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The *YELLOW VARIEGATED* (*VAR2*) Locus Encodes a Homologue of FtsH, an ATP-Dependent Protease in *Arabidopsis*

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Variegated leaves are often caused by a nuclear recessive mutation in higher plants. Characterization of the gene responsible for variegation has shown to provide several pathways involved in plastid differentiation. Here we describe an *Arabidopsis* variegated mutant isolated by T-DNA tagging. The mutant displayed green and yellow sectors in all green tissues except for cotyledons. Cells in the yellow sector of the mutant contained both normal-appearing and mutant chloroplasts. The isolated mutant was shown to be an allele of the previously reported mutant, *yellow variegated* (*var2*). Cloning and molecular characterization of the *VAR2* locus revealed that it potentially encodes a chloroplastic homologue of FtsH, an ATP-dependent metalloprotease that belongs to a large protein family involved in various cellular functions. *ftsH*-like genes appear to comprise a small gene family in *Arabidopsis* genome, since at least six homologues were found in addition to *VAR2*. Dispensability of *VAR2* was therefore explained by the redundancy of genes coding for FstHs. In the yellow regions of the mutant leaves, accumulation of photosynthetic protein components in the thylakoid membrane appeared to be impaired. Based on the role of FtsH in a protein degradation pathway in plastids, we propose a possibility that *VAR2* is required for plastid differentiation by avoiding partial photooxidation of developing chloroplasts.

Key words: *Arabidopsis thaliana* — FtsH — Plastid — T-DNA tagging — Variegated mutant.

Abbreviations: BAC, bacterial artificial chromosome; EMS, ethyl methanesulfonate; EtBr, ethidium bromide; GFP, green fluorescent protein; Km, kanamycin; Mn-SOD, manganese superoxide dismutase; MS, Murashige and Skoog; PBS, phosphate-buffered saline; PSI, photosystem I; PSII, photosystem II; SRH, second region of homology; TAIL-PCR, thermal asymmetric interaced PCR; UTR, untranslated region.

Introduction

Differentiation of plastids into chloroplasts is accompanied by a series of biochemical and morphological changes in response to both internal and environmental signals. Upon perception of light, plastids undergo synthesis and import of the proteins and photo-receiving pigments to form photosynthetically competent membrane structures with the multiprotein complexes (Taylor 1989). While plastids per se synthesize some constituents of such protein complexes, all the other proteins are nuclear-encoded (Sugita and Sugiura 1996). Thus, coordinated regulation is necessary between the two organelles during plastid differentiation. Little is known about such mechanisms in higher plants, although several nuclear loci affecting the expression of chloroplast genes have been identified in unicellular organisms (Rochaix and Erickson 1988, Rochaix 1996). Given the limited capacity of the plastid genome, most of the genes encoding developmental regulatory factors may exist in the nuclear genome.

To study chloroplast differentiation, we focus on leaf-variegated mutants. A variegated mutant (often called as a stripe mutant in monocots) has long been a subject of genetic studies in many plant species because of its easily detectable phenotype (Rédei 1963, Stubbe and Herrmann 1982, Newton and Coe 1986, Coe et al. 1988, Martínez-Zapater 1993, Hess et al. 1994). These mutations are generally nuclear recessive, and the mutants exhibit green and yellow sectors in chloroplast-containing tissues. Compared to a recessive albino mutant that is phenotypically lethal, the viable variegated mutants have a certain advantage that enables us to follow the fate of deficient chloroplasts in green and yellow sectors. Several nuclear genes responsible for the variegation have been identified and characterized at the molecular level, including *iojap* (*ij*) in maize (Han et al. 1992) and *immutans* (*im*) in *Arabidopsis* (Carol et al. 1999, Wu et al. 1999). In addition to the nuclear loci, a maternally inherited variegation has also been described in which the rearrangement of the organelle genome along with the concomitant alteration of the gene expression give rise to sectoring chloroplasts (Sears and Herrmann 1985, Newton and Coe 1986). These observations on the variegated mutants demonstrate that a block at the various steps of signaling and metabolic pathways may affect plastid differentiation.

In *Arabidopsis*, four recessive mutations have been reported to genetically cause leaf variegation (Rédei 1963, Rédei 1967, Martínez-Zapater et al. 1992). Each mutant is distinguishable based on the variegation pattern and some other phenotypes. Among them, *chloroplast mutator* (*chm*) is characterized in that the variegation is inherited in a non-Mendelian fashion (Rédei 1963). Characterization of the *chm* mutants by

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us and others has shown that a specific mitochondrial DNA rearrangement with the deficient mitochondrial gene expression is associated with the appearance of the yellow sector, suggesting that chloroplast differentiation may require functional mitochondria (Martínez-Zapater et al. 1992, Sakamoto et al. 1996).

Unlike *chm*, three other mutants (*im* and two *yellow variegated* mutants, *var1* and *var2*) exhibit strict Mendelian inheritance (Martínez-Zapater 1993, Wetzel et al. 1994, Chen et al. 1999). Formation of yellow sectors in these mutants is sensitive to light intensity and temperature (Wetzel et al. 1994, Wetzel and Rodermel 1998, Chen et al. 1999). Microscopic observation of the *im* and *var2* mutants has revealed that cells in the yellow tissues contain both deficient and normal plastids. This heteroplastidity implies that a part of the plastids included in the same cell can transiently undergo normal differentiation, and that a mutation at the *IM* or *VAR2* loci influences plastid development in a chloroplast-autonomous manner. Particularly in *im*, impairment of carotenoid biosynthesis appeared to correlate with the variegated phenotype, because yellow sectors in the mutant accumulated phytoene, a precursor of the carotenoid pathway (Wetzel et al. 1994, Wetzel and Rodermel 1998). Molecular cloning of the *IM* locus has shown that it encodes a putative chloroplast terminal oxidase with a homologous motif with a mitochondrial alternative oxidase (AOX), which plays a role in a cyanide-insensitive pathway of respiratory electron transport (Carol et al. 1999, Wu et al. 1999). Based on these results, *IM* has been proposed to function early in chloroplast development as a cofactor of the respiratory component responsible for phytoene desaturation. Redundancy of such a chloroplastic oxidase activity may explain the variegated phenotype, since the impaired phytoene desaturation results in yellow sectors with increased photooxidative damage.

Here we describe a variegated mutant allelic to *var2*, isolated by T-DNA tagging. Molecular characterization of the *VAR2* locus showed that it potentially encodes a homologue of FtsH ATP-dependent metalloprotease in *E. coli*, which belongs to a large protein family involved in various cellular functions such as cell division and heat shock response (Akiyama et al. 1994, Herman et al. 1995, Tomoyasu et al. 1995). We revealed that VAR2 is a chloroplastic protein and may participate in degradation of photooxidatively damaged complexes localized in thylakoid membranes. Database search revealed the presence of highly homologous *ftsH*-like genes in *Arabidopsis* genome, suggesting that a mutation in *VAR2* is dispensable because of the redundancy, but that the decrease of the net FtsH proteins below the threshold level may lead to the variegated phenotype.

Materials and Methods

Plant materials

Arabidopsis thaliana ecotype Columbia (Col) was used as a wild type in this study. A variegated mutant F204 has a Col background, and was isolated from our T-DNA tagging library generated by in planta vacuum infiltration (Bechtold and Pelletier 1998) using *Agrobacterium tumefaciens* strain C58C1Rif^r/pGV2260 harboring a pBI121-borne binary vector. Segregation analysis of kanamycin (Km) resistance, T-DNA insertion, and the variegated phenotype were carried out in T2 generation, and the genotype of Km resistance in the T2 population was determined by scoring T3 progeny (Km^R for resistance and Km^S for sensitivity). A variegated mutant F204 characterized in this study was allelic to *var2* and hence designated to *var2-6*. Two other alleles, *var2-1* (stock number N272) and *var2-7* (identified in this study, stock number CS3166) were kindly provided from Nottingham *Arabidopsis* Stock Centre (NASC, The University of Nottingham) and *Arabidopsis* Biological Resource Center (ABRC, Ohio State University). PL20 (designated as *var2-8* in this study) is a variegated mutant (Col background) isolated from M2 seeds mutagenized by ethyl methanesulfonate (EMS). All plants were grown for segregation analysis on agar plates containing MS medium supplemented with 1.5% (w/v) sucrose at 22°C with 14 h of light/10 h of darkness, otherwise plants were grown on soil in the same light (about 70 μmol m^{-2} s^{-1}) and temperature conditions. For light intensity analysis, Col was also grown under low-intensity light condition (about 15 μmol m^{-2} s^{-1}).

