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INTRODUCTION

Variegation mutants have been defined as "any plant that develops patches of different colors in its vegetative parts" (Kirk and Tilney-Bassett, 1978). Some of the most common variegations have green and white (or yellow) sectors in normally-green tissues and organs of the plant. Whereas cells in the green sectors typically contain normal-appearing chloroplasts, cells in the white (or yellow) sectors contain plastids that are deficient in chlorophyll and/or carotenoid pigments. These plastids appear to be blocked at various steps of chloroplast biogenesis because they frequently lack organized internal membrane structures and/or contain only rudimentary lamellae. Despite their widespread occurrence, relatively few variegations have been characterized at the molecular level.

Variegation mutants have played a prominent role in the history of genetics (reviewed by Granick, 1955; Kirk and Tilney-Bassett, 1978). As a notable example, the finding that transmission of the variegation trait does not always obey Mendel's laws led to the discovery of non-Mendelian inheritance in the early 1900's. Whereas variegations have long been associated with differences in plastid form and function, it was not possible to gain insight into the molecular basis of this phenomenon until the 1960's and early 1970's, when compelling molecular evidence was presented that mitochondria and chloroplasts contain their own DNA and protein synthesis systems, and that organellar proteins are the products of genes in the chloroplast and mitochondrion, as well as the nucleus (reviewed by Bogorad, 1981).

Nuclear-organelle interactions

Nuclear DNA-encoded proteins that are destined for chloroplasts and mitochondria are translated as precursors on 80S ribosomes in the cytosol and transported into the organelle post-translationally, whereas proteins encoded in the mitochondrial and plastid genomes are translated on prokaryotic-like 70S ribosomes in the organelle itself (reviewed by Goldschmidt-Clermont, 1998; Leon et al., 1998). Mitochondria and plastids are derived from prokaryotic endosymbionts, and during the process of symbiogenesis most of the genes of the symbiont were lost or transferred to the host genome (Gray, 1992; Doolittle, 1998; Cavalier-Smith, 2000). For instance, current-day plastid genomes in higher plants code for less than 100 of the estimated 1900 to 2500 proteins in a typical chloroplast (Abdallah et al., 2000; Martin and Herrmann, 1998). Plastid and mitochondrial genomes are polyploid, and in higher plant cells they are dispersed among many plastids and mitochondria. For example, a typical mesophyll cell contains from 1,000 to 10,000 identical plastid DNAs distributed among 100 or more chloroplasts (Bendich, 1987).

It is thought that the dispersal of genes for plastid and mitochondrial proteins between two different compartments was the central driving force that led to the evolution of mechanisms that integrate nuclear and organellar gene expression (reviewed by Bogorad, 1981). A contributing factor might also have been the vast disparity in copy number between genes in the nuclear and organellar compartments. Much of the regulatory traffic between the nucleus and the organelle is anterograde, i.e., from the nucleus to the organelle, in the form of nuclear gene products that control the transcription and translation of mitochondrial and plastid genes (reviewed by Goldschmidt-Clermont, 1998; Leon et al., 1998). Yet, much of this traffic is also retrograde, i.e., from the organelle to the nucleus (reviewed by Oelmüller, 1989; Taylor, 1989; Mayfield, 1990; Susek and Chory, 1992; Gray et al., 1995; Hess et al., 1997; Rodermel, 2001).

Perhaps the best understood examples of retrograde

trafficking in plants involve the transcriptional regulation of nuclear genes for photosynthetic proteins by plastid-to-nucleus signaling mechanisms initiated by a variety of "plastid signals" (reviewed by Rodermel, 2001). The plastid signals identified to date are intermediates or by-products of photosynthetic metabolism. Yet, poorly understood retrograde signals also control the expression of nuclear genes for a number of non-plastid proteins (reviewed by Oelmüller, 1989; Barak et al., 2001), as well as the transcription of mitochondrial genes, cell differentiation and leaf morphogenesis (reviewed by Hess et al., 1997; Hedtke et al., 1999; Rodermel, 2001). Consequently, plastid-to-nucleus signaling plays a central role in coordinating gene expression in the nucleus, plastid and mitochondrion, and in integrating pathways of cellular metabolism and development. Presumably, there is crosstalk between retrograde signaling and other signal transduction pathways (e.g., light, hormones, sugars, developmental factors) that also play a role in coordinating nuclear and organelle gene expression (e.g., Neff et al., 2000; Oswald et al., 2001).

Mechanisms of variegation

Variegations can arise by many different mechanisms (reviewed by Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1975). Some variegations are induced by external agents and are not heritable. For instance, chlorotic leaf sectors can be generated by preferential shading, pathogen attack, and nutritional deficiencies. Heritable variegations, on the other hand, mostly arise from mutations in nuclear, plastid and/or mitochondrial genes. Many of these are green-white variegations in which plastids in the white sectors fail to accumulate (or aberrantly accumulate) photosynthetic pigments. All the steps of chlorophyll and carotenoid biosynthesis occur in the plastid by nuclear gene products that are imported into the organelle post-translationally (reviewed by Bartley and Scolnik, 1995; vonWettstein, 1995). Consequently, heritable variegations arise from mutations in any compartment that affect pigment synthesis or accumulation in the plastid, either directly or indirectly.

Many variegations are caused by mutations in nuclear genes that generate defective plastids in some, but not all, cells of the plant. These variegations are Mendelian-inherited. In many cases, the defective plastids (and the cells that contain them) replicate normally, or nearly so, and sort out to produce clones of cells containing morphologically normal chloroplasts (green sectors) or abnormal plastids (white or yellow sectors) (reviewed by Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1984, 1989; Hagemann,

1986). The abnormal plastids are often, but not always, "permanently-defective". Permanently-defective plastids are inherited in a non-Mendelian fashion, i.e. maternally (in the majority of angiosperms) or biparentally (Tilney-Bassett, 1975; Connett, 1987). In cases of maternal inheritance, the probability of transmission of a permanently-defective organelle is related to the extent of variegation of the mother plant.

In principle, permanently-defective plastids can arise in many different ways. One common way is by the generation of mutations in the plastid genome by defective nuclear gene products, as in some "chloroplast mutator" lines (reviewed by Tilney-Bassett, 1975; Kirk and Tilney-Bassett, 1978; Börner and Sears, 1986; Hagemann, 1986; Chang et al., 1996). Because plastid genomes are multicopy, it is thought that chloroplast mutator and other "plastome mutants" (the general term for mutants that contain chloroplast DNA lesions) are variegated because mutant and normal plastid chromosomes, following replication, sort out to form clones of plastids and cells containing either all-normal plastid DNAs (green sectors) or all-mutant plastid DNAs (white sectors) (Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1975; Birky, 1983). Plastids containing a single type of plastid DNA are termed "homoplasmic", whereas ones with different types are termed "heteroplasmic".

Another way that permanently-defective plastids can arise is by the loss of plastid 70S ribosomes, as in the *iojap* mutant of maize and the *albostrians* mutant of barley (Walbot and Coe, 1979; Börner and Sears, 1986; Coe et al., 1988; Han et al., 1992; Hess et al., 1992, 1993, 1994a, 1994b; Zubko and Day, 1998; Hedtke et al., 1999). The plastids in the white sectors of these mutants are permanently-defective because plastid ribosome biogenesis requires the ability to translate 70S ribosomal proteins encoded in the plastid genome. Hence, once a plastid has lost its ribosomes, it can't regain them even if returned to a wild type nuclear background. The primary lesions in *iojap* and *albostrians* are not well-understood, but *IOJAP* has been cloned and appears to code for a component of the 50S subunit of the plastid ribosome (Han et al., 1992; Han and Martienssen, 1995).

In addition to variegations that are caused by the induction of permanently-defective plastids by mutations in nuclear genes, variegations can also be caused by mutations in nuclear genes that generate permanently-defective mitochondria. Notable examples include the *nonchromosomal stripe* (*NCS*) mutants of maize and *chloroplast mutator* of *Arabidopsis* (Rédei, 1973; Martínez-Zapater et al., 1992; Sakamoto et al., 1996; Leon et al., 1998). As discussed in greater detail later, these mutants are variegated because the abnormal mitochondria secondarily affect the phenotype of the plastids in the cell. Hence, these sorts of variegations are Mendelian-inherited, but the defective

mitochondria are maternally (or biparentally) inherited.

There are several other prominent mechanisms by which nuclear genes produce variegations. One is chimerism, in which different histological regions of a plant meristem, and consequently the tissues that derive from them, have different genotypes (reviewed by Kirk and Tilney-Bassett, 1978; Tilney-Bassett, 1986). This results in variegation if the genotypic differences affect pigment accumulation in the plastid. A second mechanism involves transposable element activity. In this mechanism, transposon insertion interrupts a nuclear gene required for normal chloroplast biogenesis (white sectors), while element excision reconstitutes wild type gene expression (green sectors) (reviewed by Federoff, 1989). In *Arabidopsis*, variegations of this type have been reported in transposon-tagging experiments using heterologous elements, such as the autonomous maize *En*-1 element, or the maize *Dissociation* (*DS*) transposable element/ *Activator* (*Ac*) transposase system (Wisman et al., 1998; Klimyuk et al., 1995).

A final type of nuclear gene-induced variegation that will be mentioned is homology-dependent gene silencing, viz., transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (reviewed by Waterhouse et al., 2001). It is thought that double stranded RNAs (dsRNAs) play a role in regulating both TGS and PTGS: in TGS they regulate the methylation of promoters of target genes, whereas in PTGS they control target RNA degradation. Although the mechanisms are obscure, variegations are thought to arise when a nuclear gene required for normal chloroplast biogenesis is silenced in some cells but not others (Waterhouse et al., 2001).

Related to gene silencing is the phenomenon of variable gene expression by antisense transgenes. A well-known example of this is the production of variegated flowers in transgenic petunia that contain an antisense chalcone synthase gene (e.g., van der Krol et al., 1988; 1990). Antisense RNAs are thought to regulate sense gene expression by annealing to complementary sequences of the sense RNA, resulting in dsRNAs that affect sense RNA stability, transcription or translation (reviewed by Terryn and Rouzé, 2000). An inhibition at any of these levels of gene expression can result in reduced production of the target protein. It has been proposed that sense: antisense RNA duplexes cause variegation by serving as dsRNA signals to control TGS and PTGS.

In addition to nuclear gene-induction mechanisms, variegations can be caused by mutations in plastid or mitochondrial genomes that arise without the participation of defective nuclear gene products. For example, some plastome mutations arise spontaneously or following treatment with various chemical mutagens (reviewed by Tilney-Bassett, 1975; Börner and Sears, 1986). Plastome mutants can also be produced by chloroplast transforma-tion (e.g., Kanevski and Maliga, 1994; Rochaix, 1997). Such "transplastomic" lines can be engineered by the integration of an antibiotic cassette into the plastid genome by homologous recombination via flanking plastid DNA sequences in the vector; the transformed plastid DNAs subsequently sort out to form homoplasmic lines. It might be anticipated that mutations in many plastid genes would generate permanently-defective plastids, because plastid genomes primarily code for core components of the photosynthetic apparatus and for proteins required for plastid gene expression (reviewed by Goldschmidt-Clermont, 1998).

It should be emphasized that in cases where variegations are caused by plastome mutations, transposable element activity, or chimerism, the mechanism of variegation is relatively straightforward and can be explained by the fact the green and white sectors have different genotypes. Variegations caused by gene silencing can be explained in a similar manner, i.e, a given gene is silenced in some cells but not others. These mechanisms stand in contrast to cases of nuclear gene-induced variegations in which the green and white cells have the same nuclear (mutant) genotype, but the mutant phenotype has limited penetrance and is seemingly expressed only in a subset of the cells (i.e., cells in the white sectors). These latter variegations will be the focus of this review.

Arabidopsis variegations

Mutagenesis experiments in *A. thaliana* typically generate a low frequency of color mutants (e.g., McKelvie, 1963, Reiter et al., 1994; Wisman et al., 1998). For instance, Reiter et al. (1994) identified nearly 300 color mutants in a collection of ~8000 T-DNA tagged *Arabidopsis* (3.75% frequency). These mutants had a uniform pigmentation and no variegations were reported. Of 78 color mutants uncovered in a collection of 10,950 T-DNA and *En*-tagged lines (0.75% frequency), four were variegations (0.04% frequency) (Wisman et al., 1998; D. Maiwald, unpublished observations). In contrast to the relatively low percentage of variegations that arise in tagging experiments, McKelvie (1963) found that approximately 10% of the color mutants generated by EMS and X-ray mutagenesis of *A. thaliana* were variegated.

Röbbelen (1968) reported that the *immutans* variegation mutant (discussed below) arises with a frequency of approximately 2×10^{-5} in the M2 progeny from X-ray treated *A. thaliana* seeds and with a frequency of 1.4×10^{-3} in the M2 progeny from EMS-treated seeds. *immutans* did not arise spontaneously in any of Röbbelen's experiments.

