

Tobacco *VDL* Gene Encodes a Plastid DEAD Box RNA Helicase and Is Involved in Chloroplast Differentiation and Plant Morphogenesis

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The recessive nuclear *vdI* (for *variegated* and *distorted leaf*) mutant of tobacco was obtained by T-DNA insertion and characterized by variegated leaves and abnormal roots and flowers. Affected leaf tissues were white and distorted, lacked palisadic cells, and contained undifferentiated plastids. The variegation was due to phenotypic, rather than genetic, instability. Genomic and cDNA clones were obtained for both the mutant and wild-type *VDL* alleles. Three transcripts, resulting from alternate intron splicing or polyadenylation, were found for the wild type. The transcripts potentially encode a set of proteins (53, 19, and 15 kD) sharing the same N-terminal region that contains a chloroplast transit peptide capable of importing the green fluorescent protein into chloroplasts. The predicted 53-kD product belongs to the DEAD box RNA helicase family. In the homozygous *vdI* mutant, T-DNA insertion resulted in accumulation of the shortest transcript and the absence of the RNA helicase-encoding transcript. Genetic transformation of the homozygous mutant by the 53-kD product-encoding cDNA fully restored the wild-type phenotype. These data suggest that a plastid RNA helicase controls early plastid differentiation and plant morphogenesis.

INTRODUCTION

Compared with other eukaryotic cells, plant cells contain a unique class of organelles, plastids, that differentiate according to developmental stage and cell localization. The function of each type of plastid (chloroplasts, amyloplasts, and chromoplasts) is correlated with the particular physiological role of the cell type and is accompanied by modification of plastid morphology and enzymatic machinery. All plastids differentiate from the undifferentiated proplastids in meristems. In green tissues, chloroplasts are the major form of plastids; they perform not only photosynthesis but also other metabolic processes, such as synthesis of lipids, amino acids, and hormones. Chloroplast development from undifferentiated proplastids occurs by a series of biological processes involving a large number of proteins. Because of the limited coding capacity of the chloroplast genome, most chloroplast proteins are encoded in the nucleus. The biogenesis of photosynthetic complexes and thylakoid assembly during chloroplast development, therefore, are regulated by coordination between the chloroplast and nuclear genomes (Mullet, 1988; Taylor, 1989; Susek et al., 1993; Surpin and Chory, 1997; Somanchi and Mayfield, 1999).

The identification and characterization of nuclear mutants affecting chloroplasts or other plastids constitute an inter-

esting approach to identifying the nuclear genes required for plastid biogenesis and function and to understanding nucleus-plastid interactions. Many genes involved in light perception and signaling have been identified (reviewed in Goldschmidt-Clermont, 1998; Khurana et al., 1998). If we consider genes that encode chloroplast proteins, several nuclear mutants that affect chloroplast development have been subjected to molecular analysis (see, e.g., Han et al., 1992; Keddie et al., 1996; Sundberg et al., 1997; Carol et al., 1999; Wu et al., 1999). However, most nuclear genes that encode chloroplast proteins, especially regulatory proteins essential for the early development of plastids, remain uncharacterized.

Plastid biogenesis is tightly coupled with temporal and spatial stages of plant development. Chloroplast development and leaf differentiation are both stimulated by light and are initiated in a coordinated fashion. However, certain mutants in which the light transduction pathway is blocked develop leaves in the dark in the absence of chloroplast development (Cabrera y Poch et al., 1993; Li et al., 1994), which suggests that these coordinated developmental processes can be separated. On the other hand, considerable evidence indicates that chloroplast development also regulates leaf differentiation, especially mesophyll morphogenesis. When chloroplast development is blocked at an early stage, formation of the palisade layer is also affected (Hudson et al., 1993; Reiter et al., 1994; Mandel et al., 1996), suggesting that a signal produced by the chloroplast during the early developmental stage affects leaf differentiation,

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particularly the full expansion of mesophyll cells (Surpin and Chory, 1997; Goldschmidt-Clermont, 1998). The isolation and characterization of mutations affecting chloroplast and leaf differentiation, therefore, constitute an interesting approach to understanding the early mechanisms involved in these developmental steps.

In this article, we report the identification of a tobacco T-DNA insertion mutant named *vdI* (for *variegated* and *distorted leaf*) that affects development of the leaves, flowers, and roots. The *VDL* locus encodes a putative chloroplast RNA helicase belonging to the DEAD box superfamily. These enzymes, which are present in both prokaryotes and eukaryotes, play important roles post-transcription, notably in cell growth and differentiation (Luking et al., 1998; De la Cruz et al., 1999). The possible role of this RNA helicase homolog in plastid differentiation and plant development is discussed.