Microscopic observation

The images of chloroplasts in a mesophyll layer of the mutants were directly observed by a confocal laser-scanning microscope (MRC-600, BioRad). Epifluorescence from chlorophyll was excised with a Kr/Ar laser at 488 nm and detected simultaneously at 514 nm. For electron microscopic observation, sections were prepared from yellow and green portions of true leaves in the variegated mutant embedded in a Spurr resin. Ultrathin sections were prepared using an Ultracut N ultratome (Reichert-Nissei, Tokyo). The sections were stained with uranyl acetate and lead citrate before examination in an electron microscope (model H-7000B, Hitachi, Tokyo).

Nucleic acid manipulation and PCR analysis

Genomic DNA was isolated as described (Saghai-Maroof et al. 1984). For PCR analysis, the following standard conditions were used; denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and extension at 72°C for 2 min (30 cycles). Total RNA was isolated as described (Logemann et al. 1987) and separated in a 1% denatured formaldehyde-agarose gel. Thermal asymmetric interaced PCR (TAIL-PCR) was performed as described (Liu et al. 1995) with some modifications. We amplified the left border of an inserted T-DNA using either one of three primers (TL1, TL2, and TL3) and a degenerated primer (TAIL2). Southern and northern hybridization analyses and subsequent detection experiments were performed as described (Sambrook et al. 1989). Probes for hybridization analysis were labeled with a digoxigenin (Boehringer) by PCR using the following two sets of primers: KT101 and KT102 to amplify the entire cDNA; KT101 and KT210 to amplify a 5′ portion of the cDNA (encoding a putative transit peptide sequence) for generating a gene-specific probe. Probes for chloroplast genes *psbA*, *rbcL*, and *atpB* were prepared by PCR using rice chloroplast DNA clones (kindly provided from Dr. M. Nakazono, Tokyo University) and the gene-specific primers. RT-PCR was conducted using primers KT101 and KT102 to amplify the *VAR2* locus. A 20-μl mixture containing 5 μg of total RNA and the primer KT102 in 1×RT buffer (10 mM DTT, 0.5 units μl^{-1} RNase inhibitor, 0.2 units μl^{-1} reverse transcriptase (SuperScriptII, Gibco/BRL), 0.5 mM dNTPs) was incubated at 43°C for 50 min. The reaction was subjected to PCR amplification with a standard condition after ethanol precipitation. PCR products were cloned into *Bam*HI and *Sac*I sites of pBluescript SK+ (Stratagene). All the primers used in this study are listed in Table 1.

Table 1 Primers used in this study

Primer	Sequence	Location of annealing site in VAR2	Strand
KT101	5′-TTGGATCCATGGCAGCTTCATCAGCTTGTC-3′	1–22	Sense
KT102	5′-TTGAGCTCTTAGACAGCAGCTGGTGTTGGT-3′	2,373–2,352	Antisense
KT103	5′-TTGTCGACATGGCAGCTTCATCAGCTTGTC-3′	1–22	Sense
KT104	5′-TTCCATGGCTTCTTGCTTCTTCTTCCCATC-3′	172–151	Antisense
KT105	5′-GGTACCATTTGCTTTCCCACTTGCAATT-3′	243–222	Antisense
KT201	5′-TGAATTGGGTAACCGGGTTGAGAGAGT-3′	375–401	Sense
KT202	5′-GGTGCCAAGATCCCGAAAGGTGTTCT-3′	766–791	Sense
KT203	5′-AGGACGGTTTGACCGGCAGGTACTAT-3′	1,155–1,180	Sense
KT204	5′-GTGAGAAACATATTAAAAGCCTGT-3′	1,577–1,600	Sense
KT205	5′-ATGGTAACAACATTTGGAATGTCTGA-3′	2,014–2,039	Sense
KT206	5′-ATTCAACAGTCTCAAACACTCAGTTTC-3′	1,998–1,972	Antisense
KT207	5′-TAAGCTACCAAGCTTTTGCTCTTGCCAT-3′	1,545–1,518	Antisense
KT208	5′-ATAGTACCTGCCGGTCAAACCGTCCT-3′	1,180–1,155	Antisense
KT209	5′-GAATCTCTCAGGTTTCTTCAAAAACTC-3′	756–730	Antisense
KT210	5′-GAAACAGCTTCCACAATAGCTATAGT-3′	371–346	Antisense
KT301	5′-TGAAGAAAGAGCAGAACGAGAGAGCT-3′	2,921–2,892	Antisense
TL1	5′-CCCTATCTCGGGCTATTCTTTTGA-3′	T-DNA left border	Antisense
TL2	5′-TATAAGGGATTTTGCCGATTTCGG-3′	T-DNA left border	Antisense
TL3	5′-AACCACCATCAAACAGGATTTTC-3′	T-DNA left border	Antisense
TAIL2	5′-TC[A/T]TCIG[A/C/T/G]ACIT[GC]CTC-3′	Degenerate primer	Antisense

Cloning, sequencing, and database analysis

The region corresponding to exons and introns of *VAR2* was amplified from *var2-1* allele and other variegated mutants by PCR with a set of primers KT101 and KT102, and was cloned into *Sac*I-*Bam*HI sites of pBluescript SK+. Nucleotide sequences of the clones were determined using Bigdye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems) using the primers described in Table 1. Nucleotide sequences were analyzed using the GENETYX-Mac software (ver. 10, Software Development). Database search was conducted by BLAST and the details of the bacterial artificial chromosome (BAC) clones were obtained from the following WWW sites: AtDB; http://genome www.stanford.edu/Arabidopsis/index.old.html and *Arabidopsis* genome displayer; http://www.kazusa.or.jp/arabi/displayer/.

Transient assay by particle bombardment

The putative transit peptide from *VAR2* was amplified by PCR using primers KT103 and KT104, or KT103 and KT105. The PCR-amplified fragments, coding respectively for the 58 and 81 N-terminal amino acids of *VAR2* was cloned into the *Sal*I-*Nco*I sites of p35S-sGFP, consisting of a synthetic green fluorescent protein (GFP) under the control of cauliflower mosaic virus 35S promoter (Heim et al. 1995). The resulting constructs (p35S-104-sGFP and p35S-105-sGFP) were introduced into leaves of *Nicotiana cleverandii* by particle bombardment. The leaves of *N. cleverandii* were cut and placed onto 0.8% agar plates containing Murashige and Skoog (MS) medium, and particle bombardment was performed by using BioRad PDS1000/He (1,100 pounds per square inches (77.4 kg cm^{-2}), second shelf from the top) with 1.0-μm gold particles precipitated with 5 μg of the plasmid DNA. Bombarded leaves were incubated at 22°C for approximately 12 h under light (70 μmol m^{-2} s^{-1}). Cells expressing GFP were observed with a confocal laser-scanning microscope (LSM510, Zeiss). Signals of GFP were detected with a B-excitation filter set, and chlorophyll autofluorescence was detected with a G-excitation filter set.