The frequency of EMS-generated *immutans* in these experiments is higher than the average per locus mutation frequency estimated for EMS mutagenesis in *Arabidopsis* (0.5×10^{-3} to 0.5×10^{-4}) (Koorneef et al., 1982; Haughn et al., 1988; Vizir et al., 1996). Consistent with this finding, Rédei and Röbbelen claimed (as of 1975) that there were more alleles of *immutans* than any other *Arabidopsis* gene (greater than 100) (Röbbelen, 1968; Rédei, 1975). In summary, the *Arabidopsis* mutagenesis experiments, while not exhaustive, are illustrative of the general principle that variegations arise at a low, but variable frequency in *Arabidopsis*, and that this frequency is influenced by the type of mutagen used.

There are roughly 200 variegation mutants in the *Arabidopsis* stock centers at Ohio State and Notttingham. Many of these mutants are from the Rédei and Röbbelen collections. However, most of these lines (including putative "*immutans*" lines) have been characterized only superficially. For instance, allelism tests have been conducted with relatively few of the mutants, so it is unclear whether the various lines represent alleles of known genes or novel variegation loci. Complicating matters, we have found that some differently-labeled lines are, in fact, identical (discussed later, see Table 3). Therefore, the diversity of variegations represented in the stock centers is not clear, but merits further study.

In the remainder of this review I wish to summarize what is known about specific *Arabidopsis* variegation mutants. I will restrict my attention to nuclear gene-induced variegations in which the mutant genotype is uniform in the plant. While I will focus on those that have been characterized at the molecular level, several others that have been characterized to a certain extent will also be discussed. These mutants are listed in Table 1. Several questions are of particular relevance in surveying each mutant. One would first like to know the identity of the gene product defined by each mutant locus, how it functions in plastid biogenesis, and why plastids are defective in the mutant. A second question concerns the mechanism of variegation. The fact that so few variegation mutants of this type have been documented raises the possibility that they have some underlying mechanism in common. Why are these mutants variegated rather than albino or uniformly-pigmented?

The first mutant that will be discussed is *immutans*. It is among the oldest *Arabidopsis* mutants to be described and is represented by over 25 papers in the literature, spanning nearly half a century of work. *immutans* will be discussed in much greater detail than the other variegation mutants. One reason for this is that the methods used to study *immutans* serve as a paradigm of the sorts of experiments that have been conducted to characterize other variegation mutants. The discussion of *immutans* will also provide a contextual framework within which to understand the other mutants.

immutans

Phenotype of im

The *immutans* (*im*) mutant of *Arabidopsis thaliana* was first described and partially characterized nearly 40 years ago by Rédei, Röbbelen and co-workers (Chung and Rédei, 1974; Chung et al., 1974; Rédei, 1963, 1967a, 1967b, 1967c, 1975; Rédei et al., 1974; Röbbelen, 1968) (Figure 1). These studies showed that sectoring in *im* is caused by a nuclear recessive gene, and that white sector formation is enhanced when plants are grown in elevated temperatures and light intensities or when illuminated with red (versus blue) light (Rédei, 1963; Röbbelen, 1968).

Progeny from the selfing of *im/im* plants recapitulate the variegation of the parent, regardless of whether they are derived from all-green or all-white inflorescences (Rédei, 1967a). This indicates that the mutant seeds have a uniform genetic constitution, and that transposable element activity is not responsible for the *im* variegation. If transposition were the cause, progeny from green inflorescences would be predominantly wild type, while progeny from white branches would be white and/or variegated, depending on the frequency of the excision event. The selfing experiments also suggest that white plastids are capable of being converted into chloroplasts and vice versa, i.e., that the plastid phenotype is reversible. Consistent with this idea, abnormal plastids are not maternally inherited in *im*, suggesting that the plastid defect is "cured" before or during reproduction (Wetzel et al., 1994). This presumably occurs during the plastid dedifferentiation and redifferentiation events that take place during the reproductive process (e.g., during the formation of reproductive cells or during early embryo development), and which ultimately give rise to proplastids in the meristem tissues; proplastids are undifferentiated plastids that are the source of most plastid types in the mature plant (Tilney-Bassett, 1989). The phenotypic reversibility of plastids in *im*, and the apparent inability of the mutant to convert permanently from an all-green ("wild type-like") to a variegated ("mutant") phenotype led Rédei (1975) to name the mutant *immutans* (for "immutable").

Recent studies have revealed that the white tissues of *im* are heteroplastidic and contain cells with abnormal plastids, as well as rare, normal-appearing chloroplasts (Wetzel et al., 1994) (Figure 2). The abnormal plastids are vacuolated and lack organized lamellar structures (Figure

Table 1. *Arabidopsis* nuclear gene-induced variegation mutants

Mutant	Phenotype	Alleles	Maternal Inheritance	Mutagen	Protein	Plastid Morphology	Leaf Morphology	References
am	White-green variegated	One	Yes	X-ray	?	**White sectors:** vacuolated plastids lacking lamellae **Green sectors:** normal-appearing chloroplasts	?	Röbbelen, 1966
atd2	White leaves – green cotyledons	One	?	T-DNA	Glutamine 5-phospho ribosylpyrophosphate amidotransferase (Atase2)	**White sectors:** small vesiculated plastids that lack organized lamellae **Green sectors:** normal-appearing chloroplasts	**White sectors:** lack of palisade cells **Green sectors:** normal	van der Graaff, 1997
chm	White-green-yellow variegated	Three	Yes	EMS & Somaclonal variation	?	**White sectors:** a range of abnormal plastid types **Green sectors:** normal-appearing chloroplasts	?	Rédei, 1973 Rédei and Plurad, 1973 Martinez-Zapater et al., 1992 Sakamoto et al., 1996
cue1	White-green reticulate; light-sensitive	Eight	?	EMS & T-DNA	Plastid phospho enolpyruvate/phosphate translocator (PPT)	**Mesophyll cells:** small plastids with few grana **Bundle sheath cells:** normal-appearing chloroplasts	**Pale green sectors:** decreased number of palisade cells; decreased chloroplast sizes **Green sectors:** normal	Li et al., 1995 López-Juez et al., 1998 Streatfield et al., 1999
dov1	White-green reticulate	One	?	EMS	?	**Mesophyll cells:** small plastids with vacuoles and no grana **Bundle sheath cells:** normal-appearing chloroplasts	**Pale green sectors:** normal numbers and sizes of mesophyll cells; decreased number of plastids **Green sectors:** normal	Kinsman and Pyke, 1998
immutans	White-green variegated; temperature- and light-sensitive	100?	No	EMS, X-ray & T-DNA	Plastid AOX homolog	**White sectors:** heteroplastidic for normal chloroplasts and large, vacuolated plastids lacking lamellae **Green sectors:** normal-appearing chloroplasts	**White sectors:** lack of palisade cell expansion **Green sectors:** enlarged mesophyll cells	Rédei, 1967a Wetzel et al., 1994 Meehan et al., 1996 Wu et al., 1999 Carol et al., 1999 Aluru et al., 2001
pac	Pale green (*pac-1*) and white-green variegated (*pac-2*)	Two	?	T-DNA	Plastid 36 kD protein, role in plastid mRNA maturation	*pac-1:* rudimentary lamellae	*pac-1:* normal early leaf development, then palisade cells decrease in size and epidermal cells enlarge	Reiter et al., 1994 Grevelding et al., 1996 Meurer et al., 1998 Tirlapur et al., 1999
var1	White-green variegated leaves; temperature sensitive	One	No	Somaclonal variation	?	?	?	Martinez-Zapater, 1993
var2	White-green variegated	Ten	No	EMS, X-ray & T-DNA	FtsH-like zinc metallo-protease	**White sectors:** heteroplastidic for normal chloroplasts and vacuolated plastids with rudimentary lamellae **Green sectors:** normal-appearing chloroplasts	?	Martinez-Zapater, 1993 Chen et al., 1999 Chen et al., 2000 Adam et al., 2001

Table 2. Proposed nomenclature for FtsH proteases from *Arabidopsis*

Protein Name	cDNA Accession No.	Precursor (aa)	Mature Protein (aa)	Transit Peptide (aa)	Chromosomal Location and Accession No.	Cellular Location	Reference
FtsH1	X99808	709	662	48	I (AC007980) (protein id: AAD50055)	C	(Lindahl et al., 1996)
FtsH2	AF135189[1]	695	648	47	II (AC004669) (protein id: AAC20729)	C	(Chen et al., 2000)
FtsH3	AV550192[2]	807	753	54	II (AC005315) (protein id: AAC33234)	M	
FtsH4	AI994723[2]	627			II (AC004747) (protein id: AAC31223)	?	
FtsH5	AV521115[2]	761	703	58	V (AB023032)[3]	C	
FtsH6	none	687	612	75	V (AL353993) (protein id: CAB89335)	C	
FtsH7	BE523403[2]	802	747	55	III (AL133292) (protein id: CAB61952)	C	
FtsH8	AI995330[2]	662	625	37	I (AC007592) (protein id: AAF24819)	C	
FtsH9	AV529278[2]	806	744	62	V (AB016885) (protein id: BAB09632)	C	
FtsH10	AV550192[2]	843	817	26	I (AC022464) (protein id: AAF79577)	M	
FtsH11	AV528850[2]	806	743	63	V (AB025622) (protein id: BAB08420)	?	

[1]Designated also VAR2
[2]EST
[3]The coding sequence spans nucleotides 41203–43350

Abbreviations: C, chloroplast; M, mitochondria. Predicted locations are in italics. (This table is adapted from Adam et al., 2001).

Table 3. *var2* alleles

Allele	Mutant Designation	Mutagen	Mutation	Reference
var2-1	VAR2	EMS	Nonsense (Q597*)	Martinez-Zapater, 1993; Chen et al., 2000; Takechi et al., 2000
var2-2	VAR4	EMS	Missense (R191K)	Martinez-Zapater, 1993; Chen et al., 2000;
var2-3	CS3154 (same as CS279, CS3640, CS3647, 69-2043b)	EMS	Missense (G267D)	Chen et al., 1999; 2000
var2-4	405-D-2357 (*yellow variegated*) (same as CS3320)	X-ray	Splicing	Chen et al., 1999; A. Manuell and S. Rodermel, unpublished
var2-5	405-D-2569 ("greener" *immutans*) (same as CS3681)	X-ray	Missense (P320L)	Chen et al., 1999; 2000
var2-6	F204	T-DNA	Insertion	Takechi et al., 2000
var2-7	CS3166 (?)	EMS	Frameshift	Takechi et al., 2000
var2-8	PL20	EMS	Unknown	Takechi et al., 2000
var2-9	CS3166	EMS	Splicing	A. Manuell and S. Rodermel, unpublished
var2-10	CS3622 (same as CS3621, CS3654, CS3659)	EMS	Splicing	A. Manuell and S. Rodermel, unpublished

3). Heteroplastidic ("mixed") cells are uncommon in plants and usually arise from the incomplete sorting out of different plastid types during development (Tilney-Bassett, 1975). Complete sorting out generates homoplastidic cells with plastids of one type or the other. The finding of heteroplastidic cells indicates that plastids respond differently to factors from the nucleus-cytosol in *im* cells, and hence that *im* is "plastid autonomous" (Wetzel et al., 1994). An early idea was that some plastids receive threshold amounts of the IM protein (green chloroplasts), while others do not, thus blocking their development (white plastids) (Wetzel et al., 1994). As discussed below, this interpretation has been called into question because some *im* alleles are apparently null (Wu et al., 1999).

