H

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   125        135        145        155        165        175
Wogon-Sugi   TCATCATTTA GATAGATCTG GTTCCTCCGA ACCAACGGAA ATTTTAGTTT CTAATTTTTT
               S  S  F   R  *  I   W  F  L  R   T  N  G   N  F  S   F  *  F  F
Wild type    TCATCATTTA GATAGATCTG GTTCCTCCGA ACCAACGGAA ATTTTAGTTT CTAATTTTTT
             D  H  H  L   D  R  S   G  S  S   E  P  T  E   I  L  V   S  N  F

             ....|....| ....|....| ....|....|
                   185        195        205
Wogon-Sugi   GAGTTTCCTA ACTGTAAAAC GTTCAATTCG
               E  F  P   N  C  K   T  F  N  S
Wild type    GAGTTTCCTA ACTGTAAAAC GTTCAATTCG
             L  S  F  L   T  V  K   R  S  I
```

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 *ycf*2 coding region, none of the variants caused a change in the reading frame. Similarly, the *ycf*1 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 frameshift 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

Fig. 2 Repetitive indel mutations in the *ycf*2 coding region of *Wogon*-Sugi and four wild-type chloroplast genomes. The nucleotide and deduced amino acid sequences of part of the *ycf*2 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

```
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    5         15         25         35         45         55
Type I      CAATTGACAA AAGTATGGAA TAAATACTAT TTGGAATTAC AAAAAGTATG GAATAAATAC
              Q  L  T   K  V  W   N  K  Y  Y   L  E  L   Q  K  V   W  N  K  Y
Wogon-Sugi  CAATTGACAA AAGTATGGAA TAAATACTAT TTGGAATTAC AAAAAGTATG GAATAAATAC
              Q  L  T   K  V  W   N  K  Y  Y   L  E  L   Q  K  V   W  N  K  Y
Type II     CAATTGACAA AAGTATGGAA TAAATACTAT TTGGAATTAC AAAAAGTATG GAATAAATAC
              Q  L  T   K  V  W   N  K  Y  Y   L  E  L   Q  K  V   W  N  K  Y
Type III    CAATTGACAA AAGTATGGAA TAAATACTAT TTGGAATTAC AAAAAGTATG GAATAAATAC
              Q  L  T   K  V  W   N  K  Y  Y   L  E  L   Q  K  V   W  N  K  Y
Type IV     CAATTGACAA AAGTATGGAA TAAATACTAT TTGGAATTAC AAAAAGTATG GAATAAATAC
              Q  L  T   K  V  W   N  K  Y  Y   L  E  L   Q  K  V   W  N  K  Y

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   65         75         85         95        105        115
Type I      TATTTGGAAT TACAAAAAGT ATGGAATAAA TACTATTTGG AATTACAAAA AGTATGGAAT
              Y  L  E   L  Q  K   V  W  N  K   Y  Y  L   E  L  Q   K  V  W  N
Wogon-Sugi  TATTTGGAAT TACAAAAAGT ATGGAATAAA TACTATTTGG AATTACAAAA AGTATGGAAT
              Y  L  E   L  Q  K   V  W  N  K   Y  Y  L   E  L  Q   K  V  W  N
Type II     TATTTGGAAT TACAAAAAGT ATGGAATAAA TACTATTTGG AATTACAAAA AGTATGGAAT
              Y  L  E   L  Q  K   V  W  N  K   Y  Y  L   E  L  Q   K  V  W  N
Type III    TATTTGGAAT TACAAAAAGT ATGGAATAAA TACTATTTGG AATTACAAAA AGTATGGAAT
              Y  L  E   L  Q  K   V  W  N  K   Y  Y  L   E  L  Q   K  V  W  N
Type IV     TATTTGGAAT TACAAAAAGT ATGGAATAAA TACTATTTGG AATT------ ----------
              Y  L  E   L  Q  K   V  W  N  K   Y  Y  L   E