Protein gel blot analysis

First and second true leaves from Col and F204 (50 mg) were homogenized in liquid nitrogen, and total cell proteins were isolated in extraction buffer (100 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2% (v/v) β-mercaptoethanol, 2% (v/v) Tween 20). Green and pale yellow sectors in the first and second true leaves of the F204 mutant were separated using a razor blade and subjected to protein extraction. Insoluble proteins were harvested by centrifugation at 20,000×*g* for 10 min at 4°C and then were solubilized in 175 μl of SDS sample buffer (7% (w/v) SDS, 2% (v/v) β-mercaptoethanol in phosphate-buffered saline (PBS), pH 8.0). The extracted proteins (5 μl per lane) were electrophoresed through a 12.5% SDS-Urea acrylamide gel. The antibodies raised against CPI (reaction center polypeptides of photosystem I (PSI)), cytochrome b_6, and D1 proteins (a reaction center protein of photosystem II (PSII)) were generously provided by Dr. Y. Takahashi (Okayama University). Each antibody was used at dilution of 1 : 1,000. Proteins were electroblotted onto clear blot membrane-p (Atto). Immunodetection of the western blots was performed by ECL system (Amersham) as described by the manufacturer's protocol using a secondary antibody conjugated to horseradish peroxidase.

Results

Phenotypic characterization of a variegated mutant F204

From the stock of our T-DNA-tagged library, a mutant line F204 was found in which green and pale yellow variegated leaves segregated in the T2 generation. Scored numbers of Km^R (variegated) : Km^R (normal) : Km^S was 28 : 109 : 74, respectively. T3 progeny of the variegated individuals were all Km^R/variegated. Km^R and variegation were also linked in the T3 progeny of Km^R (normal) plants, suggesting that the varie-

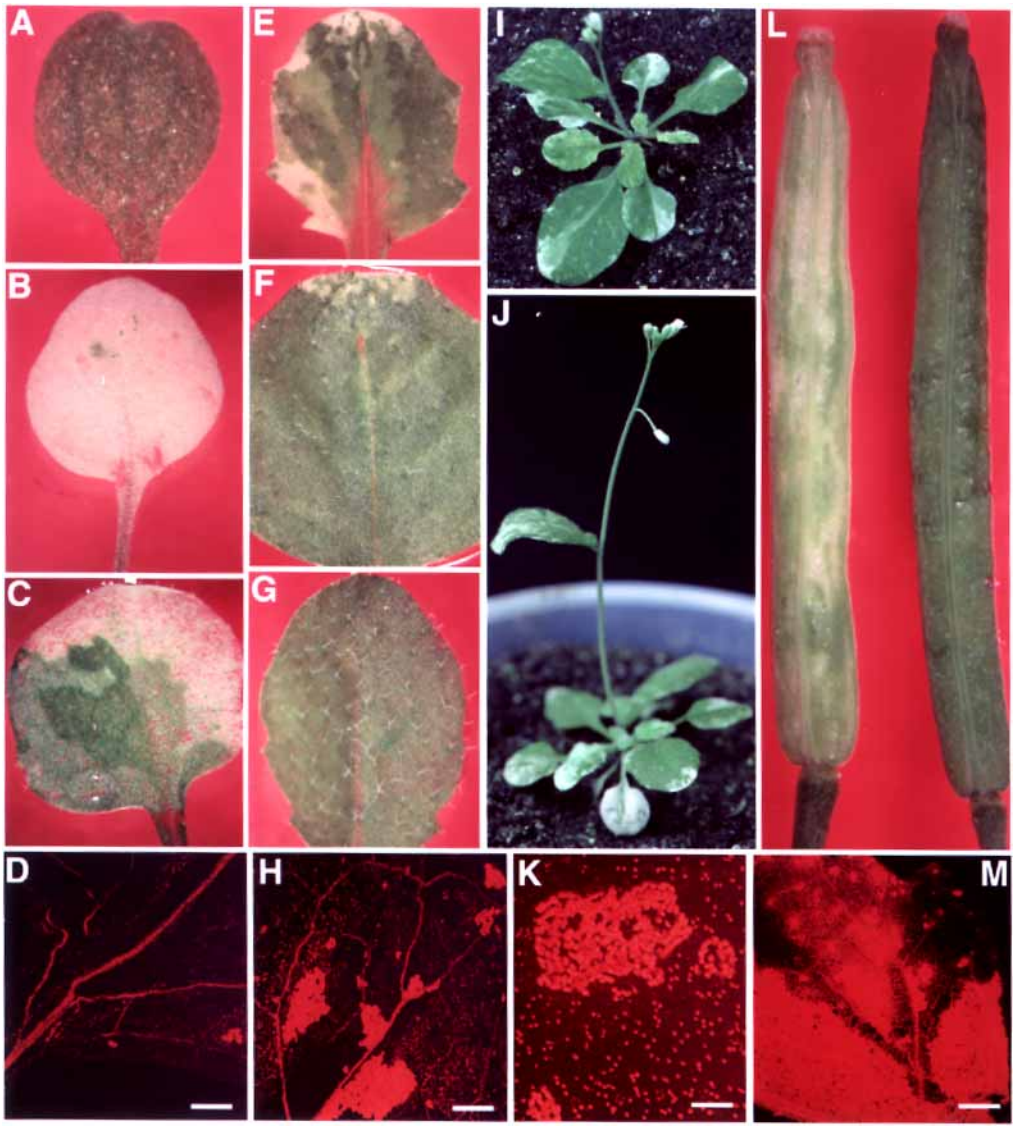


Fig. 1 Phenotype of F204 mutant. (A) Cotyledon with no variegation. (B) Primary leaf with a green cell cluster. (C) Half area of a secondary leaf containing chloroplasts. (E) Tertiary leaf. (F and G) Cauline leaves. (I) and (J) Whole plants after bolting. (L) Silique of F204 (Left) and Col (Right). (D, H, K, and M) Images of chlorophyll autofluorescence by a confocal laser-scanning microscope. Bar = 50 μm in (D) and (M), 10 μm in (H), and 3.3 μm in (K).

gation was caused by a single T-DNA insertion event.

F204 was characterized by the appearance of the ivory-colored sectors in green tissues like leaves, stems, and siliques, but not in cotyledons (Fig. 1A–C, L). Their growth rate was slower than that of the wild type, but the variegation seemed not to influence the morphology of leaves and other organs (Fig. 1I, J). Upon the development of normal-appearing cotyledons, the yellow true leaves emerged with an occasional small green spot. The yellow leaves turned greener as they expanded, although yellow/green boundaries were retained. Influence of the variegated phenotype appeared to be less severe, as the vegetative stage proceeded (Fig. 1E–G). While the first few true leaves were highly affected at both distal and proximal parts including petioles, the tip and edge of the lateral rosette leaves tended to have less and limited yellow variegation. The extent of variegation tended to be enhanced when F204 plants were grown under high-intensity light (data not shown).

Autofluorescence and electron microscopic observation of the mutant plastids

Observation of the mesophyll cells in variegated leaves by a confocal laser-scanning microscope showed that the chloro-