Biochemical analyses have demonstrated that *im* white sectors accumulate phytoene, a colorless C_{40} carotenoid intermediate (Wetzel et al., 1994). This suggests that the mutant is impaired in the activity of phytoene desaturase (PDS), the plastid enzyme that converts phytoene to zeta-carotene (Bartley et al., 1991). Yet, *IMMUTANS* is not the

PDS structural gene (Wetzel et al., 1994), nor does *IM* affect *PDS* expression at the level of mRNA or protein accumulation (Wetzel and Rodermel, 1998). The finding that carotenoid biosynthesis is blocked in the white tissues of *im* suggests that these tissues are susceptible to chlorophyll-mediated photooxidation (Oelmüller, 1989). In support of this idea is the observation that white sector formation in *im* can be modulated by illumination intensity. Also consistent is the finding that RNAs from various nuclear photosynthetic genes are preferentially reduced in abundance in the white *im* tissues (Wetzel et al., 1994). This is similar to the situation in other carotenoid-deficient, photooxidized tissues (the "plastid signal" hypothesis, discussed above) (reviewed by Oelmüller, 1989; Taylor, 1989; Mayfield, 1990; Susek and Chory, 1992; Gray et al., 1995; Hess et al., 1997; Rodermel, 2001). By contrast, the levels

Figure 1. The *immutans* variegation mutant of *Arabidopsis*. The green sectors contain normal-appearing chloroplasts, whereas the white sectors are heteroplastidic and contain abnormal plastids that lack pigments and lamellar structures, as well as rare normal chloroplasts (Wetzel et al., 1994). The white sectors accumulate the carotenoid precursor phytoene (Wetzel et al., 1994), and white sector formation is promoted by increased light intensity and temperature (Rédei, 1963; Röbbelen, 1968).
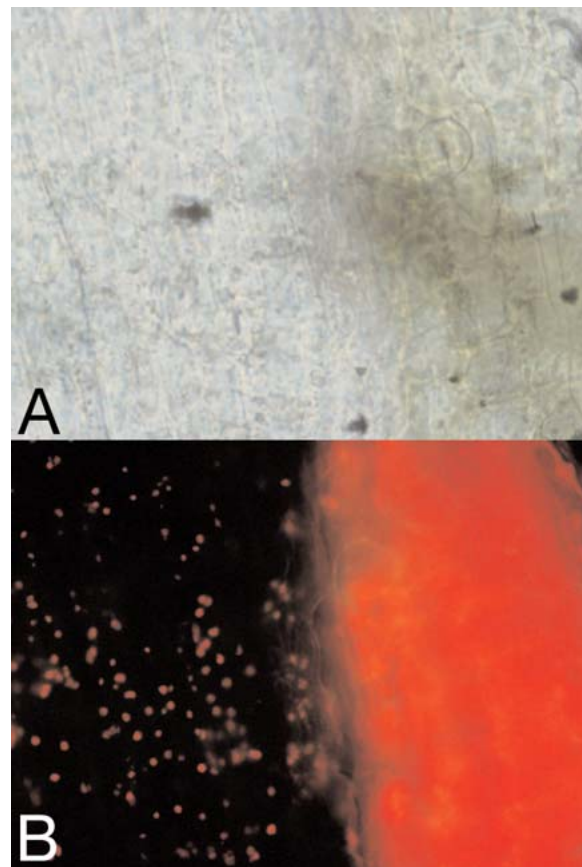

Figure 2. Cells in *immutans* white sectors are heteroplastidic.
Light **(A)** and corresponding chlorophyll autofluorescence images **(B)** of variegated *immutans* tissue, showing a green-white interface. Chloroplasts appear red in the fluorescence photographs. Magnification is 80X.
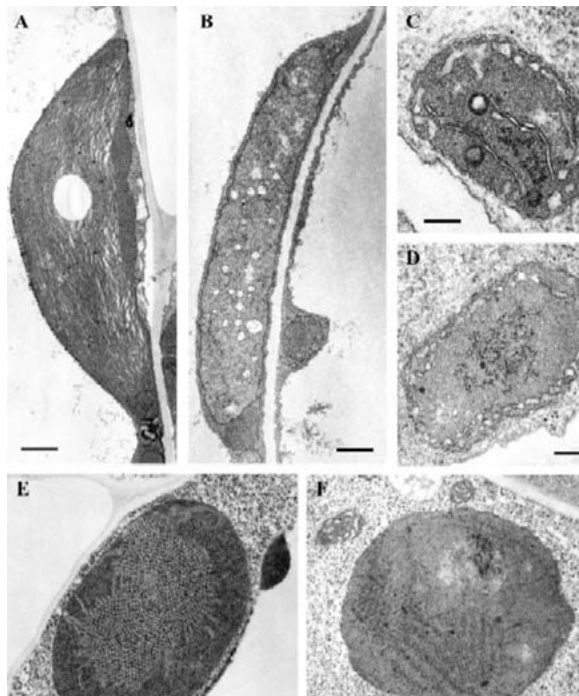
Figure 3. Plastid ultrastructure in *immutans*.
Wild-type and *im* seedlings were grown on MS plates for 7 days under normal light conditions (A, B, C, D) or in darkness (E, F).
(A) chloroplast from a wild type cotyledon, similar to chloroplasts from leaves (Wetzel et al., 1994) (Bar = 500 nm).
(B) plastid from a white sector of an *im* cotyledon, similar to plastids from white leaf sectors of *im* (Wetzel et al., 1994) (Bar = 500 nm).
(C) amyloplast from a wild-type root (Bar = 200 nm).
(D) amyloplast from an *im* root (Bar = 200 nm).
(E) etioplast from a wild-type cotyledon (Bar = 200 nm).
(F) etioplast from an *im* cotyledon (Bar = 200 nm).
(This figure is from Aluru et al., 2001).

Figure 4. The *gh* variegation mutant of tomato.

of RNAs from photosynthetic genes are normal in *im* green tissues (Wetzel et al., 1994).

In early studies, Rédei (1967a, 1975) found that the activity of a cytoplasmic acid RNase is enhanced in *im*, and he suggested that the primary lesion in the mutant resides in RNA metabolism. Wetzel et al. (1994) confirmed these results but found, in addition, a similar enhancement of acid RNase activity in photooxidized *Arabidopsis* leaf tissues produced by treatment with norflurazon, an inhibitor of PDS. It was concluded that increased cytoplasmic acid RNase activities are most probably a secondary effect of photooxidation rather than the primary

lesion in the mutant. This was demonstrated conclusively by the isolation of the *IM* gene.

Cloning and identification of IM

We cloned the *IM* gene by map-based methods and found that it is a plastid member of the alternative oxidase (AOX) class of inner mitochondrial membrane proteins (Wu et al., 1999); a transposon-tagged allele of *IM* has also been isolated (Carol et al., 1999). AOX functions as a terminal oxidase in the alternative (cyanide-resistant) pathway of mitochondrial respiration, where it generates water from ubiquinol (reviewed by Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997). AOX is found in all higher plants and in some algae, fungi and protists. The fact that IM bears similarity to AOX suggests that IM is a redox component of a phytoene desaturation pathway

A
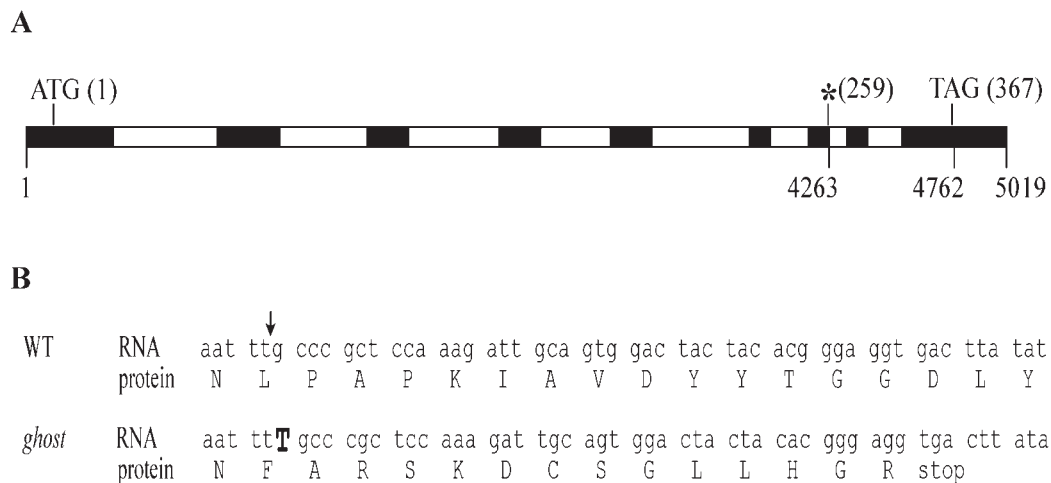

B

Figure 5. *gh* mRNA has an insertion mutation.
(A) Schematic of the *GH* genomic sequence (deposited in Genbank as Accession number AF302932). Exons are shown as filled boxes, introns as open boxes. The numbering below the gene refers to base pairs (bp) in the genomic sequence (5.01 kbp), commencing with the first base of the *GH* cDNA. The numbering above the gene diagram refers to the codon position in the translated sequence. *, site of the insertion mutation in *gh*.
(B) The translated sequence of the *IM* homolog has a T insertion in *gh/gh* plants, resulting in a premature stop codon.

involving PDS, plastoquinol and oxygen as a final electron acceptor (Beyer et al., 1989; Mayer et al., 1990, 1992; Schulz et al., 1993; Nievelstein et al., 1995; Norris et al., 1995). Consistent with this interpretation, IM has quinol:oxygen oxidoreductase activity when expressed in *E. coli* (Josse et al., 2000).

IM and GH are orthologous genes

We and others (Wetzel et al., 1994; Bartley and Scolnik, 1995) reported that there are phenotypic similarities between *im* and the well-known *ghost* (*gh*) variegation mutant of tomato (Rick et al., 1959) (Figure 4). Like *im*, variegation arises in *gh* due to the action of a nuclear recessive gene (Rick et al., 1959); the white *gh* sectors accumulate phytoene (Rick et al., 1959; Mackinney et al., 1956; Scolnik et al., 1987); and white sector formation in *gh* is promoted by elevated light intensities (Rick et al., 1959; Scolnik et al., 1987).

The *im* and *gh* mutants appear to arise from mutations in orthologous genes (Josse et al., 2000; R. Bae, C. Wetzel, and S. Rodermel, unpublished observations). In our experiments, we isolated a cDNA from tomato with 67%

amino acid identity to *IM* (Genbank accession number AF302931), and found that this cDNA is from a single copy tomato gene. Mapping of the cDNA (the *IM* homolog) using a collection of F2 plants from an interspecific cross between *Lycopersicon esculantum* (L.) Mill. and *L. pennelli* (Correll) D'Arcy revealed that it maps to the *gh* locus. Figure 5 shows that transcripts from the *IM* homolog contain a T nucleotide insertion mutation in the *gh* background. It would be anticipated this would generate a premature stop codon 40 nucleotides downstream from the site of insertion. Taken together, these data suggest that GH and IM are orthologous proteins.

Proposed structural model of IM, GH and AOX proteins

Phylogenetic analyses revealed that *IM* is a distantly related member of the AOX class of inner mitochondrial membrane proteins (Wu et al. 1999) (Figure 6). The substrates of AOX are ubiquinol and dioxygen, and iron is essential for activity (Siedow and Umbach, 1995; Vanlerberghe and McIntosh, 1997). Structural models of AOX are based on the "RNR R2" class of di-iron carboxylate proteins (named

after the R2 subunit of ribonucleotide reductase) (Siedow et al., 1995; Moore et al., 1995; Andersson and Nordlund, 1999). The active sites of RNR R2-type proteins consist of
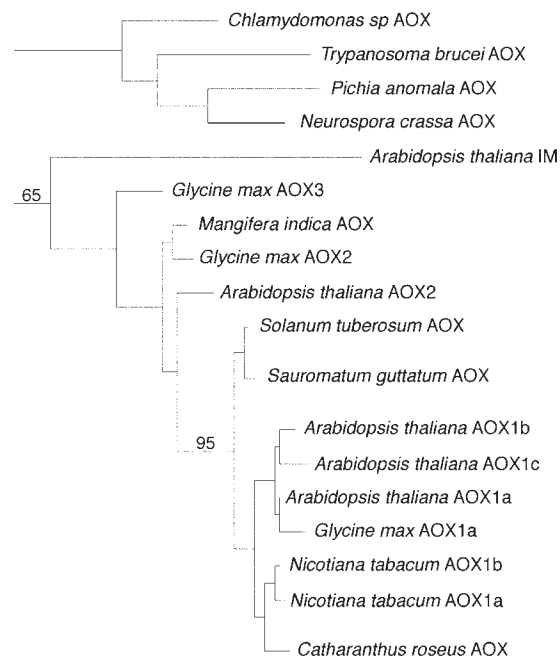

Figure 6. Phylogenetic relationships of AOX and IMMU-TANS amino acid sequences.
Relationships were determined by the neighbor-joining distance algorithm (described in Wu et al., 1999). Bootstrap values (100 replicates) are indicated above the branch nodes. The sources of the various AOX sequences are listed in Wu et al. (1999). For tree construction, amino acid sequences were limited to the conserved hydrophobic domains and the first two iron binding motifs (see Figure 7 sequence alignments). *IMMUTANS* and all plant *AOX* sequences are well-separated from the *AOX* genes of algae, fungi, and protists (in 65% of bootstrap replicates). This suggests that *IMMUTANS* shares a more recent evolutionary history with plant *AOX* genes. Most plant *AOX* sequences form a single, well-supported clade (95% bootstrap support) (the nine sequences at the bottom of the figure). Genes within this branch share approximately 75-95% amino acid identity. Although more distantly-related *AOX* genes are found in plants (namely, *Arabidopsis AOX*2, *Glycine AOX*2 and *AOX*3, and *Mangifera AOX*), the relationships of these genes to one other and to the other *AOX* genes is less clear. Even though *IMMUTANS* shares many conserved structural motifs with plant *AOX* genes, it is distantly related to all of them (as indicated by its long branch length). This suggests that IM is a novel type of AOX. (The figure is from Wu et al., 1999)

a binuclear iron center coordinated by two histidines and four carboxylate residues. An early model of AOX, proposed by Siedow and colleagues (Moore et al., 1995; Siedow et al., 1995), suggested that the protein contains two transmembrane domains, with the N- and C-termini exposed to the matrix side of the membrane (Figure 7A). Of three "EXXH" motifs in the C-terminal portion of the protein (B1, B2 and B3), B2 and B3 were proposed to form part of the di-iron center because only these two would reside on the same side of the membrane. It has proven difficult to test the Siedow model, and it enjoys little unequivocal experimental support (Andersson and Nordlund, 1999).