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  125        135        145        155        165        175
Type I      AAATACTATT TGGAATTACA AAAAGTATGG AATAAATACT ATTTGGAATT ACAAAAAGTA
              K  Y  Y   L  E  L   Q  K  V  W   N  K  Y   Y  L  E   L  Q  K  V
Wogon-Sugi  AAATACTATT TGGAATTACA AAAAGTATGG AATAAATACT ATTTGGAATT ACAAAAAGTA
              K  Y  Y   L  E  L   Q  K  V  W   N  K  Y   Y  L  E   L  Q  K  V
Type II     AAATACTATT TGGAATTACA AAAAGTATGG AATAAATACT ATTTGGAATT A---------
              K  Y  Y   L  E  L   Q  K  V  W   N  K  Y   Y  L  E
Type III    AAATACTATT TGGAATT--- ---------- ---------- ---------- ----------
              K  Y  Y   L  E
Type IV     ---------- ---------- ---------- ---------- ---------- ----------

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  185        195        205        215        225        235
Type I      TGGAATAAAT ACTATTTGGA ATTCAATTTT GAATTACGAA AAATATTAAA TAGAATCTAT
              W  N  K   Y  Y  L   E  F  N  F   E  L  R   K  I  L   N  R  I  Y
Wogon-Sugi  TGGAATAAAT ACTATTTGGA ATTCAATTTT GAATTACGAA AAATATTAAA TAGAATCTAT
              W  N  K   Y  Y  L   E  F  N  F   E  L  R   K  I  L   N  R  I  Y
Type II     ---------- ---------- ---CAATTTT GAATTACGAA AAATATTAAA TAGAATCTAT
                                     F  N  F   E  L  R   K  I  L   N  R  I  Y
Type III    ---------- ---------- ---CAATTTT GAATTACGAA AAATATTAAA TAGAATCTAT
                                     F  N  F   E  L  R   K  I  L   N  R  I  Y
Type IV     ---------- ---------- ---CAATTTT GAATTACGAA AAATATTAAA TAGAATCTAT
                                     F  N  F   E  L  R   K  I  L   N  R  I  Y
```

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 *mat*K 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 *mat*K, *ycf*2, and *ycf*1 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 *mat*K reading frame. On the other hand, the indels of *ycf*2 and *ycf*1, 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 *mat*K 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 *mat*K 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 *mat*K coding region has not been reported in other plant species. On the other hand, a similar indel mutation within *ycf*1 and *ycf*2 has been reported in intraspecies comparisons of *Oenothera hookeri* (Blasko et al. 1988) and between plants from

Fig. 3 Repetitive indel mutations in the *ycf*1 coding region of *Wogon*-Sugi and four wild-type chloroplast genomes. The nucleotide and deduced amino acid sequences of part of the *ycf*1 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

```
             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  5         15         25         35         45         55         65
Type I       TTTAGGGAGA AACTTAGAAA ATTTCTAACG GAAAGTAGAG ATTCTCTTCC 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 II      TTTAGGGAGA AACTTAGAAA ATTTCTAACG GAAAGTAGAG ATTCTCTTCC 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   TTTAGGGAGA AACTTAGAAA ATTTCTAACG GAAAGTAGAG ATTCTCTTCC 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     TTTAGGGAGA AACTTAGAAA ATTTCTAACG GAAAGTAGAG ATTCTCTTCC 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      TTTAGGGAGA AACTTAGAAA ATTTCTAACG GAAAGTAGAG ATTCTCTTCC 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

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 75         85         95        105        115        125        135
Type I       GGGAGAAACT TAGAAAATTT CTAACGGAAA GTAGAGATTC TCTTCCAAAA TTGATAAGTT CTTTTAGGGA
              R  E  K   L  R  K  F   L  T  E   S  R  D   S  L  P  K   L  I  S   S  F  R
Type II      GGGAGAAACT TAGAAAATTT CTAACGGAAA GTAGAGATTC TCTTCCAAAA TTGATAAGTT CTTTTAGGGA
              R  E  K   L  R  K  F   L  T  E   S  R  D   S  L  P  K   L  I  S   S  F  R
Wogon-Sugi   GGGAGAAACT TAGAAAATTT CTAACGGAAA GTAGAGATTC TCTTCCAAAA TTGATAAGTT CTTTTAGGGA
              R  E  K   L  R  K  F   L  T  E   S  R  D   S  L  P  K   L  I  S   S  F  R
Type III     GGGAGAAACT TAGAAAATTT CTAACGGAAA GTAGAGATTC TCTTCCAAAA TTGATAAGTT CTTTTAGGGA
              R  E  K   L  R  K  F   L  T  E   S  R  D   S  L  P  K   L  I  S   S  F  R
Type IV      GGGAGAAACT TAGAAAATTT CTAACGGAAA GTAGAGATTC TCTTCCAAAA TTGATAAGTT CTTTTAGGGA
              R  E  K   L  R  K  F   L  T  E   S  R  D   S  L  P  K   L  I  S   S  F  R