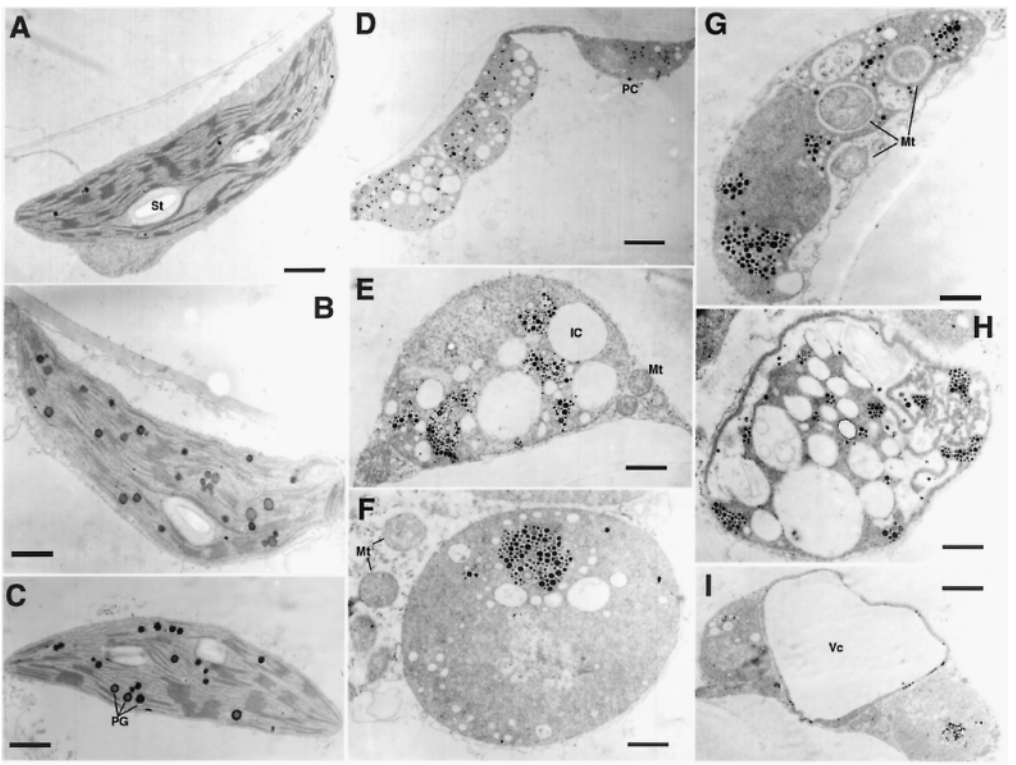


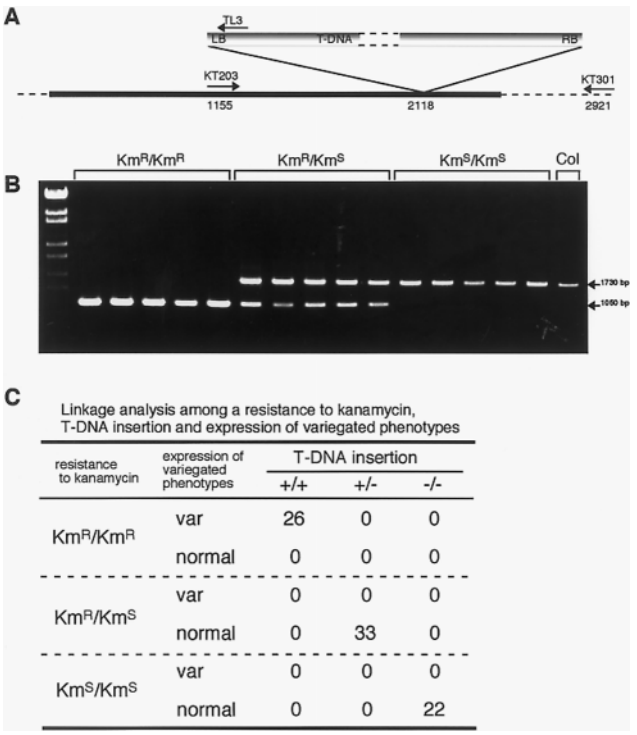
Fig. 2 Electron micrographs of plastids from Col and F204 leaves. (A) Chloroplast from a Col leaf. (B) Chloroplast from a green sector of a F204 leaf. (C) Chloroplast from a green cell cluster of a variegated leaf. (D-F, H, and I) Plastid highly vacuolated and lacking inner membrane. (G) Chloroplast-like plastid with rudimentary lamellar structure. Examples of starch (St), plastoglobule (PG), mitochondrion (Mt), inner cytoplasm (IC), vacuole (Vc), and plastid with chlorophyll (PC) were shown. Bars = 1 μm.

phyll autofluorescence was present not only in the green but also yellow sectors (Fig. 1D, H, M). Most of the autofluorescences were detected on the green sectors and leaf veins, where cells contained a number of normal-appearing chloroplasts. Chloroplasts with autofluorescence were also detected in the yellow sectors, although their number and size were apparently decreased. Electron microscopic observation showed that these cells contained vacuolated plastids lacking an internal membrane structure, with a few plastids that retained primary lamellae and likely corresponded to the autofluorescent bodies (Fig. 2D). Thus, deficient and normal-appearing chloroplasts coexisted in the yellow sectors of variegation, suggesting that the impact of the mutation in F204 is plastid-autonomous.

Further analysis of green and yellow sectors in primary leaves of the F204 mutant was performed by electron microscopy (Fig. 2). Cells in the green sectors contained morphologically normal chloroplasts whose thylakoid membrane developed comparable extent to those of the wild type. Granal stacks in the mutant appeared less dense, and a considerable number of highly dense-stained globular structures, likely representing plastoglobuli, were detected (Fig. 2B, C). In contrast, most of the plastids in the yellow tissue lacked granal membranes, and instead contained various numbers and sizes of vacuolated structures and clustered plastoglobuli (Fig. 2D–I). Given that the variable sizes of plastids were observed in these tissues, we considered that the F204 mutant caused abnormal development of internal membrane structures in chloroplasts at various steps of chloroplast differentiation. We also examined mitochondrial structure carefully, since the altered mitochondrial potential can be associated with the variegated phenotype. Mitochondria in both the green and yellow tissues appeared normal and retained indistinguishable morphology with well-developed inner membranes.

Linkage analysis and allelism tests

To examine whether T-DNA insertion in the F204 mutant is linked to the variegated phenotype, we isolated a DNA region flanked by the inserted T-DNA using TAIL-PCR analysis. With the specific primers for the left border of T-DNA, a 2.0-kb fragment was amplified. Sequencing and database search revealed that this fragment was included in a BAC clone F7F1 mapped at 61 cM on chromosome 2. Based on the sequence of the BAC clone, we designed the following PCR experiment to confirm that the variegation was linked to the T-DNA insertion. Total DNA was isolated from 81 individual F204 plants in the T2 generation, and PCR was performed using the DNA



C

Linkage analysis among a resistance to kanamycin, T-DNA insertion and expression of variegated phenotypes

resistance to kanamycin	expression of variegated phenotypes	T-DNA insertion +/+	+/-	-/-
Km^R/Km^R	var	26	0	0
	normal	0	0	0
Km^R/Km^S	var	0	0	0
	normal	0	33	0
Km^S/Km^S	var	0	0	0
	normal	0	0	22

Fig. 3 (A) Schematic representation of the genomic region flanked by the T-DNA insertion. Shown are the annealing sites of the primers used for PCR analysis. (B) Agarose gel electrophoresis of PCR fragments. PCR was performed with the three primers indicated in (A) using total DNA from T2 plants. Segregation of the variegated phenotype and the genotype for Km was shown in (C).

samples with the three primers KT203, KT301 and TL3, as schematically shown in Fig. 3A. If a plant were homozygous for the T-DNA insertion, then only a single PCR fragment of 1,050 bp could be detected with the primers KT203 and TL3. Because the integrated T-DNA is too long to be amplified, no PCR fragment should be detected with the primers KT203 and KT301. Likewise, a plant without the T-DNA insertion should generate only a 1,730-bp fragment from a wild-type DNA context, and a hemizygous plant should generate both fragments. The result indicated that all Km^R plants contained the 1,050-bp fragment, and that the variegation occurred only when the insertion is homozygous (Fig. 3B, C). The observed linkage between the T-DNA insertion and the variegated phenotype strongly suggested that the variegation in F204 was tagged by T-DNA.

Identification of the causative T-DNA integration at 61 cM on chromosome 2 directed our attention that *var2* is genetically mapped close to this site (43.5 cM) (Martínez-Zapater 1993). In fact, the observed phenotype of the F204 mutant resembled that of *var2*. To examine the allelism between the two mutants, F204 was crossed with *var2-1* (obtained from NASC as N272) and other variegated mutants. Since all the F1 plants from a cross between *var2-1* and F204 showed Km^R and a variegated phenotype (data not shown), we concluded that F204 is allelic to *var2*. We also found that two other variegated isolates are alleles of *var2*. These were CS3166 from ABRC, and PL20 isolated in this study. Both were originated from EMS mutagenesis. The variegated phenotypes of CS3166 and PL20 were similar to that of F204, showing no variegation in cotyledons, clearly visible variegated sectors in true leaves, and the enhancement of the yellow sectors under high-light intensity (data not shown). F204 was designated as *var2-6*, and similarly CS3166 and PL20 were designated as *var2-7* and *var2-8*, respectively.