Taking advantage of a larger number of AOX sequences than were available when the Siedow model was proposed, Andersson and Nordlund (1999) proposed a revised structural model of AOX. They hypothesized that the hydrophobic regions of AOX are not transmembrane segments but rather, as with other RNR R2 proteins, that AOX is an interfacial membrane protein with an active site contained within a four helix bundle, with helices 1 and 3 (and helices 2 and 4) oriented anti-parallel to one another. In this model, the active site consists of a di-iron center coordinated by the B1 and B3 "EXXH" motifs on the paired second and fourth helices (Figure 7A), while the other two carboxylates are contributed by the paired first and third helices. It was proposed that these carboxylate residues are E183 on helix 1 and E274 on helix 3, based on the spacing between helices 1 and 2 (usually 30 amino acids) and between helices 3 and 4 (also, usually 30 amino acids), as found in other RNR R2 type proteins.

Because we found that IM and GH are only distantly related to AOX (Wu et al., 1999), we reasoned that phylogenetic comparisons of these sequences might offer an opportunity to test the validity of the Andersson and Nordlund model, i.e., AOX sequences that are evolutionarily conserved in GH and IM are likely important for structure and function. In Figure 7B, the sequences of 20 AOX proteins were compared with those of IM and GH in the C-terminal two-thirds of the protein. This is the most conserved region of the three proteins. Consistent with the Andersson and Nordlund model, GH (and IM) are predicted to contain four helices. Importantly, the B1 and B3 "EXXH" sites are precisely conserved between AOX, GH, and IM; the B2 site is not conserved. This suggests that these sequences provide four of the six expected Fe-ligands. The only conserved carboxylates in helices 1 and 3 are E147 and E238, respectively, suggesting that these residues serve as the other two Fe-ligands.

The strict conservation of the B1 and B3 sequences (on helices 2 and 4, respectively) indicates that if these sequences provide four of the six Fe-ligands, they must reside on the same side of the membrane to form a binuclear iron center. Considered together with the precise
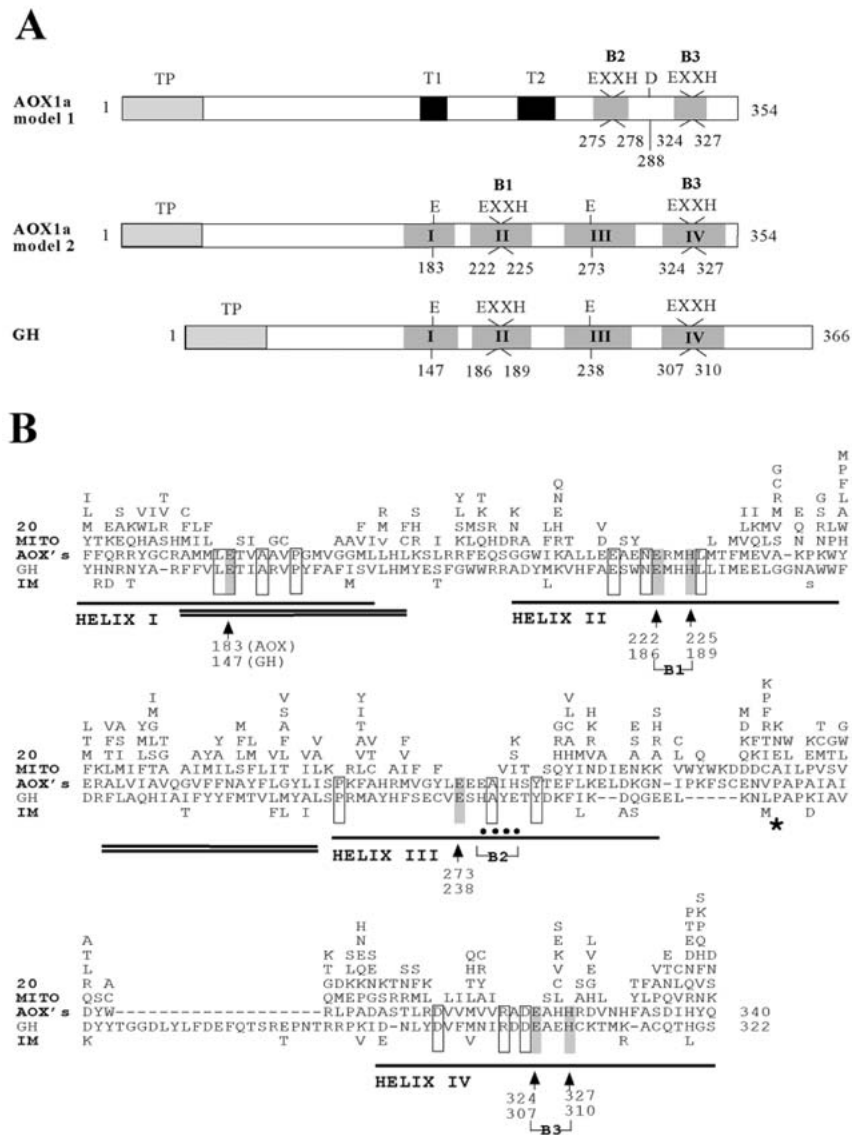
Figure 7. Structural and functional domains of AOX, GH and IM.
(A) The predicted structure of AOX according to the Siedow model (top) (Siedow et al., 1995; Moore et al., 1995) and to the Andersson and Nordlund (1999) model (middle). The structure of GH (bottom) is modeled after the Andersson and Nordlund model. Shaded boxes (I-IV) are alpha helical regions; T1 and T2 are putative transmembrane segments; "EXXH" (B1, B2 and B3), "D" and "E" are putative Fe-binding carboxylate ligands; TP are putative transit peptides. Size of the protein is from initiating ATG (codon 1) to the termination codon.
(B) Comparison of AOX, GH and IM derived amino acid sequences. Non-identical residues are shown. The sequences of GH and IM are compared downstream from codon 135 in the GH sequence. The sequences were compared with 20 AOX sequences from GenBank (*Arabidopsis thaliana* AOX1a*, Arabidopsis thaliana* AOX1b*, Arabidopsis thaliana* AOX1c*, Arabidopsis thaliana* AOX2*, Glycine max* AOX1*, Glycine max* AOX2*, Glycine max* AOX3*, Nicotiana tabacum* AOX1*, Nicotiana tabacum* AOX2*, Oryza sativa* AOX1a*, Oryza sativa* AOX1b*, Sauromatum guttatum* AOX1*, Catharanthus roseus* AOX*, Mangifera indica* AOX1*, Zea mays* AOX*, Chlamydomonas reinhardtii* AOX1*, Neurospora crassa* AOX*, Hansenula anomala* AOX*, Trypanosoma brucei brucei* AOX*, Chlamydomonas sp* AOX). Open and shaded boxes, identical amino acids among all three sequences; the six perfectly conserved Fe-ligands are indicated by the shaded boxes with the arrows underneath. Alpha helices are single-underlined; hydrophobic regions are double-underlined. *, site of the insertion mutation in *gh*. —, gaps in the alignment.
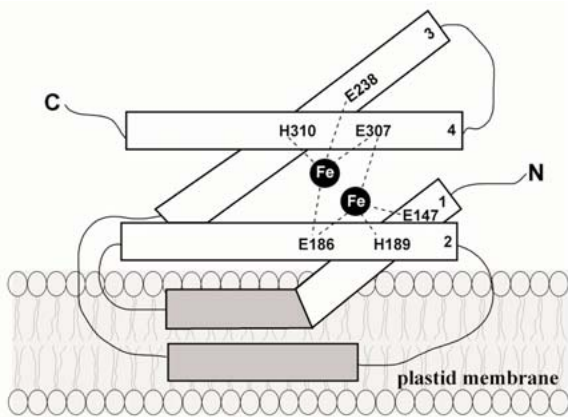
Figure 8. Structural model of GH.
GH is proposed to be an interfacial membrane protein with a di-iron center coordinated by two EXXH motifs on helices 2 and 4 (oriented anti-parallel to one another), and two carboxylates on helices 1 and 3 (also oriented anti-parallel to one another).

conservation of the E147 and E238 sequences (on helices 1 and 3, respectively), our data thus lend striking support to the Andersson and Nordlund hypothesis: the four helices in AOX are oriented anti-parallel to one another, as in other RNR R2 proteins, and AOX is an interfacial membrane protein, also as in other RNR R2 proteins. We propose that IM and GH are similar in structure to AOX, as proposed by Andersson and Nordlund (Figure 8). As with AOX, we hypothesize that the hydrophobic portions of GH and IM insert only partially through the lipid bilayer, providing a hydrophobic surface for protein/protein interactions within the bilayer, e.g., AOX is a dimer (Siedow et al., 1995). A similar structural model for IM has been proposed by Berthold et al. (2000).

Function of IM

As discussed above, phylogenetic analyses provide compelling evolutionary evidence for the validity of the Andersson and Nordlund model (1999) of the structure of AOX. Not only are the active site helices conserved between AOX, GH, IM, and RNR R2 type di-iron proteins, but evolutionary filtering allowed us to identify precisely the six carboxylates that likely act as coordinating Fe-ligands. The conservation of active site residues between

GH, IM and AOX argues strongly that GH is a quinol oxidase, and suggests that these proteins have similar reaction mechanisms. This confirms biochemical analyses showing that IM has quinol oxidase activity when expressed in *E.coli* (Josse et al., 2000). The question arises, how does IM function in the plastid (in which biochemical pathways?) and why do lesions in *IM* give rise to variegated plants? Patterns of *IM* expression have been examined as a first approach to address these questions.
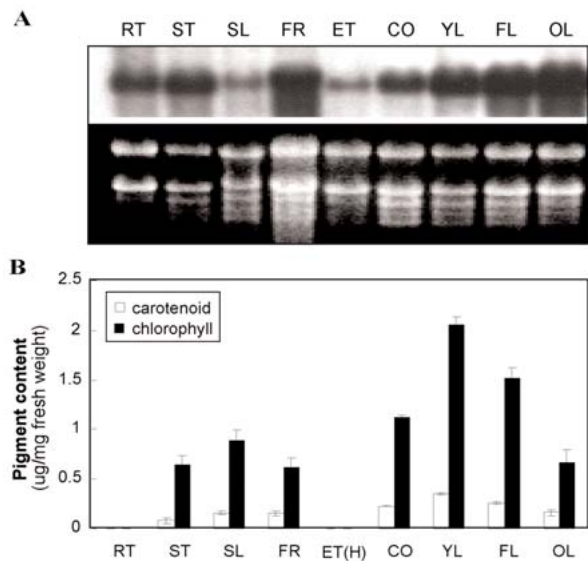

Figure 9. Expression analysis of *IM* mRNA and pigment levels.
(A) Equal amounts of total cell RNA were electrophoresed through formaldehyde gels and blotted onto a membrane filter (Aluru et al., 2001). The RNA gel was stained with ethidium bromide to show rRNA (loading control). The blot was probed with a radiolabeled *IM* cDNA (Wu et al., 1999).
(B) Total carotenoids and chlorophylls were extracted from *Arabidopsis* (Aluru et al., 2001). Values are an average of three separate experiments ± SD. The samples in A and B are from 4-5 wk old plants grown under normal light conditions (100 μmol·m^{-2}·s^{-1}); exceptions are samples from dark-grown seedlings (ET). RT, root; ST, stem; SL, green silique; FR, flowers (petals + green sepals); ET, 7-day-old etiolated seedling (cotyledon + hypocotyl); ET(C), cotyledons from 7-day old etiolated seedlings; CO, 7-day-old cotyledon; YL, young leaf (5 mm length); FL, just fully-expanded leaf (40 mm length); OL, senescing, late-fully expanded leaf. (This figure is from Aluru et al., 2001).

IM expression

Light shift experiments by Röbbelen (1968) and Wetzel et al. (1994) showed that the *IM* gene product is first active during a discrete phase of cotyledon development. This phase is coincident with seed coat breakage, and during this phase the phenotype of the cotyledons is irreversibly determined by the light intensity perceived by the growing seedling.

A powerful way to gain insight into IM function is to examine the phenotype of *im* plants. Three null *im* alleles have been reported (Wu et al., 1999), and thus the function of IM can be assessed in plants that completely lack IM activity. As discussed above, studies on *im* have focused on leaf variegation; in leaves, IM affects the functioning of the carotenoid biosynthetic pathway and appears to play a role in chloroplast biogenesis. Recent studies have shown that other organs of *Arabidopsis* (both green and non-green) are impaired in *im*, suggesting that IM is required for normal plant growth and development (Aluru et al., 2001). This impairment is due, in part, to a blockage of plastid differentiation in diverse cell types, including cells from cotyledons, roots and etiolated seedlings (Figure 3).