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                145        155        165        175        185        195        205
Type I       GAAACTTAGA AAATTTCTAA CGGAAAGTAG AGATTCTCTT CCAAAATTGA TAAGTTCTTT TAGGGAGAAA
             E  K  L  R   K  F  L   T  E  S   R  D  S  L   P  K  L   I  S  S   F  R  E  K
Type II      GAAACTTAGA AAATTTCTAA CGGAAAGTAG AGATTCTCTT CCAAAATTGA TAAGTTCTTT TAGGGAGAAA
             E  K  L  R   K  F  L   T  E  S   R  D  S  L   P  K  L   I  S  S   F  R  E  K
Wogon-Sugi   GAAACTTAGA AAATTTCTAA CGGAAAGTAG AGATTCTCTT CCAAAATTGA TAAGTTCTTT TAGGGAGAAA
             E  K  L  R   K  F  L   T  E  S   R  D  S  L   P  K  L   I  S  S   F  R  E  K
Type III     GAAACTTAGA AAATTTCTAA CGGAAAGTAG AGATTCTCTT CCAAAATTGA TAAGTTCTTT TAGGGAGAAA
             E  K  L  R   K  F  L   T  E  S   R  D  S  L   P  K  L   I  S  S   F  R  E  K
Type IV      GAAACTTAGA AA-------- ---------- ---------- ---------- ---------- ----------
             E  K  L  R

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                215        225        235        245        255        265        275
Type I       CTTAGAAAAT TTCTAACGGA AAGTAGAGAT TCTCTTCCAA AATTGATAAG TTCTTTTAGG GAGAAACTTA
               L  R  K   F  L  T   E  S  R  D   S  L  P   K  L  I   S  S  F  R   E  K  L
Type II      CTTAGAAAAT TTCTAACGGA AAGTAGAGAT TCTCTTCCAA AATTGATAAG TTCTTTTAGG GAGAAACTTA
               L  R  K   F  L  T   E  S  R  D   S  L  P   K  L  I   S  S  F  R   E  K  L
Wogon-Sugi   CTTAGAAAAT TTCTAACGGA AAGTAGAGAT TCTCTTCCAA AATTGATAAG TTCTTTTAGG GAGAAACTTA
               L  R  K   F  L  T   E  S  R  D   S  L  P   K  L  I   S  S  F  R   E  K  L
Type III     CTTAGAAA-- ---------- ---------- ---------- ---------- ---------- ----------
               L  R
Type IV      ---------- ---------- ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                285        295        305        315        325        335        345
Type I       GAAAATTTCT AACGGAAAGT AGAGATTCTC TTCCAAAATT GATAAGTTCT TTTAGGGAGA AACTTAGAAA
              R  K  F   L  T  E  S   R  D  S   L  P  K   L  I  S  S   F  R  E   K  L  R
Type II      GAAA------ ---------- ---------- ---------- ---------- ---------- ----------
              R
Wogon-Sugi   GAAA------ ---------- ---------- ---------- ---------- ---------- ----------
              R
Type III     ---------- ---------- ---------- ---------- ---------- ---------- ----------
Type IV      ---------- ---------- ---------- ---------- ---------- ---------- ----------