Genomic organization of the VAR2 gene

In the *var2-6* mutant, T-DNA was inserted into the fourth exon of the *VAR2* candidate gene. This gene was predicted to consist of four exons with three introns (termed F7F1.16 in the BAC clone F7F1, accession number AC004669). To confirm the open reading frame (ORF), we designed a pair of primers (KT101 and KT102) based on the sequence, and subsequently performed RT-PCR. Sequencing of several cDNA clones revealed the predicted ORF was indeed transcribed (data not shown). We also found an EST clone (OS062, accession number U74127) containing an identical sequence, which defined an additional intron (289 bp) at the 5′ untranslated region (UTR) and the length of 5′ UTR to be 51 bp.

Insertion of the T-DNA in the *var2-6* mutant disrupted the genomic sequence at +2,118 (from A of the initiation codon ATG), resulting in a truncated ORF. Similarly, cloning and sequencing analysis of the corresponding genomic region from *var2-1* and *var2-7* revealed a mutation at the same locus (summarized in Fig. 4). *var2-1* contained a nonsense mutation with a nucleotide substitution (C to T) at +1,789. *var2-7* contained a frameshift with the deletion of a nucleotide C at +5 resulting in a generation of an unrelated 14 amino-acid peptide. No sequence difference was found within the investigated region of *var2-8*, suggesting that the responsible mutation lay in a flanking regulatory element. Based on these results, we concluded that the predicted ORF represents the *VAR2* gene.

VAR2 encodes a potential FtsH homologue

The deduced amino acid sequence from the *VAR2* cDNA consisted of 695 amino acid residues and was predicted to have a calculated molecular mass of 74.1 kDa. Database search showed that it is homologous with FtsH, an ATP-dependent metalloprotease and a member of AAA-protein family (ATPase associated with diverse cellular activities), from *Escherichia coli*, yeast, red and brown algae, bell pepper, tobacco and *Arabidopsis* (Fig. 4). It contained the AAA module composed of Walker A and B motifs as an ATP-binding domain at amino acid positions 261–276 and 322–329, respectively (Fig. 4). In addition, a single AAA-family signature domain, the second region of homology (SRH) was contained at amino acids 366–384 and a zinc-binding domain char-

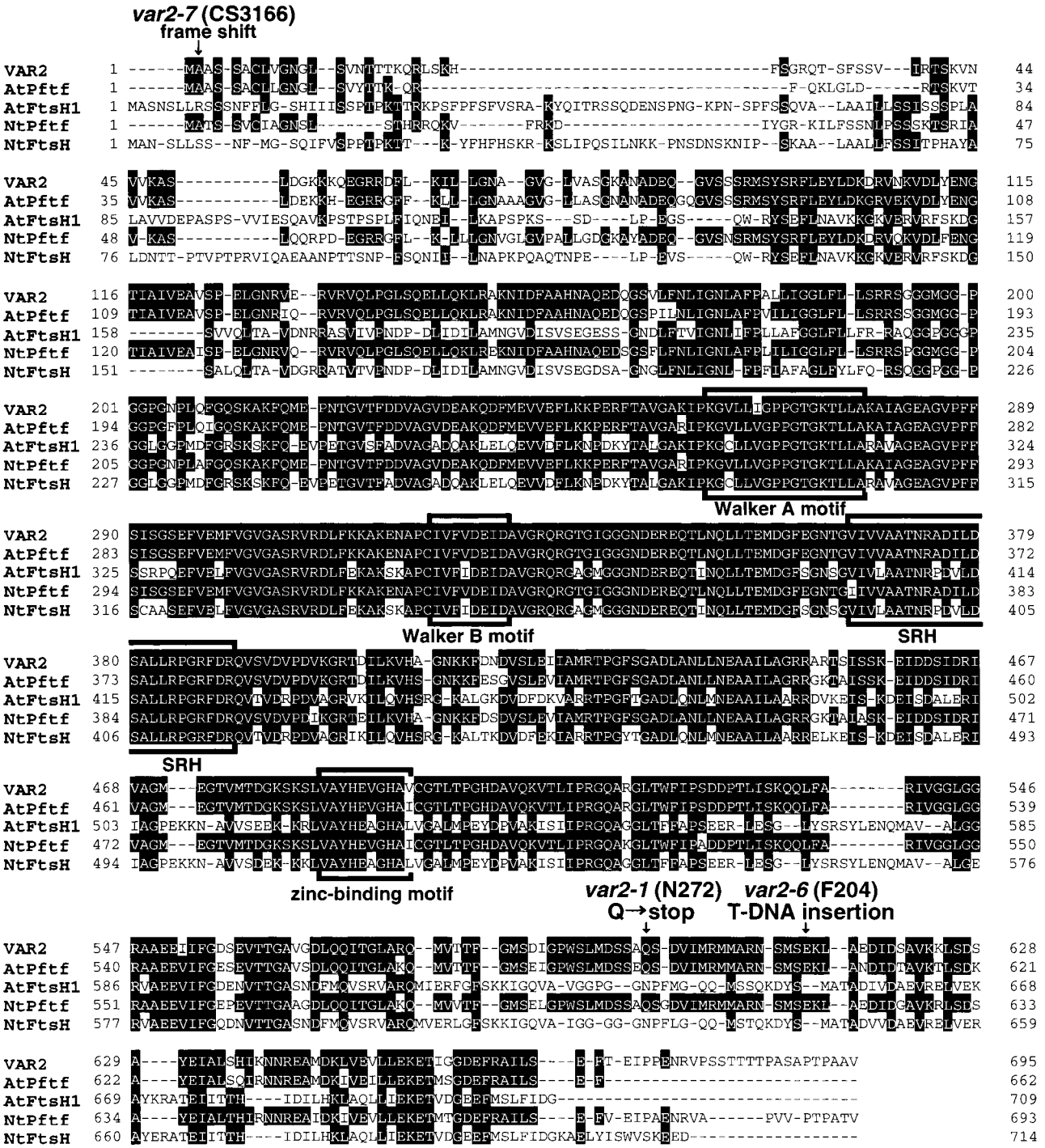


Fig. 4 Multiple alignment of the deduced amino acid sequence from VAR2 and the related proteins. Arrows indicate sites of the mutations in the *var2* mutants. Boxes represent specific motifs in the AAA protein family indicated at the bottom. Accession number of the presented proteins; AtPftf (AC007592), AtFtsH1 (AA68141), NtPftf (AF117339), and NtFtsH (BAA33755).

acteristic of zinc-dependent metalloproteases at residues 485–494.

Phylogenetic analysis revealed that VAR2 can be grouped together with Pftf (plastid fusion and/or translocation factor protein) in *Capsicum annuum* (Hugueney et al. 1995), and its homologue, NtPftf in *Nicotiana tabacum* (Summer and Cline 1999) (homology 84.7% with Pftf and 85.6% with NtPftf, respectively, shown in Fig. 5). It should be noted that there is an-

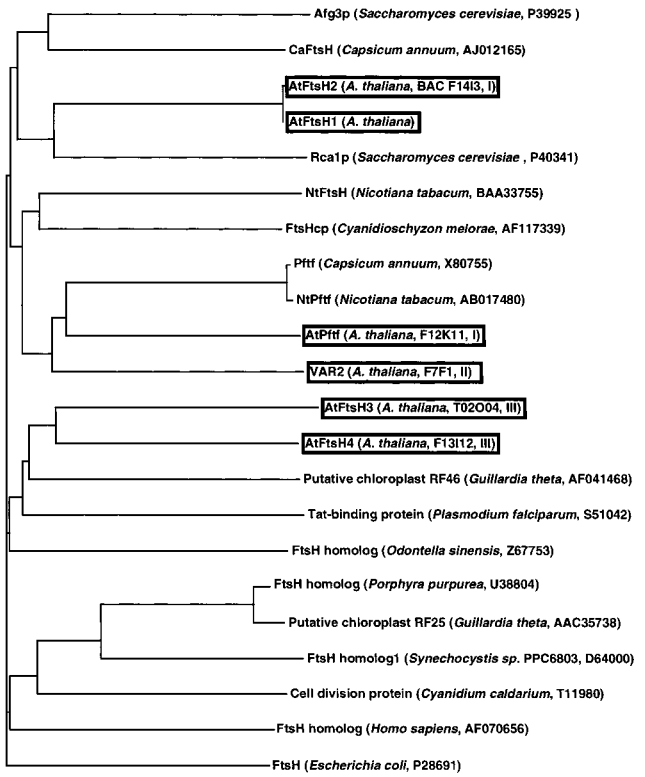


Fig. 5 Phylogenetic analysis of the proteins that belong to the FtsH family and show similarity to VAR2. The phylogenetic tree was constructed according to the NJ method using the full-length amino acid sequences from each protein. Accession numbers of the analyzed proteins are indicated in parentheses, and the *Arabidopsis* proteins are highlighted by open box.