In support of the notion that IM plays a role in many types of plastids, RNA gel blot experiments (Figure 9) and analyses of transgenic plants with *IM* promoter:GUS reporter gene fusions have revealed that *IM* expression is ubiquitous in *Arabidopsis* tissues and organs (Aluru et al., 2001). Expression levels are generally high in tissues that accumulate carotenoids, such as leaves and cotyledons (Figure 9), consistent with the idea that IM is a redox component involved in carotenogenesis. However, IM is also expressed at appreciable levels in some tissues, such as roots, that accumulate only trace carotenoid amounts. This raises the possibility that IM is a general electron sink in plastid membranes. In agreement with this hypothesis, recent evidence in *Chlamydomonas* suggests that IM serves as a terminal oxidase in chlororespiration (Cournac et. al., 2000).

Leaf morphogenesis and plastid signals

Mesophyll cell morphogenesis is affected in both the green and white leaf sectors of *im* (Figure 10). The white sectors have a normal leaf thickness but the palisade cells fail to expand normally. As mentioned above, a considerable body of evidence supports the notion that the transcription of some nuclear genes, especially those for photosynthet-

ic proteins, is controlled by the developmental and/or metabolic state of the plastid (the "plastid signal" hypothesis) (reviewed by Oelmüller, 1989; Taylor, 1989; Mayfield, 1990; Susek and Chory, 1992; Gray et al., 1995; Hess et al., 1997; Rodermel, 2001). Consistent with this hypothesis, we have reported that plastids in the white sectors of *im* have reduced rates of *Lhcb* transcription and decreased *Lhcb* mRNA levels (Meehan et al., 1996).

In addition to the regulation of transcription of nuclear photosynthetic genes, analyses of a handful of nuclear gene-induced pigment mutants, including *im* (Aluru et al., 2001), have led to the hypothesis that plastid signals also control cell differentiation and thereby affect tissue and organ morphogenesis (reviewed by Rodermel, 2001). These mutants include *dag* of *Antirrhinum* (Chatterjee et al., 1996), *dcl* of tomato (Keddie et al., 1996), and several *Arabidopsis* mutants, including *cla1* (Mandel et al., 1996; Estévez et al., 2000), *cue1* (Li et al., 1995; Streatfield et al., 1999) and *pac* (Reiter et al., 1994; Meurer et al., 1998). Like *im*, the white leaf tissues of these mutants have abnormal plastids and cells, abnormal cell sizes and/or numbers, and altered palisade and/or spongy mesophyll
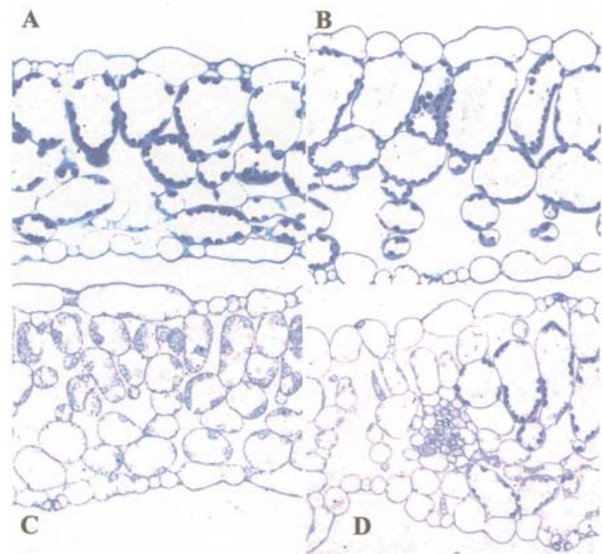

Figure 10. Anatomy of leaves of *immutans* and wild type plants.
Light microscopy was conducted on fully-expanded leaves from wild-type and *im* plants grown under normal light conditions. A magnification of 25X applies to all panels. The white sectors stain less intensely than green sectors because their plastids are deficient in internal structures. **(A)** wild-type. **(B)** green leaf sector of *im*. **(C)** white leaf sector of *im*. **(D)** adjacent green and white sectors of *im*. (This figure is from Aluru et al., 2001).

cell layer organizations. Because the products of the genes defined by these mutations reside in the plastid, it has been argued that these proteins are not required independently for plastid development, cell differentiation and leaf morphogenesis, but rather that the affects on cell differentiation and leaf morphogenesis are a consequence of incomplete chloroplast differentiation. The molecular details are not understood.

If cellular differentiation and development are affected by plastid-to-nucleus signaling, are plastid "developmental" signals the same as the plastid signals that regulate photosynthetic gene expression? Most of the mutants discussed above express *Lhcb* at markedly reduced levels in their white tissues. The one exception is *pac*, which has normal *Lhcb* mRNA accumulation (Reiter et al., 1994; Meurer et al., 1998). This suggests that plastid "developmental" signaling pathways can be separated from the plastid-to-nucleus signaling pathways involved in regulating nuclear photosynthetic gene expression.

Recent experiments in *immutans* have revealed that the green leaf sectors, as well as the white leaf sectors, have an aberrant anatomy (Aluru et al., 2001). In particular, the blades are thicker than normal due to a dramatic enhancement in mesophyll and epidermal cell sizes and to an increase in intercellular air space volume (Figure 10). Analyses of fluorescence-activated cell-sorter (FACS)-purified cells have demonstrated that cells from green *im* leaf sectors have more chlorophyll than similarly-sized cells from wild-type plants (Meehan et al., 1996). The green sectors also have significantly elevated photosynthetic rates (Aluru et al., 2001). These observations point towards a complex mechanism whereby the photosynthetic potential of the green sectors is enhanced to compensate for a lack of photosynthesis in the white sectors. It has yet to be tested whether part of this compensating mechanism involves feedback on cell differentiation by the metabolic state of the plastid.

We also found that the green *im* cells have significantly higher chlorophyll *a/b* ratios than wild-type cells under normal light conditions. High chlorophyll *a/b* ratios are typically found in "sun" versus "shade" plants and are indicative of smaller light harvesting complexes and/or an altered stoichiometry of PSI and PSII (reviewed by Stitt, 1991). These are typically adaptations to avoid light stress. Our working hypothesis is that a lack of IM gives rise to morphological and biochemical adaptations in the green sectors that make the leaf more "sun"-like, perhaps as a way to avoid photooxidative damage.

Mechanism of im variegation: the threshold model for

phytoene desaturation capacity

Taken together with the observation that *immutans* plants are heteroplastidic and variegated, not albino, the finding of null *immutans* alleles suggests that there is a redundant function able to compensate for the absence of IMMU-TANS activity in the green plastids. Computer searches and low stringency hybridizations have failed to detect *IMMUTANS*-related sequences in the *Arabidopsis* genome (Wu et al., 1999). Hence, the redundant or parallel redox component is unlikely to be another IMMUTANS-like AOX protein. Given the likelihood that IM acts as a quinol oxidase, one possibility is that the redundant function is a redox component downstream of the PQ pool.

According to our working model (Figure 11), different pathways of electron transport function in phytoene desaturation at different stages of development, and with different efficiencies, depending on which electron transport components are available (Wu et al., 1999). A fundamental assumption is that IMMUTANS is one of these components and is required for carotenoid synthesis during early chloroplast biogenesis, i.e., when thylakoid membranes are being elaborated in growing chloroplasts, following division of either progenitor proplastids in the meristem or mature chloroplasts in the expanding leaf (reviewed by Mullet, 1988). This is consistent with expression studies showing that IM is expressed ubiquitously in *Arabidopsis* tissues (Aluru et al., 2001). We hypothesize that PDS is unable, or only minimally able, to carry out phytoene desaturation during the early stages of thylakoid development when IMMUTANS is absent and the redundant or parallel pathway is not yet fully-functional. Phytoene would accumulate because of over-reduction of the PQ pool, causing a blockage in carotenoid synthesis. The plastids would thus be in a state vulnerable to high-light induced photooxidation by newly accumulating chlorophylls. In essence, a developmental race would occur between, on the one hand, photooxidation due to a lack of carotenoid photoprotection, and on the other hand, the development of an efficient mechanism of electron transport away from phytoene to accommodate PDS activity and the synthesis of enough carotenoids to afford photoprotection. Low light and thus lower photooxidative pressure would allow more plastids to survive the race through the vulnerable stage, accumulate chlorophylls and turn green. In the presence of a functional IMMUTANS, electron transport would not be inhibited during early development and carotenoid synthesis would proceed unhindered, thus avoiding photooxidative vulnerability.

In sum, our model in Figure 11 invokes a threshold of electron transport capacity for phytoene desaturation that is required for carotenoid synthesis and the develop-

ment of green chloroplasts (Wu et al., 1999). Below this threshold, carotenoids cannot be made in a sufficient enough quantity to prevent light-induced photodestruction. Consequently, the outcome of development in IMMUTANS-deficient plastids is either a white, photooxidized state or a fully-functional, green state. If the inner membrane structure has been destroyed along with the resident pigments then there will be no electron transport from phytoene and consequently no carotenoid synthesis (white plastids). If, on the other hand, enough electron transfer from phytoene can occur to support colored carotenoid accumulation, then wild type levels of pigments can accumulate, as observed in green *immutans* plastids and cells (Wetzel et al., 1994; Meehan et al., 1996). White plastids are capable of division because the requisite components are imported from the nucleus-cytoplasm (Tilney-Bassett, 1975). Therefore, the *immutans* mutation is effectively plastid autonomous, and each round of plastid division and differentiation carries the same risks of photooxidation.

Summary of immutans

In light of our current knowledge about *im*, several observations about the phenotype of *im* made by Rédei and co-workers in the 1960's and 1970's bear keeping in mind, particularly when considering the putative activity that compensates for a lack of IM in the green tissues of the mutant. As noted above, an acid RNase is overproduced in the white sectors of *im* (Rédei, 1967a). Rédei also found that two enzymes of the pyrimidine biosynthetic pathway are overproduced in the white *im* sectors, viz., orotidylic acid pyrophosphorylase (O5Ppase) and orotidylic acid decarboxlase (O5Pdase), but that growth on 6-azauracil, a pyrimidine analog that inhibits the activity of O5PDase, is able to partially normalize the *im* phenotype (Rédei, 1967b, Chung and Rédei, 1974; Chung et al., 1974). These and related observations prompted Rédei to suggest that IM is a primary regulator of RNA metabolism (Rédei, 1967a; 1975). Given the fact that *IM* codes for an AOX homolog, it would appear that the impact of IM on acid RNase activities and on pyrimidine biosynthesis are pleiotropic consequences of the lack of terminal oxidase activity in the plastid membrane. Yet, the partial phenotypic reversal by azapyrimidines is important and suggests that azapyrimidine-treatment might be a means of bypassing the primary lesion. This might provide a tool to understand compensating activities in the green *im* tissues.

Another intriguing finding by Rédei (1963) was that growth of *im* on cysteine promotes green sector formation

and enhanced pigment accumulation. Cysteine is a precursor of glutathione, which plays a major role in oxidative stress by acting as a donor of reducing equivalents for the scavenging of reactive oxygen species (May et al., 1998). Transgenic plants with elevated levels of cysteine and glutathione are resistant to oxidative stress (Foyer et al., 1995; Wellburn et al., 1998; Blaszczyk et al., 1999; Youssefian et al., 2001). Given the possibility that cells become photooxidized in the absence of IM, one hypothesis is that cysteine acts as an antioxidant buffer to counter photooxidative pressure in *immutans*.

As a third example, Rédei (1967a) reported that growth on kinetin promotes pigment production in *im*. However, the concentrations of the hormone that were used in these experiments were very high and the treated plants were stunted. Although the impact of the hormone on sector formation and plastid morphology were not reported, these findings might be related to the observation that
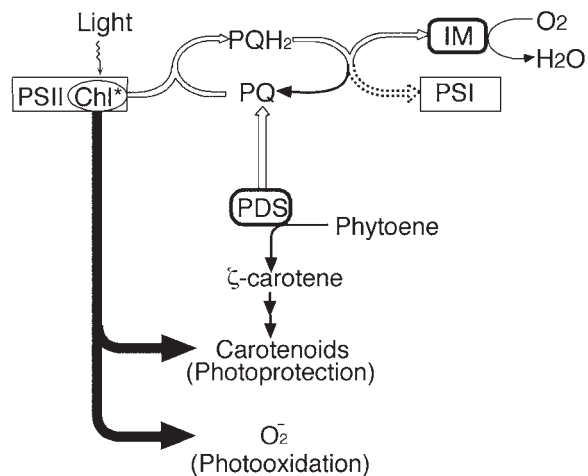

Figure 11. Working model of *immutans* variegation. Light is absorbed by the light harvesting complex of photosystem II (PSII), and energy from excited state chlorophyll can either be transferred to the reaction center or be used to form triplet chlorophyll. Unless quenched by colored carotenoids, energy from triplet chlorophyll generates oxygen radicals (O_2^-), which can lead to the photooxidation of the contents of the plastid if not detoxified by free radical scavengers (such as superoxide dismutase). The pathway of energy flow from triplet chlorophyll is indicated by shaded arrows, and the pathway of electron transport from PSII via the plastoquinone pool (PQ) to photosystem I (PSI) is shown as an open arrow. Electrons can also enter the PQ pool by the PDS-mediated desaturation of phytoene. In the model, PDS transfers electrons from phytoene to the PQ pool and IMMUTANS acts after this step to reduce molecular oxygen. In the absence of IMMUTANS, electron flow from the PQ pool to PSI serves as the redundant function to generate green plastids. (This figure is from Wu et al., 1999).

cytokinin is able to phenocopy some aspects of the *det* phenotype, viz., the development of chloroplasts in dark-grown plants (Chory et al., 1994). Cytokinin is also able to induce greening in *pac2* (Grevelding et al., 1996) and *atd2* (van der Graaff, 1997), two nuclear gene-induced variegations (see Table 1). Therefore, further insight into how cytokinins control plastid biogenesis might lend insight into *im* compensating activities.