             ....|....| ....|....| ....|....| ....|....| ....|....|
                355        365        375        385        395
Type I       GTTTCTAACG GAAAGTAGAG ATTCTCTTCT AAAAAATGGA ATCATAAGAA
             K  F  L  T   E  S  R   D  S  L   L  K  N  G   I  I  R
Type II      GTTTCTAACG GAAAGTAGAG ATTCTCTTCT AAAAAATGGA ATCATAAGAA
             K  F  L  T   E  S  R   D  S  L   L  K  N  G   I  I  R
Wogon-Sugi   GTTTCTAACG GAAAGTAGAG ATTCTCTTCT AAAAAATGGA ATCATAAGAA
             K  F  L  T   E  S  R   D  S  L   L  K  N  G   I  I  R
Type III     GTTTCTAACG GAAAGTAGAG ATTCTCTTCT AAAAAATGGA ATCATAAGAA
             K  F  L  T   E  S  R   D  S  L   L  K  N  G   I  I  R
Type IV      GTTTCTAACG GAAAGTAGAG ATTCTCTTCT AAAAAATGGA ATCATAAGAA
             K  F  L  T   E  S  R   D  S  L   L  K  N  G   I  I  R
```

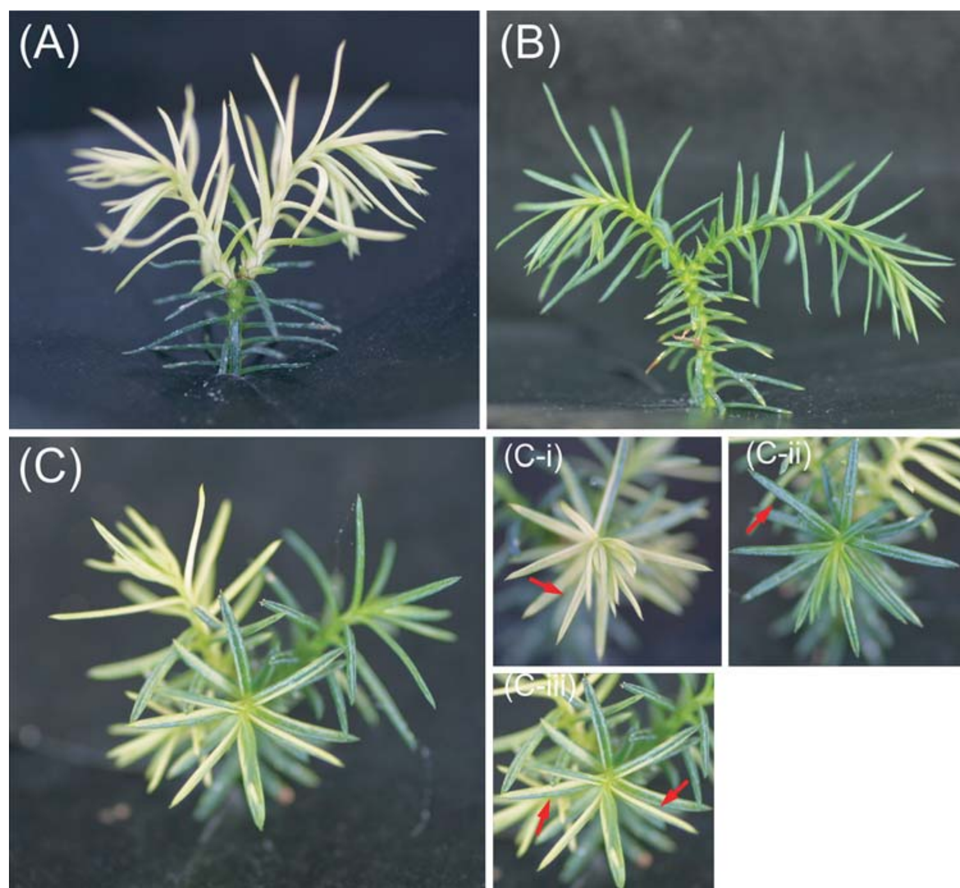

Fig. 4 The phenotypes obtained from an artificial cross between *Wogon*-Sugi (paternal parent) and wild-type (maternal parent). **a** *Wogon*-Sugi type with yellowish-white leaves. **b** Wild-type with normal green leaves. **c** Chimeric type with yellowish-white sectors and normal green sectors. In 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

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

	Inheritance pattern[a]			
	Wogon-Sugi type (Paternal)	Wild-type type (Maternal)	Chimeric type (Biparental)	Total
Number of observed seedlings[b]	114	6	11	131
Rate (%)	87.0	4.6	8.4	100

[a] The 131 offspring were obtained by an artificial cross between wild-type (Yoshiki 1; maternal parent) and *Wogon*-Sugi (paternal parent)

[b] The chloroplast genomes of each of the 131 offspring were genotyped based on the length variation of PCR product (Fig. 5)

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 fit is amplified instead. Consequently, we conducted the PCR-RFLP analysis as a supplementary analysis, namely detection of longer chloroplast regions, including *mat*K 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 *mat*K (with the insertional mutation) were only observed in *Wogon*-Sugi, and not for the nuclear paralog.