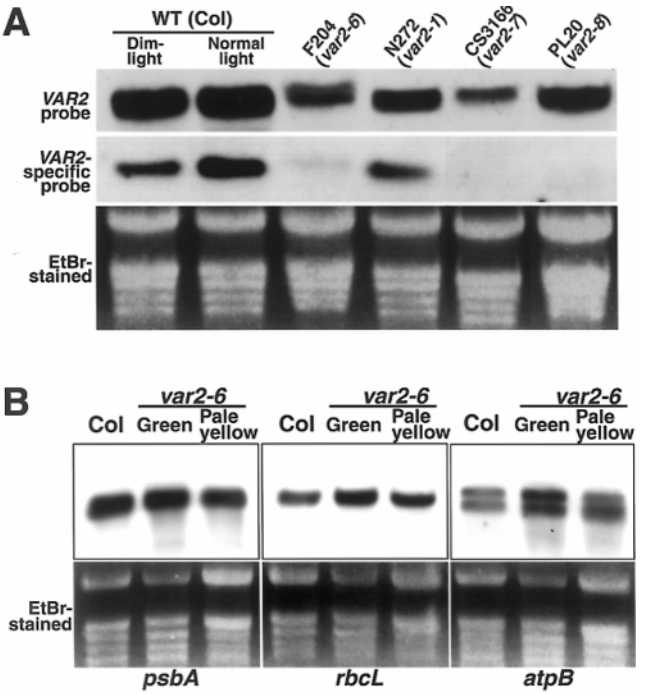


Fig. 6 (A) Northern blot analysis of Col and *var2* mutants. Expression of *VAR2* in Col was also examined under a dim-light condition. A full-length probe (*VAR2* probe) was prepared from PCR with primers KT101 and KT102, and a gene-specific probe (*VAR2*-specific probe) was prepared from PCR from primers KT101 and KT210 amplifying the 5′ portion of *VAR2* encoding a putative transit peptide sequence. (B) Expression of chloroplast genes in green and pale yellow tissues of the *var2* mutant. RNA was isolated from green and pale yellow regions of first and second true leaves of the *var2-6* mutant, and hybridized with gene-specific probes for *psbA*, *rbcL* and *atpB*. Two transcripts of 1.8 kb and 2.3 kb detected by the *atpB*-specific probe were considered to correspond to *atpB* monocistronic and *atpB-atpE* dicistronic transcripts, respectively. An ethidium bromide (EtBr)-stained gel is shown at the bottom of each panel to indicate equal loading of RNA samples.

other *Arabidopsis* gene (AtPftf in a BAC clone F12K11 on chromosome 1, accession number AC007592) coding for a protein highly homologous to VAR2. An *Arabidopsis* FtsH (here referred as AtFtsH1), that has been biochemically shown to act on the D1 protein of PSII (Lindahl et al. 1996, Lindahl et al. 2000), belongs to a different clade and shows 52.9% homology to VAR2. In addition to these homologues, three FtsH-like proteins were found in *Arabidopsis* genome. AtFtsH2 is included in the same clade as AtFtsH1. Likewise, AtFtsH3 and AtFtsH4 are 95.9% identical. AtFtsH1 was shown to contain two hydorophobic α-helices that span a membrane in its N-terminal region. Similarly, the hydropathy profiles of other FtsH-like proteins also predicted that they contain two transmembrane segments at their N-termini. Thus, the genes coding for FtsH-like proteins including *VAR2* were considered to comprise a small gene family in *Arabidopsis*, and we were able to classify six FtsH homologues into three groups.

ATP-dependent metalloproteases included in the AAA protein family have also been reported in mitochondria. For example, three proteins, Afg3p, Rca1p, and Yme1p, have been reported to play roles in various mitochondria activities in yeast (Thorsness et al. 1993, Pajic et al. 1994, Tzagaloff et al. 1994). This raises a question as to whether any of *Arabidopsis* FtsH homologues described above might be targeted into mitochondria rather than chloroplasts. Based on a computer prediction by ChloroP program (http://www.cbs.dtu.dk/services/ChloroP/), all the FtsH-like proteins except for AtFtsH3 appeared to be targeted into plastids (prediction scores more than 0.5) with their N-terminal amino acid sequences (data not shown). Localization of AtFtsH3 was uncertain but it did not appear to contain a typical chloroplastic transit peptide. On the other hand, a database search demonstrated that the genes potentially coding for a protein highly similar to Rca1p (accession number AAF79577), Yme1p (T02610) and Afg3p (T02738) were found independently from the *ftsH*-like genes in *Arabidopsis* genome. A computer prediction by PSORT program (http://psort.nibb.ac.jp/) showed that these putative mito-

chondrial homologues may be indeed targeted into mitochondria. Although such prediction lacks substantial evidence and real targeting needs to be demonstrated experimentally, these observations strongly suggest that chloroplastic FtsH proteins are encoded in several genes in *Arabidopsis* genome.

VAR2 mRNA accumulation

To examine the *VAR2* expression in the wild-type and *var2* mutant plants, we performed northern hybridization. Using the full length *VAR2* cDNA as a probe, a 2.1-kb transcript was detected in all *var2* mutant plants as well as the Col plant, most likely due to cross hybridization to transcripts from other *ftsH*-like genes (Fig. 6A). When a gene-specific probe corresponding to a 5′ portion of *VAR2* (encoding a putative transit peptide sequence) was used, signals were undetectable in all *var2* alleles. A faint signal was seen in *var2-1*, and this may be because the point mutation near the 3′ end had a modest effect on the stability of the truncated transcript. The results indicated that the *VAR2* mRNA accumulation was compromised but the redundant *ftsH*-like genes were expressed in all *var2* alleles.

Total RNA isolated from different tissues of Col plants showed that *VAR2* mRNAs accumulated in all tissues examined (rosette and cauline leaves, stems, flowers, and roots) with relatively high expression in leaves (data not shown). We then examined the *var2* mRNA accumulation in Col rosette leaves grown under relatively high- (70 μmol m^{-2} s^{-1}) and low- (15 μmol m^{-2} s^{-1}) intensity light, since the appearance of the variegated phenotype was light dependent. We used a low-intensity light instead of complete darkness, because leaf development was similar under these conditions except that leaves under low-intensity light were paler than those under high-intensity light. The *VAR2* transcripts were consistently increased several fold under high-intensity light (Fig. 6A). Likewise, the transcripts slightly decreased in rosette leaves from dark-adapted plants for several days (data not shown), suggesting that the accumulation of *VAR2* mRNA is not dramatically affected by light.