A final observation worth noting is that heavy doses of X irradiation result in an increase in the number and size of *im* green sectors (Rédei, 1967c). The reason for this is unknown.

var2

Sectoring in the *var2* variegation mutant of *Arabidopsis* is due to the action of a nuclear recessive gene (Martínez-Zapater, 1993; Chen et al., 1999). The mutant has normal-appearing cotyledons, but the first true leaves are bright yellow (Figure 12). As leaf expansion proceeds, green islands become visible and expand in size, and the yellow sectors fade to white. Sector boundaries and identity become fixed at full expansion. Whereas cells in the green leaf sectors and cotyledons of *var2* contain morphologically normal chloroplasts, cells in the yellow and white sectors are heteroplastidic and contain vacuolated plastids with few, if any, lamellae (Figure 13), as well as some normal-appearing chloroplasts (Chen et al., 1999). Similar to *immutans*, the presence of heteroplastidic cells indicates that plastids in the mutant are affected differently by the nuclear mutation, i.e., that the phenotype of *var2* is plastid autonomous. Also similar to *immutans*, defective plastids are not maternally inherited in *var2*, suggesting that the plastid defect can be "cured" during or after reproduction (Martínez-Zapater, 1993; Chen et al., 1999).

Early studies showed that carotenoid and chlorophyll precursors do not accumulate in *var2*, indicating that the primary lesion in the mutant does not involve a blockage in pigment biosynthesis (Chen et al., 1999). We recently cloned *VAR2* by map based methods and found that it codes for a 74 kDa plastid protein with high amino acid sequence similarity to the AAA protein class of Walker A/GTPases (Chen et al., 2000). AAA proteins (ATPases associated with diverse cellular activities) have one or more "AAA cassette" domains (~200-250 amino acids) (Kunau et al., 1993; Beyer, 1997; Neuwald et al., 1999). These domains contain an ATP binding site, which is composed of Walker boxes A and B, and other conserved sequences whose functions are not understood. VAR2 shows high similarity to the "metal-dependent protease"

subfamily of AAA proteins (Chen et al., 2000). The members of this family are ubiquitous among prokaryotes and eukaryotes and appear to be derived from an ancestral prokaryotic *FtsH* gene (Beyer, 1997). *FtsH*-like genes have a single AAA cassette, two transmembrane helices in their N-terminus, and a zinc binding site in their C-terminus (Beyer, 1997) (Figure 14).

FtsH has been most thoroughly studied in *E. coli*, where it is involved in a diversity of processes, including the degradation of misfolded and excess proteins (protein quality control), the export of proteins from the cell, the integration of membrane proteins, mRNA decay, and resistance to colicins (Akiyama et al., 1994; Gottesman et al., 1997; Granger et al., 1998). The genes for several FtsH homologs have also been cloned in yeast (e.g., *YME1*), and these proteins are found in mitochondrial inner membranes where they associate into multimeric complexes that degrade unassembled subunits of inner membrane components (reviewed by Suzuki et al., 1997). It has been proposed that FtsH proteins are versatile because they have an intrinsic chaperone activity in addition to their protease activity (e.g., Akiyama et al., 1994; Arlt et al., 1996; Shirai et al., 1996; Gottesman et al., 1997; Akiyama et al., 1998; Leonhard et al., 1999).

Among photosynthetic organisms, four *FtsH* homologs have been identified in the completely sequenced genome of the cyanobacterium *Synechocystis* 6803 (Kaneko et al., 1996), and a small number of *FtsH*-like sequences have been identified in eukaryotic algae and higher plants (Beyer, 1997; Chen et al., 2000; Adam et al. 2001). In higher plants these genes are found in the nuclear genome, but in brown and red algae they are present in the plastid genome. Consistent with the endosymiotic origin of plastids from a single cyanobacterium (Doolittle, 1998), this suggests that progenitor *FtsH*-like sequences were transferred from the genome of the symbiont to that of the host in the lineage of photosynthetic organisms that gave rise to higher plants, but not in the lineage that gave rise to the red and brown algae (Chen et al., 2000).

Analyses of the complete *Arabidopsis* genome sequence have revealed that *FtsH*-like genes comprise a nuclear multigene family of at least 11 members (Adam et al., 2001) (Table 2). These genes have limited sequence similarity, except in the AAA cassette portion of the protein. In fact, sequences downstream from the zinc binding site (i.e., the C-terminal one-third of the protein) cannot generally be aligned for most of the family members. The lack of sequence conservation in this region is consistent with the notion that the C-terminus confers unique biochemical activities on the various family members. Seven of the *Arabidopsis FtSH* gene family members are predicted to have chloroplast targeting sequences and two are predicted to have mitochondrial targeting sequences. Organellar targeting sequences cannot be discerned for

the other two family members (Table 2). The diversity of *FtsH* sequences in *Arabidopsis* suggests that these proteins function in multiple compartments and have varied activities.

Of the *Arabidopsis* FtsH proteins, only VAR2 (also designated FtsH2 in Table 2) and FtsH1 have been characterized to any extent (Lindahl et al., 1996, 2000; Chen et al., 2000). Both proteins appear to be localized on stromally-exposed regions of the thylakoid membrane (Figure 14),

Figure 12. The *var2* mutant of *Arabidopsis*.
The cotyledons are green and the first true leaves are yellow. The *yellow variegated* allele is shown (see Table 3).

and the expression of both genes is up-regulated during the greening of etiolated seedlings (Lindahl et al., 1996; Chen et al., 2000). These findings suggest that VAR2 and FtsH1 play a role in membrane modeling events during thylakoid biogenesis. *In vitro* studies have shown that FtsH1 catalyzes the proteolytic degradation of photooxidatively-damaged D1 proteins (the reaction center protein of PSII) (Lindahl et al., 2000). A proteolytic activity stimulated by zinc has also been implicated in the degradation of unassembled Rieske FeS protein (RISP) in the thylakoid membrane (Ostersetzer and Adam, 1997); however, it is not known whether this activity is caused by VAR2, FtsH1, another FtsH homolog, or a different type of metalloprotease. Although VAR2 substrate(s) have not yet been defined, it is possible that they differ from those of FtsH1 because sequences downstream from the zinc binding sites of these two proteins cannot be aligned. VAR2, at least, does not appear to be a general plastid membrane biogenesis factor inasmuch as etioplasts have a normal morphology in *var2* plants (Chen et al., 2000).

Of the FtsH-like proteins described to date, VAR2 most closely resembles the red pepper Pftf protein (Hugueney et al., 1995; Chen et al., 2000). The sequences of VAR2 and Pftf can be aligned over their entirety, and the fact that both proteins share a high degree of amino acid identity (nearly 79%) suggests that they might have similar activities and functions. Pftf was isolated as a soluble factor that promotes membrane fusion and/or translocation events in an *in vitro* vesicle fusion assay using chromoplast membrane vesicles from red pepper fruit (Hugueney et al., 1995). Because Pftf was isolated as a soluble factor, it was thought to reside in the stroma. However, Summer and
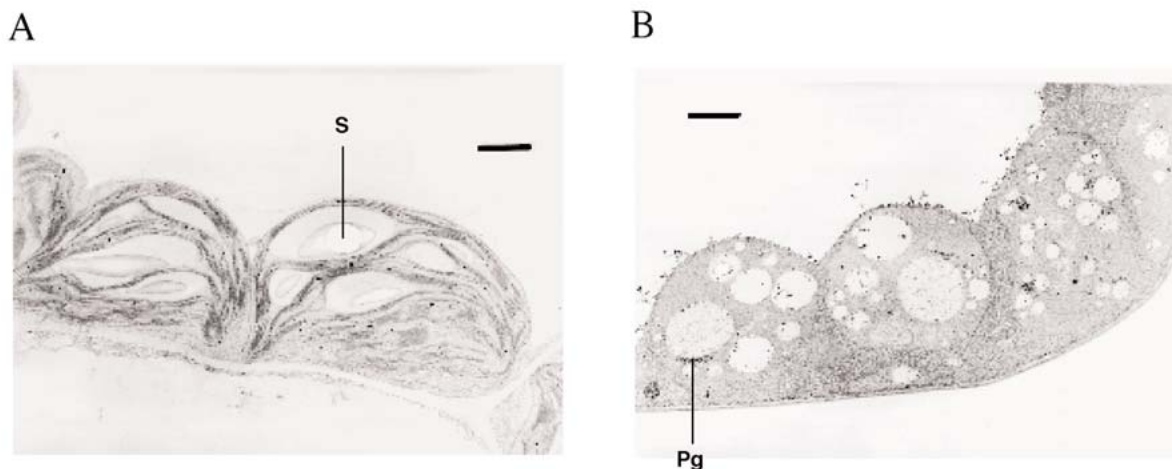

Figure 13. Ultrastructure of *var2* plastids.
Electron micrograph of representative plastids from **A**) a *var2* green sector and **B**) a *var2* yellow sector (Bar = 1 μm). Pg = plastoglobulae. S =starch. (This figure is from Chen et al., 1999).
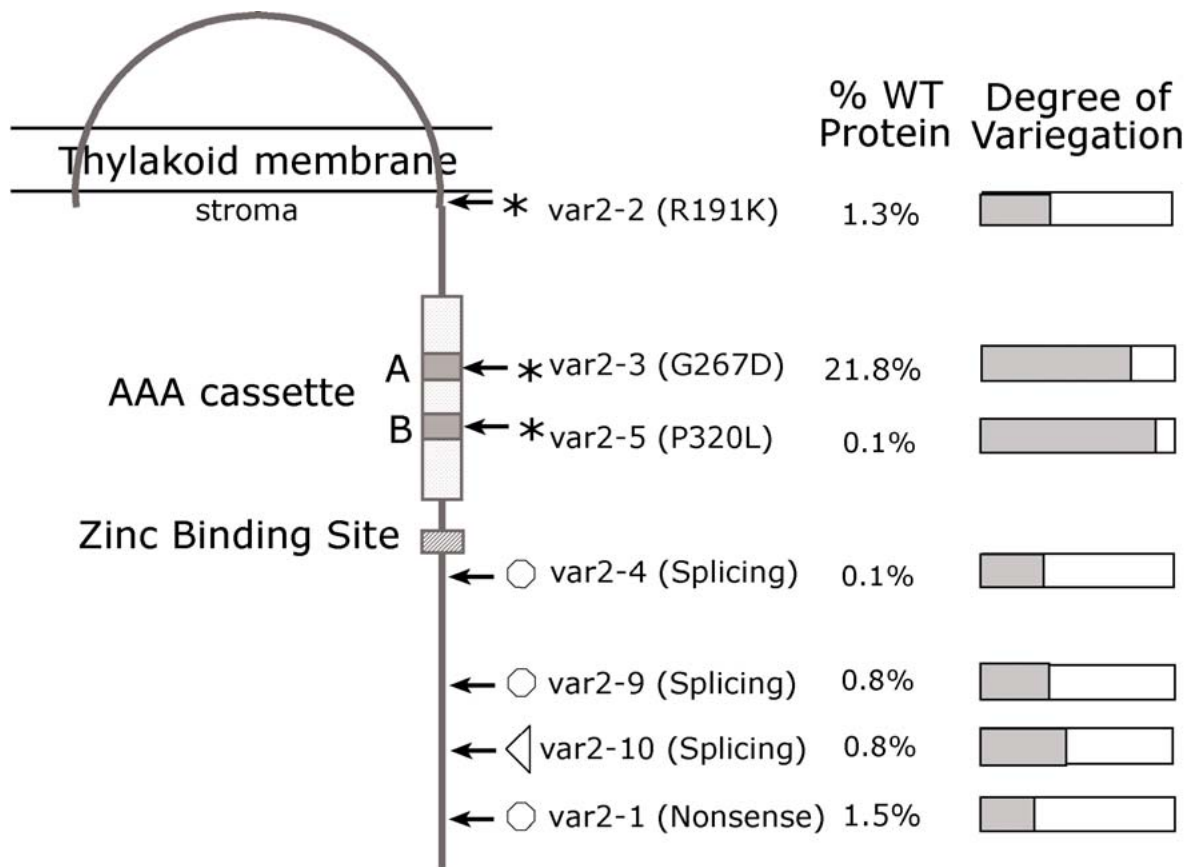
Figure 14. Topology of VAR2 in the thylakoid membrane.
Shown are the two transmembrane domains, the AAA cassette (with Walker boxes "A" and "B"), and the zinc binding site. Sites of mutations in the various *var2* alleles are illustrated (see also Table 3). Protein and pigment amounts have been reported for most of the alleles (Chen et al., 1999, 2000). Pigment contents provide a measure of the degree of variegation; hatched boxes represent the amount of pigment relative to wild type (100% green) determined on a fresh weight basis using fully-expanded first true leaves.