The *mat*K gene, which is encoded in the *trn*K 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 *mat*K, 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 *mat*K 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

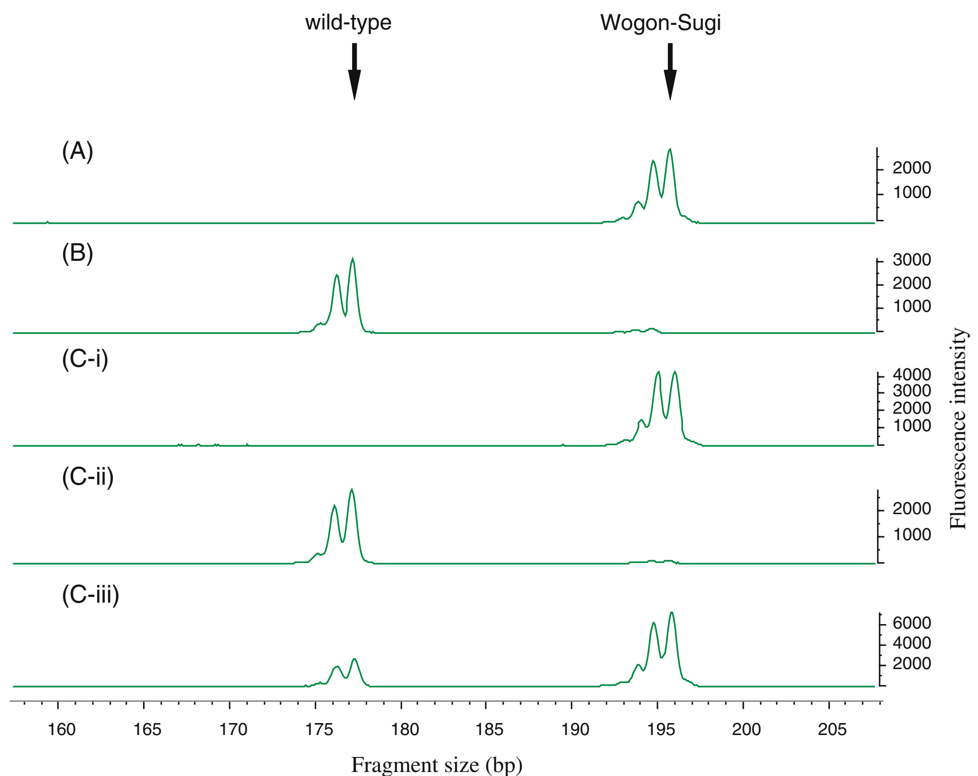

Fig. 5 Fluorescently labeled PCR products from the three seedling types of Fig. 4. The expected PCR product size of *Wogon*-Sugi type and wild-type (Yoshiki 1) is 196 bp and 177 bp, respectively. **a** Yellowish-white leaves with the *Wogon*-Sugi type *mat*K indel. **b** Normal green leaves with the wild-type *mat*K indel. **c-i** Yellowish-white sector of chimeric seedling with the *Wogon*-Sugi type *mat*K indel; **c-ii** normal green sector of chimeric seedling with the wild-type *mat*K indel; **c-iii** variegated sector of chimeric seedling with the *Wogon*-Sugi type *mat*K indel and the wild-type *mat*K indel. The *rightmost peak* of each group corresponds to the expected PCR product (196 bp and 177 bp). On the *left*, peaks corresponding to slippage of the *Taq* polymerase are visible (195 bp and 176 bp)

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 *mat*K gene in the *Wogon*-Sugi chloroplast genome. A plastid DNA marker targeting the 19-bp insertion of the *Wogon*-Sugi *mat*K 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 *mat*K insertion in the maternal chloroplast genome. These results indicate that the 19-bp insertion of the *mat*K coding region is the most likely candidate for a polymorphism underlying the *Wogon*-Sugi phenotype.

The *mat*K 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 *trn*K, *trn*A, *trn*I, *rps*12, *rpl*2, and *atp*F 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 *mat*K 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 *mat*K 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 virescence 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 virescent 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; Ostersetzer 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 virescent *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 *mat*K gene, and to determine its association with a nuclear-encoded splicing factor.

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