Targeting of VAR2 into chloroplasts with its transit peptide

VAR2 contained an N-terminal tail with several features of a plastid transit peptide with acidic residues and serine residues (von Heijne et al. 1989). To examine whether VAR2 is synthesized as a precursor with its transit peptide and targeted into chloroplasts, a transient assay using a GFP fusion protein was conducted. Based on the N-terminus of the mature Pftf protein from bell pepper (Hugueney et al. 1995), we assumed a maturation site of the precursor VAR2 protein to be at amino acid 45. We constructed two chimeric genes in which GFP was fused to the predicted transit peptide (57 amino acids in p35S-104-sGFP and 81 amino acids in p35S-105-sGFP). These genes were introduced into leaf epidermal cells by particle bombardment. Using both constructions, GFP signals were localized on the particles whose size corresponded to the amount of autofluorescence from chloroplasts (Fig. 7A–C). Without the putative transit peptide, GFP signals were dispersed in cytosol and nuclei (Fig. 7D). These results suggest that *VAR2* encodes a novel FtsH-like protein targeted into chloroplasts with its transit peptide.

Accumulation of chloroplast mRNAs and proteins in var2

Considering that VAR2 is a protease and is located in the thylakoid membrane, it is possible that *VAR2* may be involved in protein degradation of photosynthetic protein complexes. Loss of VAR2 may, in part, affect proper turnover of such multiprotein complexes and lead to a variegated phenotype with heteroplastidic cells. Because VAR2 as a protease appears to act on such process most likely at the post-translational level, steady-state levels of chloroplast proteins, rather than those of their mRNAs, may decrease in the green and/or yellow region of the mutant leaves.

To examine this, we performed northern and western blot analyses. Variegated leaves of the *var2-6* mutant were dissected and divided into green and yellow parts. Total RNAs from Col leaves and from green and pale yellow regions of the *var2-6* mutant leaves were separated by a denatured-formaldehyde gel and hybridized with probes for three chloroplast genes, *psbA* (encoding D1 protein of PSII), *rbcL* (encoding large subunit of ribulose 1,5-bisphosphate carboxylase-oxygenase), and *atpB* (encoding β subunit of chloroplast ATPase). The results, shown in Fig. 6B, showed that mRNAs transcribed from these genes accumulated substantially in both green and yellow sectors of the mutant leaves, and that their levels were comparable to those of the wild-type leaves. It is therefore suggested that loss of VAR2 did not affect expression of chloroplast genes at the level of transcription and mRNA stability.

We then tested steady-state levels of chloroplast proteins of photosynthetic complexes by western blotting and immunodetection. Total insoluble proteins from green and pale yellow sectors of the *var2-6* mutant leaves and from Col plants were isolated and probed with antibodies raised against CPI (reaction center polypeptides of PSI), cytochrome b_6 ($Cytb_6$, encoded by *petB*), and D1 proteins (reaction center protein of PSII). As shown in Fig. 8, we detected proteins of 64 kDa, 17 kDa, and 26 kDa crossreacted respectively with anti-CPI, anti-$Cytb_6$, and anti-D1 antibodies in total insoluble extracts from Col and the green regions of the mutant plants. The estimated molecular weight of D1 protein was smaller than the expected size (32 kDa), and this was considered due to the presence of urea in our SDS-PAGE. These signals were undetectable in the pale yellow regions of the mutant plant. Lack of the signals in the pale yellow sectors was not due to the inefficient protein extractions, since relatively equal intensities of a band crossreacting with a control antibody against manganese superoxide dismutase (Mn-SOD) were detected in all samples (data not shown). These results, together with data from northern analysis showed that in the yellow sectors of the mutant leaves, accumulation of proteins in the thylakoid but not their transcripts

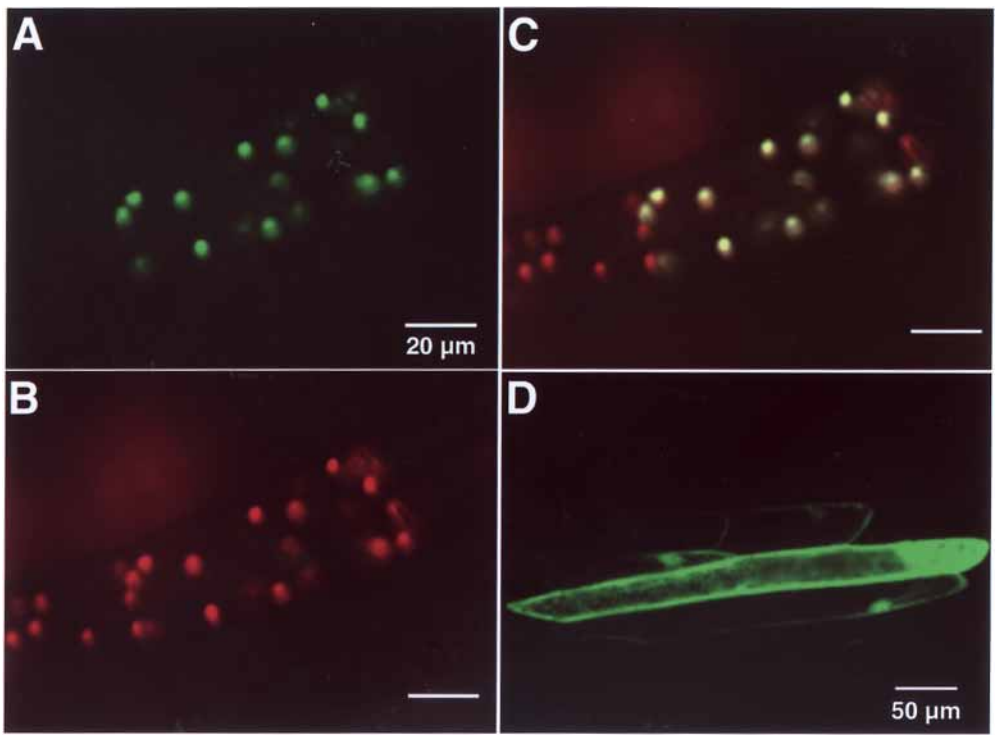


Fig. 7 Localization of GFP protein into chloroplasts with a putative transit peptide from *VAR2*. A tobacco epidermal cell in which p35S-104-GFP was introduced by particle bombardment was observed. Fluorescence from GFP (A) and from chlorophyll autofluorescence (B) was detected by a laser confocal-scanning microscope. (C) A superimposed picture of (A) and (B). GFP fluorescence from a cell expressing GFP without a targeting signal is shown in (D).

was impaired by loss of VAR2.

Discussion

FtsH has been identified from a cell division mutant in *E. coli* (Ogura et al. 1991) and shown to belong to a subgroup of AAA-protein superfamily (Patel and Latterich 1998). It is a membrane-bound ATP-dependent metalloprotease with two N-terminal transmembrane segments, and participates in various cellular processes such as the degradation of protein translocase subunit SecY, heat-shock transcription factor σ^{32} and transcriptional activator λCII (Herman et al. 1993, Herman et al. 1995, Kihara et al. 1995, Tomoyasu et al. 1995), mRNA decay (Granger et al. 1998), resistance to colincin (Gottesman et al. 1997) and the regulation of sporulation in *Bacillus subtilis* (Deuerling et al. 1997). Some FtsH proteases also appear to have a chaperone-like activity (Suzuki et al. 1997). Members of the AAA-protein family are present in many eukaryotic as well as prokaryotic organisms, including higher plants (Hugueney et al. 1995, Lindahl et al. 1996, Deuerling et al. 1997). Involvement of FtsH in chloroplasts has also been implicated, since the chloroplast genomes of some red alga and diatom contain a *ftsH*-like gene (Kowallik et al. 1995, Itoh et al. 1999). In fact, chloroplastic FtsH proteins have been identified in *Arabidopsis* and other plants (Hugueney et al. 1995, Lindahl et al. 1996). The evidence presented here demonstrates that a mutation at *VAR2*, encoding one of the proteins that belong to the AAA family, causes a leaf variegation, and therefore that the proper expression of a FtsH-like protein is essential for plastid differentiation.