Cline (1999) have provided compelling evidence that Pftf is a membrane protein. Given the high sequence similarity between Pftf and VAR2, the finding that VAR2 is also a membrane protein (Chen et al., 2000) lends support to the conclusions of Summer and Cline (1999).

var2 allelic series

We initially became interested in the *yellow variegated*

mutant of *Arabidopsis* because we thought it might be an allele of *immutans* (G.P. Redéi, personal communication). However, complementation analyses revealed that it is allelic to *var2*, not *immutans* (Chen et al., 1999). *var2* was first characterized by Martínez-Zapater (1993), who reported two alleles of the locus (designated *var2-1* and *var2-2*) (Table 3). We have identified five additional *var2* alleles from the collection of variegation mutants in the Ohio State and Nottingham stock centers. Complementation tests were performed on candidate lines (i.e., ones with a phenotype similar to *var2*), and those showing allelism to *var2* were sequenced. These alleles are designated *var2-3* through *var2-5, var2-9* and *var2-10* (Chen et al., 1999,
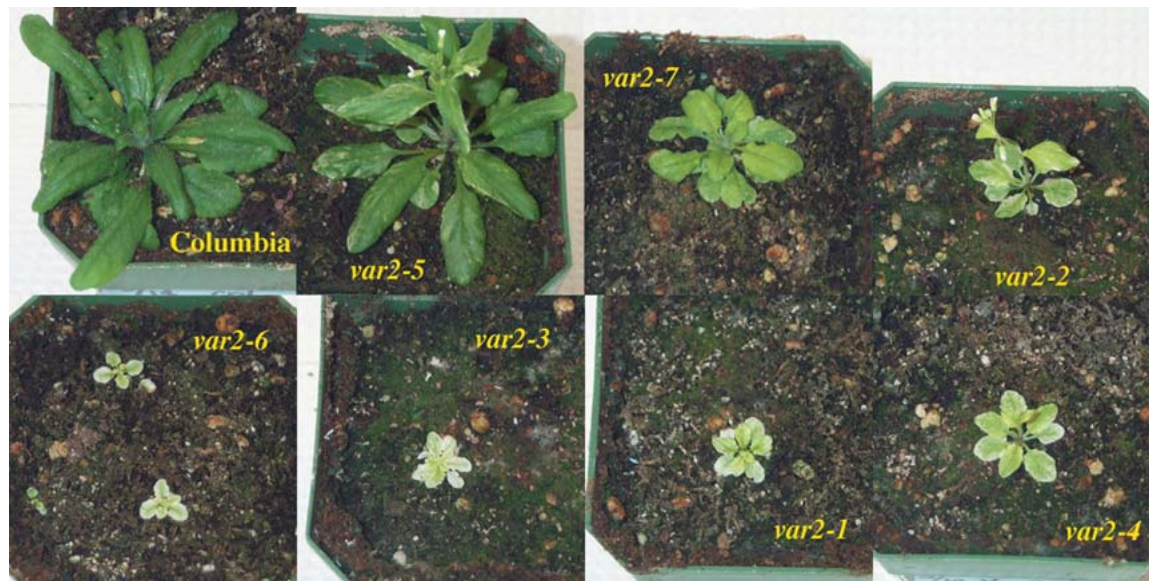
Figure 15. Alleles of *var2*.
The plants were germinated at the same time and maintained under identical conditions (100 μmol·m^{-2}·s^{-1}, continuous illumination; 22°C).

2000; A. Manuell and S. Rodermel, unpublished findings) (Table 3). Takechi et al. (2000) have isolated an additional three *var2* alleles: *var2-6*, a T-DNA insertion allele; *var2-7*, an uncharacterized line from the Ohio State stock center; and *var2-8*, an EMS-generated allele. As mentioned earlier, not all the lines in the stock centers define unique alleles. For instance, six lines that were identified as alleles of *var2* in complementation tests were found, upon sequencing, to be identical to other *var2* alleles (A. Manuell and S. Rodermel, unpublished findings) (Table 3).

We have generated a *var2* allelic series on the basis of differences in the extent of white sector formation in first leaves (Chen et al., 1999, 2000). The differences between the alleles are visually striking (Figure 15). These differences have been quantitated by determining chlorophyll concentrations in first fully-expanded leaves (Figure 14): *var2-1* is the most severe allele (leaves are predominantly white), whereas *var2-5* is the least severe allele (leaves are nearly all-green with little white-sectoring).

The lesions in nine of the ten *var2* alleles have been determined (see Figure 14 and Table 3). Two of the mutations are in the Walker ATP binding sites A and B (*var2-3* and *var2-5*, respectively), providing *in vivo* evidence that these sequences are important for VAR2 activity (Chen et al., 2000). *var2-1* has a nonsense mutation (Q597*) and is predicted to produce a truncated protein with nearly 100 fewer amino acids at the C-terminus, whereas *var2-2* (R191K) has a conservative amino acid substitution imme-

diately adjacent to the predicted end of the second transmembrane domain (Chen et al., 2000). It is not obvious how the latter mutation affects function and/or protein stability, but one possibility is that it influences integration of the protein into the membrane. *var2-6* has a T-DNA insertion in the extreme C-terminus of the coding region, and *var2-7* has a base pair deletion that causes a frameshift and the putative generation of a truncated protein (Takechi et al., 2000). No mutation has yet been found in the *var2-8* allele (Takechi et al., 2000). The mutations in the remaining three alleles (*var2-4*, *var2-9* and *var2-10*) are in intron splice sites (A. Manuell, V. Brendel and S. Rodermel, manuscript in preparation). Figure 14 shows that of the seven alleles examined, all have decreases in the abundance of the mutant VAR2 protein. Whereas this is consistent with the idea that the mutant proteins are unstable, there is evidence that decreases in protein abundance in some of the alleles might be due, in part, to decreases in *var2* mRNA abundance (Chen et al., 2000; Takechi et al., 2000).

Mechanism of var2 variegation

One of the most intriguing aspects of *var2* concerns the mechanism of variegation. Most *var2* alleles have appre-

ciable reductions in mutant VAR2 protein abundance in their green sectors, but the protein cannot be detected in the white sectors (Chen et al., 2000). Therefore, it's possible that green tissue formation in *var2* requires only limited VAR2 activity, whereas a lack of VAR2 accumulation in the white sectors might be a secondary consequence of the mutation. For example, VAR2 might be turned over if it can't be assembled properly in the membrane. Alternatively, it might be degraded if it becomes photodamaged, e.g., as a consequence of photooxidation if photoprotective carotenoids fail to accumulate in the mutant white sectors. Consistent with the latter possibility, white sector formation in *var2* is promoted by increased light intensity, although the effects are not as pronounced as with *immutans* (Martínez-Zapater, 1993; Chen et al., 1999; Takechi et al., 2000). Another possibility to explain the formation of normal-appearing green sectors in *var2* plants is that there is an activity able to compensate for a lack of VAR2, at least in some cells of the mutant. It is also possible that events catalyzed by VAR2 can occur at a low rate even in its absence or near-absence.

In addition to light, sector formation in *var2* is sensitive to temperature (Martínez-Zapater, 1993; Chen et al., 1999). We have also found that factors that promote green sector formation (i.e., increased temperature and decreased light) also depress the growth rate of the plant (Chen et al., 1999). Therefore, similar to *immutans*, we suggest that the development of a green chloroplast requires the attainment of a threshold VAR2 activity (or a VAR2 redundant activity), and that once attained, the "green" state is able to propagate itself. Accordingly, factors that promote slow growth might allow more time for threshold VAR2 activities to accumulate in developing *var2* plastids. Plastids with below-threshold activities, on the other hand, might remain white due to a blockage in chloroplast biogenesis and/or to photooxidation.

chloroplast mutator (chm)

The *chloroplast mutator* (*chm*) mutant was first isolated by Rédei (1973) following EMS mutagenesis of *Arabidopsis* seeds. He reported that recessive mutations at the *CHM* locus induce the generation of white and yellow sectors in normally-green organs of the plant. Mesophyll cell differentiation and leaf morphology are also affected in *chm*, leading to a "rough-leaf" appearance. Rédei and Plurad (1973) found that long term maintenance of lines homozygous for *chm* results in the accumulation of plastids with different morphologies and the production of heteroplastidic (mixed) cells, and they suggested that the different

plastid types are blocked at various steps of chloroplast biogenesis. Rédei (1973) showed that defective plastids are maternally inherited in *chm*, and he proposed that mutations in *CHM* cause the induction of mutations at various sites in the plastid genome, generating permanently-defective organelles. Stable *chm*-free homoplastidic lines, i.e., lines with a single type of mutant plastid, have been derived from backcrosses of the mutant and wild type (Rédei, 1973; Rédei and Plurad,1973; Mourad and White, 1992). These lines arise by the sorting out of defective plastids in *chm*-free progeny plants.

Rédei (1973) isolated two EMS-induced *chm* alleles (*chm1 and chm2*). Martínez-Zapater et al. (1992) isolated a third *chm* allele (*chm3*) from the progeny of T-DNA-tagged *Arabidopsis* regenerated from tissue culture; however, the T-DNA did not segregate with the variegation phenotype. Molecular studies showed that the mitochondrial genomes of *chm3* are rearranged, and that these rearrangements cosegregate in a maternal fashion with the variegation trait (Martínez-Zapater et al., 1992). Gross chloroplast DNA polymorphisms could not be detected in these studies. The mitochondrial genomes of most higher plants are polyploid and have a multipartite organization, with subgenomic molecules arising by intramolecular recombination (reviewed by Goldschmidt-Clermont, 1998). Mitochondrial DNAs with an abnormal organization (termed "sublimons") are also maintained at very low levels in plant mitochondria (reviewed by Leon et al., 1998). The stoichiometries of the various mitochondrial DNAs appear to be under nuclear control. One of the first demonstrations of this was the finding that cytoplasmic male sterility in common bean is associated with a mutation (*pvs-orf239*) on a subgenomic mitochondrial DNA molecule (Janska and Mackenzie, 1978). The dominant nuclear restorer gene, *Fr*, restores pollen fertility by reducing the content of this molecular species (reviewed by Leon et al., 1998).

Martínez–Zapater et al. (1992) suggested that *CHM* acts in a manner similar to *Fr* and prevents the amplification of mutant subgenomic mitochondrial DNA molecules that cause variegation. Support for this proposal has come from the observation that mutant mitochondrial DNAs are abundant in *chm* plants, and that these DNAs are also present in wild type plants, but at extremely low levels (Sakamoto et al., 1996). These conclusions were based on experiments with *maternal distorted leaf* (*MDL*), a mutant derived from a cross between *chm1* and wild type plants; these plants lack the *chm* allele (Sakamoto et al., 1996). *MDL* plants are not variegated, but they grow slowly and have rough (distorted) leaves and aborted floral organs. The plants are homoplastidic for normal-appearing plastids, but the mitochondria have an abnormal ultrastructure. Mitochondrial DNAs in *MDL* are rearranged in regions of the chromosome coding for ribosomal proteins (*rps3* and

rpl16), and expression from these genes is reduced in the mutant. The rearranged DNAs, which are abundant in the mutant, are also present in very low amounts in the wild type, as assayed by quantitative PCR. Consistent with the suggestion of Martínez–Zapater et al. (1992), these results led Sakamoto et al (1996) to propose that the function of *CHM* is either to suppress the amplification of mutant mitochondrial DNAs or to maintain a high level of master mitochondrial genomes that contain the complete complement of genetic information. The isolation of *CHM* has yet to be reported.