In the course of preparing this paper, molecular cloning of the *VAR2* locus by chromosome walking has been reported by Chen et al. (2000). In addition to several *var2* alleles characterized in this study, they have identified mutations in three other *var2* alleles (*var2-2*, *2–3*, and *2–5*). These three alleles contained a missense mutation in the conserved region of a transmembrane domain and ATP binding motifs. They also showed by a protein import assay that VAR2 is targeted into chloroplast and located in the thylakoid. The presented data by them and us are consistent and further support the notion that loss of VAR2 results in a variegated phenotype. Furthermore, we demonstrated that several chloroplast proteins composing complexes of photosynthetic electron transport were at the undetectable level in the yellow sectors of the *var2-6* mutant leaves, despite the fact that chloroplast mRNAs accumulated substantially. VAR2 may be involved in a protein degradation pathway of photosynthetic complexes at the post-translational level, although biochemical evidence that it encodes a protease needs

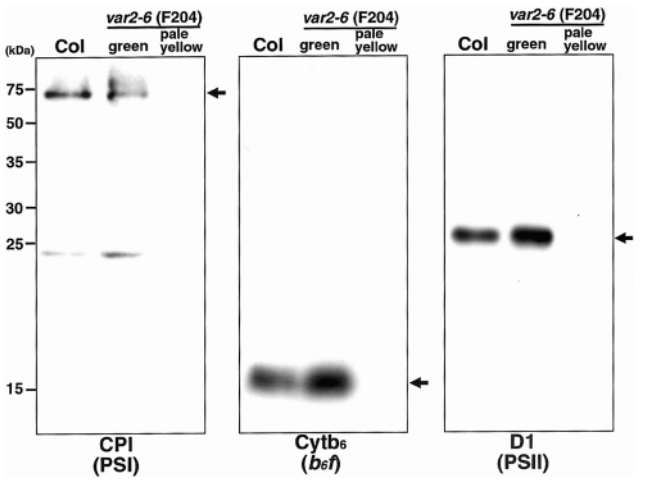


Fig. 8 Accumulation of chloroplast proteins in leaves of *var2*. First and second true leaves of *var2-6* were divided to green and pale yellow regions, and the isolated total proteins were subjected to western analysis. Proteins isolated from equal amounts of the fresh tissues (50 mg) were electrophoresed through a 12.5% SDS-Urea acrylamide gel, and immunoblot analysis was carried out using anti-CPI, anti-$Cytb_6$, and anti-D1 antibodies. Position and size of molecular weight markers are indicated on the left. Arrows indicate positions of the signals corresponding CPI, $Cytb_6$, and D1 proteins. A 21-kDa band detected by the anti-CPI antibody was considered to be a degradation product.

to be investigated. An alternative possibility is that VAR2 activity may be required for synthesis of chloroplast proteins.

In chloroplasts, the formation of photosynthetic protein complexes in the thylakoid requires coordinated expression and assembly of the subunit proteins encoded both in the chloroplast and nuclear genomes (Rochaix 1996, Sugita and Sugiura 1996). If not assembled correctly or damaged, the complexes together with unassembled subunit proteins are then rapidly degraded (Rochaix and Erickson 1988, de Vitry et al. 1989, Sieburth et al. 1991, Takahashi et al. 1991). A biochemical search and in vitro analysis of a responsible enzyme showed a possibility that a chloroplastic FtsH, located in thylakoid membrane with its catalytic domain faced to stroma, acts as a protease in this degradation pathway (Lindahl et al. 1996, Lindahl et al. 2000). For example, unassembled Reiske-FeS proteins, a component of the b_6f complex that is synthesized and imported from cytosol, appeared to be degraded by this mechanism (Ostersetzer and Adam 1997). Similarly, addition of AtFtsH1 to isolated thylakoids in vitro accelerated the breakdown of a 23-kDa degradation intermediate of D1 protein in PSII reaction center that was generated by excess exposure to light. Given that D1 protein is a major site exposed to photo damage and in fact turns over very rapidly (Mattoo et al. 1984), these results suggest that AtFtsH1 plays a role in maintaining the complexes by degrading an inactive subunit and/or correctly replacing it with a newly synthesized protein (Shipton and Barber 1991). Partial loss of such activities in the *var2* mutants perhaps leads to accumulation of the inactive complexes, consequently disturbing a proper flow of electron with the production of toxic oxygen species. Such an oxidative damage known as photoinhibition often causes bleaching of chloroplasts (Wetzel and Rodermel 1998), as we observed in the yellow sectors of the *var2* mutants. Supporting this idea, the appearance of yellow sectors was dependent on light intensity.

Among the FtsH proteins identified to date, VAR2 highly resembles Pftf rather than the other FtsHs. Pftf has been biochemically isolated as a factor to promote vesicle formation during the transition of chloroplasts into chlomoplasts in bell pepper fruits (Hugueney et al. 1995). This indicates that FtsH-like activities are responsible in part for plastid differentiation by supporting the degradation and/or fusion of inner plastid membranes to organize plastid-type competent membranes. Pftf was originally shown to be a stromal protein, but a recent study in pea indicated that Pftf is localized in thylakoids as shown in other FtsHs (Summer and Cline 1999). It should be noted that *Pftf* and *VAR2* are the only examples in which their expression was directly shown to affect plastid differentiation. These observations suggest that a subset of FtsH-like proteins including Pftf and VAR2 may be needed depending on the developmental status of each plastid. However, the following observations contradict with this view. First, *VAR2* expression is not strictly regulated by such status (slightly affected by light intensity), and similarly Pftf is constitutively expressed. Second, normal chloroplast differentiation occurs without VAR2 in the green sectors of the *var2* mutants. Such a leaky effect of VAR2 levels on plastid differentiation may be explained by the possibility that the developmental requirement of VAR2 is subtle and compensated by other FtsH-like proteins in certain plastids.

Based on these observations, we propose that *VAR2* plays a role in the developmental regulation of membrane formation and maintenance in plastids by preferentially degrading photooxidatively damaged protein complexes. Loss of the VAR2 function results in bleaching of chloroplasts but its function can be partially substituted by other FtsH proteins, thus showing variegation. The demand of such a mechanism represented by VAR2 depends on the developmental status of plastids rather than those of the host cell, thus showing heteroplasticity. The basal level of FtsHs can be retained because of their redundant expression, whereas a rather drastic transition of plastids into the chloroplast during leaf expansion may be secured by accumulation of VAR2. The proposed function of VAR2 protein is consistent with the observation by Chen et al. (2000) that a mutated version of VAR2 in *var2-3* accumulated in the green sectors but not in the yellow sectors of the variegated leaves.

Several questions remain to be answered. First, the primary substrate(s) of VAR2 needs to be determined. Redundant expression of the FtsH proteins raises a possibility that each FtsH might have a specific target protein. Conversely, broad substrates shared with each FtsH may explain our threshold hy-

pothesis. Members of the AAA protein family have been shown to form a hetero-oligomer (for example, Afg3p and Rca1p in yeast mitochondria, Arlt et al. 1996). VAR2 may interact with other FtsH homologues identified in this study, since N-terminal transmembrane regions required for the multimeric formation are retained in all AtFtsH proteins. Second, whether VAR2 has a chaperone-like activity as well as a protease activity needs to be examined. Like some of the bacterial and mitochondrial FtsH proteins (Akiyama et al. 1994, Suzuki et al. 1997), such activity rather than protease may be important for plastid development. Third, we still pause on the fact that cotyledons never showed variegation. Regardless of the precise function of VAR2, this clearly implies that some other factors, most likely in a tissue-specific manner, regulate plastid development in concert with the *VAR2* activity.

Characterization of the *VAR2* locus in our study demonstrated a novel mechanism by which variegation occurs in higher plants. It strengthened our assumption that the leaf variegation is caused by an important mechanism regulating a crosstalk between the two organelles. *var2* is currently the only mutation known to cause the variegation among the six genes encoding the putative FtsH proteins in *Arabidopsis*. Therefore, it may be possible that *VAR2* is one of the most essential FtsHs for plastid development in *Arabidopsis*. To examine the influence of chloroplastic FtsHs on plastid development genetically, we are in search for a knock-out mutant in which these *ftsH*-like genes are disrupted.

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