If *chm* causes the production of permanently-defective mitochondria, why are some *chm* plants variegated? The reason for this appears to be that the defective mitochondria secondarily affect chloroplast function. This might not be surprising in light of the fact that mitochondria and chloroplasts exchange a variety of metabolites (Raghavendra et al., 1994). Support for such exchange comes from the *non chromosomal stripe* (*NCS*) mutants of maize (Newton and Coe, 1986). These mutants have pale-green or yellow leaf stripes, reduced growth, and sectors of aborted kernels on the ears. The morphology and function of both mitochondria and chloroplasts are defective in the pale-green and yellow sectors of the *NCS* mutants (Roussell et al., 1991; Gu et al., 1993). Chloroplast DNA mutations have not been detected in these mutants, but lesions in the mitochondrial DNA have been observed, e.g., deletions in the cytochrome oxidase subunit 2 gene (*Cox2*) in *NCS5* and *NCS6* (Lauer et al., 1990; Newton et al., 1990; Gu et al., 1993), a deletion in genes encoding ribosomal proteins in *NCS3* (Hunt and Newton, 1991), and a chimeric *nad4-nad7* gene in *NCS2* (Marienfeld and Newton, 1994). The mutant and normal forms of these genes are present in the green sectors of *NCS* mutants, but the pale-green or yellow sectors are homoplasmic, or nearly so, for the mutant DNAs (Gu et al., 1993). *NCS* mutants are viable because of mitochondrial DNA heteroplasmy in the green sectors.

Although both mitochondria and chloroplasts are defective in *NCS* plants, the variegation phenotype cosegregates with the mitochondrial DNA defect in a maternal fashion. This suggests that the primary mutation in the mutants is the mitochondrion, and that the defective mitochondria secondarily affect chloroplast form and function, generating cells with pigment-deficient plastids. It is proposed that sectors arise during development because of somatic segregation of the mutant and normal mitochondria. Similar explanations may hold for sectoring in *chm* plants.

cue1

The *cue* (*C*AB *u*nder*e*xpression) mutants of *Arabidopsis* were generated by mutagenesis of transgenic *Arabidopsis* bearing an *Lhcb* promoter/ GUS reporter gene fusion (Li et al., 1995; López-Juez et al., 1998). Plants were selected with lower than normal levels of GUS activity, suggesting that they were also impaired in *Lhcb* transcription. Consistent with this idea, RNA gel blot assays revealed that the *cue* mutants underexpress *Lhcb* mRNAs, as well as transcripts from other nuclear genes for plastid proteins.

The *cue* mutants define seven complementation groups and fall into several phenotypic classes; only *CUE1* has been cloned (Streatfield et al., 1999). Some *cue* mutants are allelic to known phytochrome-deficient mutants (*hy*1 and *phy*B); some are virescent (i.e., young leaves are pale, mature leaves are green), some are uniformly pale, and one (*cue1*) has pale-green mesophyll cells and dark-green bundle sheath cells that align the veins (a reticulate phenotype). The paraveinal regions of *cue1* have normal bundle sheath cells, normal plastids and normal *Lhcb* mRNA expression, whereas the interveinal regions contain reduced numbers of palisade mesophyll cells, smaller than normal chloroplasts and reduced *Lhcb* expression (Li et al., 1995). The reductions in palisade cell number and chloroplast size explain why the interveinal regions of *cue1* are pale-green.

Streatfield et al. (1999) cloned *CUE1* and found that it codes for the plastid phosphoenolpyruvate/ phosphate translocator (PPT). PPT is localized on the inner envelope and imports PEP in exchange for inorganic phosphate. PEP is the first substrate of the shikimate pathway, which produces aromatic amino acids and a variety of secondary metabolites, including UV light protectants, photosynthetic pigments, quinones and other phenolic redox compounds. These components are reduced in concentration in the pale-green sectors of *cue1*, and the mutants have reduced photosynthetic efficiencies. The fact that the mutant has normal-appearing bundle sheath cells and plastids, but abnormal mesophyll cells and plastids, indicates that there is a mesophyll cell-specific requirement for PPT in *Arabidopsis* leaves. Because PPT appears to be a member of a multigene family, one possibility is that expression of *CUE1* is mesophyll cell-specific, while another PPT gene is bundle sheath cell-specific. Another possibility is that PPT is not required in bundle sheath cells. These hypotheses have yet to be tested.

It is interesting that reductions in *Lhcb* transcription in the mesophyll cells of *cue1* are accompanied by decreases in plastid size and in chlorophyll content per cell, i.e., the mesophyll cells have the same number of chloroplasts

but a reduced chloroplast plan area (Streatfield et al., 1999). Therefore, *cue1* cells might have less *Lhcb* transcription because their nuclei perceive a lower than normal chloroplast plan area, perhaps via a reduction in plastid-to-nucleus signaling. An alternative hypothesis is provided by the finding that the reticulate phenotype of *cue1* is light-sensitive, with growth in high light promoting the formation of white (versus pale-green) sectors. One reason for this sensitivity might be that *cue*1 plastids lack sufficient carotenoids to afford protection against chlorophyll-sensitized photooxidation (reviewed by Oelmüller, 1989). *Lhcb* expression in *cue1* is also sensitive to light, with white sectors having less transcription than pale-green sectors. One hypothesis to explain these findings comes from the observation that *cue1* plastids have decreased quinone concentrations and an altered redox state of the quinone pool (Streatfield te al., 1999). It has been demonstrated in several systems that the redox state of the plastoquinone pool serves as a plastid signal to regulate the transcription of nuclear photosynthetic genes (reviewed by Huner et al., 1998; Pfannnschmidt et al., 2001; Rodermel, 2001). This might also be the case for *cue1*.

One final comment about plastid signaling and *cue1* comes from the finding that palisade cells are reduced in number in the mutant. Like other mutants perturbed in leaf morphogenesis (discussed above), this suggests that the *CUE1* gene product (PPT) is required for normal palisade cell differentiation, and consequently for the normal mor-phogenesis of the mesophyll cell layer (Streatfield et al., 1999). In some manner, the lack of PPT disrupts one or more plastid-to-nucleus signaling pathways that regulate palisade cell division.

dov, re

In addition to *cue1*, other reticulate mutants have been reported in *Arabidopsis*, and several of these have been partially characterized. These include *reticulata* (*re*) (Rédei and Hirono, 1964) and *dov1* (differential development of vascular associated cells) (Kinsman and Pyke, 1998). Both of these mutants have green vasculature on a pale-green lamina (similar to the phenotype of *cue*1). *dov1* was selected for study from the large collection of reticulate mutants (over 200) in the *Arabidopsis* stock centers (Kinsman and Pyke, 1998). It is a nuclear recessive gene and is not allelic to *re* or *cue1*. *dov1* has normal bundle sheath cells and chloroplasts. Mesophyll cell sizes and numbers are also normal, but the plastids are reduced in size and number. This provides an explanation for the pale-green phenotype of the interveinal regions of *dov1* leaves. Chloroplast morphology is also disrupted in *dov1*: the mutant plastids are vacuolated and lack grana. *re* and *dov1* have not been cloned.

Table 4. Genes cited in this review and their GenBank accession numbers.

Gene	Accession Number	Reference
AOX (*Arabidopsis*)	D89875, AB003175, AB003176	Saisho et al., 1997
ATD2	AB006210	van der Graaf, 1997
CLA1	U27099	Mandel et al., 1996
COX2	NC_001284	Lauer et al., 1990; Newton et al., 1990
CUE1	U66321	Streatfield et al., 1999
DET2	U53860	Chory et al., 1994
FtsH1 to *FtsH11* (*Arabidopsis*)	(see Table 2)	Adam et al., 2001
FtsH (*E. coli*)	P28691	Beyer, 1997
GHOST	AF302932	Josse et al., 2000; R. Bae and S. Rodermel, unpublished
HY1	AB021858	Neff et al., 2000
IMMUTANS	AF098072	Wu et al., 1999; Carol et al., 1999
IOJAP	Z15063	Han et al., 1992
LHCB (*CAB3*)	X15222	Meehan et al., 1996
NAD4	NC_001284	Marienfeld and Newton, 1994
NAD7	NC_001284	Marienfeld and Newton, 1994
PAC	X96480, X96481, X96482	Reiter et al., 1994; Grevelding et al., 1996
Pftf	CAA09935	Hugueney et al., 1995
PHYB	X1732	Neff et al., 2000
VAR2	AF135189	Chen et al., 2000
YME1	P32795	Beyer, 1997

pale cress

Two T-DNA-tagged alleles of the *pale cress* (*pac*) mutant have been isolated (Reiter et al., 1994; Grevelding et al., 1996). One allele is pale-green (*pac1*) and the other has green/white variegated leaves (*pac2*). Plastids in the pale-green sectors of *pac1* appear to be blocked early in chloroplast biogenesis. They contain rudimentary lamellae and low levels of chlorophylls and carotenoids. Leaf morphology is also markedly altered in *pac1*. Early leaf development is normal, but in later stages the palisade cells fail to elongate and the air spaces increase in size. As with other mutants discussed above (e.g., *im*, *dcl*, *dag*), this suggests that *pac* is disrupted in the transmission of a plastid signal that regulates palisade cell differentiation. Ultrastructural and anatomical studies of *pac2* have not been reported.

The *PAC* locus has been cloned in both *pac1* (Reiter et al., 1994) and *pac2* (Grevelding et al., 1996). In *pac1*, at least three unique cDNAs correspond to the locus (*PAC1, 2* and *3*) (Reiter et al., 1994). The three are products of alternative splicing and have the capacity to encode different proteins. By contrast, a single cDNA is found in the *pac2* allele (Grevelding et al., 1996). This cDNA corresponds to the most abundant cDNA (*PAC3*) in *pac1*, and codes for a 36 kD protein of unknown function. It is not understood why *PAC* expression patterns differ in *pac1* and *pac2*, but it might be related to differences in ecotype (*pac1* is in the Wassilewskija background, and *pac2* is in Columbia).

The putative PAC3 protein has a plastid transit sequence, and translational fusions of the N-terminus of PAC3 with the green fluorescent protein (GFP) are imported into plastids in transgenic *Arabidopsis* (Tirlapur et al., 1999). These findings demonstrate that PAC is a chloroplast protein. Meurer et al. (1998) found that specific plastid mRNAs are altered in abundance and in their pattern of maturation in *pac* plants, suggesting that PAC is a nuclear factor required for plastid mRNA maturation and accumulation.

The phenotypic differences of the *pac* alleles are intriguing. Particularly interesting is why one allele is variegated and the other is not. This might be related to differences in *PAC* expression, or to other background–specific factors. Nevertheless, the *pac* mutants are an ideal system to explore the molecular mechanisms of nuclear-gene induced variegation in which only some cells of the mutant plant exhibit the mutant phenotype. Of relevance in this context is the observation that cytokinin is able to bypass PAC function and to induce greening in *pac* plants (Grevelding et al., 1996). An understanding of how suppressors of variegation (such as cytokinin) work should also lend insight into the variegation mechanism.

var1

The *var1* mutant was isolated from the progeny of an *Arabidopsis thaliana* plant (Columbia) regenerated from tissue culture (Martínez-Zapater, 1993). The mutant is nuclear recessive and resides on chromosome 5. Defective plastids are not maternally inherited. Variegation is limited to the leaves, and the extent of variegation is temperature-sensitive: plants grown below 20°C appear normal, but at 25°C the leaves have chlorotic sectors. Leaves that develop at the low temperature, then transferred to the higher temperature, develop chlorotic sectors in newly-differentiated tissues. By contrast, chlorotic cells, once produced, are unable to revert to a normal-appearing state, even if the temperature is reduced. *var1* has not been further characterized.

atd2

The *atd2* (*glutamine 5-phosphoribosylpyrophosphate amidotransferase 2-deficient*) mutant was generated by T-DNA mutagenesis (van der Graaff, 1997; Leon et al., 1998). It has white leaves and green cotyledons. Whereas cells in the cotyledons have normal-appearing chloroplasts, plastids in cells of the white leaves are smaller than normal, are vesiculated, and lack organized lamellae. The anatomy of the cotyledons is normal, but the palisade cells fail to expand in the white leaves. This suggests that *atd2*, like some of the other mutants discussed above (e.g., *im*, *dcl*, *dag*), is disrupted in the transmission of a plastid signal that regulates palisade cell differentiation.

The *ATD2* locus codes for glutamine 5-phosphoribosylpyrophosphate amidotransferase (Atase2). Atase2 is one of two isoenzymes (the other is Atase1) that catalyzes the first step of purine biosynthesis. Because there are two Atase isoenzymes, it is possible that *atd2* mutants are variegated because Atase1 is expressed in cotyledons and Atase2 is expressed in leaves. This has yet to be tested.

albomaculans

The *albomaculans* (*am*) variegation mutant was isolated by Röbbelen (1966) following X-ray treatment of *Arabidopsis* pollen. The mutation is inherited in a Mendelian fashion and sectoring is observed only in homozygous recessive individuals. The mutation generates permanently-defective, maternally inherited plastids. The abnormal plastids are vesiculated, lack lamellae and contain plastoglobulae (lipid bodies). Like *immutans* and *var*2, "mixed" cells are found in the white sectors of *am*. These cells are heteroplastidic for abnormal plastids and normal-appearing chloroplasts, and are probably products of incomplete sorting out. Tilney-Bassett (1975) suggested that *albomaculans* is an example of a plastome mutator (i.e, a nuclear gene that causes chloroplast DNA mutations), but this has yet to be demonstrated.

ACKNOWLEDGMENTS

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