RESULTS

Identification of the *vdI* Mutation

The *vdI* mutant was originally identified in distorted and variegated plants that appeared in the self-pollinated F_1 progeny of a transgenic tobacco plant (E6A) obtained by transformation with an *Agrobacterium* T-DNA-derived vector containing the yeast nuclear gene *MIP1*, which encodes a mitochondrial DNA polymerase. Because E6A was the only transgenic plant to give rise to progeny with this phenotype, we suspected that the phenotype resulted from T-DNA insertion.

When germinated in vitro, the self-pollinated F_1 progeny of E6A segregated into normal green seedlings and slow-growing, distorted seedlings characterized by albino or variegated cotyledons and leaves (Figure 1A). Later, the extent of distortion and variegation varied markedly (see Figures 1B to 1D for details). Green sectors or spots usually stood out, suggesting better cell proliferation in these areas. The root growth of the mutants was severely limited, even in plants with leaves that were almost completely green (Figure 1E).

In soil-grown plants, the phenotype varied according to the plant and the leaf (examples are shown in Figure 1F). Some albino leaves developed a main vein of normal length with very narrow and curled lamina (Figures 1G and 1H). Severely affected plants grew extremely slowly in soil, and most died before flowering; the survivors often had albino flower buds (Figure 1I) that fell off before opening. A few flowers developed variegated and distorted petals (Figure 1J) and had very poor male fertility. F_1 seed could be obtained by pollinating with wild-type pollen, yielding germinated F_1 seedlings that were 100% normal plants. In a few cases, self-fertilized seed was obtained from less-affected flowers; however, the germination rate was very low and the seedlings all displayed a mutant phenotype.

Cell Morphology

Closer inspection of the leaf morphology of the *vdI* mutant showed that the palisade layer was present in green sectors (Figure 2A) but absent from white sectors (Figure 2B). In narrow and curled leaves, spongy cells were irregularly shaped and the lamina thickness was variable (Figure 2C). The correlation between the absence of palisadic structure and albino phenotype was clear at junctions between green and albino sectors (Figure 2D) or at a white spot within a green sector (Figure 2E). Root structure, too, was disorganized (Figures 2F and 2G).

Electron microscopic analysis of cells from white leaf sectors (Figure 3B) showed undeveloped plastids that lacked thylakoids and had vesiculated internal membranes. In green sectors, the chloroplasts were just as well developed as they were in the wild type (cf. Figures 3A and 3C). These data suggest that the *vdI* mutation affects both plastid differentiation and organ morphogenesis.

The *vdI* Mutation Is Genetically Stable

A somatic unstable phenotype is often associated with non-Mendelian mutation or transposon-generated nuclear mutation. Although difficult to obtain, the self-fertilized progeny of *vdI* mutant plants were 100% mutant, whereas the progeny of *vdI* mutant plants crossed with wild-type tobacco were 100% normal (data not shown). In the self-fertilized offspring of the latter cross, mutant and normal plants typically were observed in a ratio of 214:750 (for example), which is close to the theoretical 1:3 ratio expected for a single recessive nuclear mutation. This was confirmed in subsequent crosses (data not shown).

Genetic instability of the mutation, for example, as induced by transposon excision, could be ruled out, because normal plants were never found in the self-fertilized progeny of a homozygous mutant when seed was obtained. In addition, when stem sections obtained from a phenotypically normal area of a mutant plant were grown in vitro, the new leaves that developed from the axillary bud adjacent to a normal leaf were always albino with green edges (Figure 1K). Finally, when placed on in vitro regeneration medium, leaf discs from mutant plants produced variegated or albino shootlets, regardless of whether the discs came from normal-looking green leaves (Figure 1L), green sectors of albino leaves, or albino leaves. Therefore, these data show that the *vdI* mutation is genetically stable even though its expression in the phenotype is variable.

Cloning and Characterization of the *vdI* Locus and Its Transcripts

To demonstrate that the *vdI* mutation was induced by T-DNA insertion, DNA isolated from 36 plants produced by a cross

between heterozygous *vdI* and wild-type (+) seedlings (18 +/+ and 18 +/-*vdI* plants, as deduced from their self-crossed progeny) was digested with EcoRI or XbaI and subjected to DNA gel blotting with a T-DNA left border fragment as probe. One 7.2-kb XbaI fragment and two EcoRI fragments (1.6 and 1.0 kb) cosegregated with plants producing the mutant phenotype in their self-fertilized progeny (data not shown).

Specific primers corresponding to the T-DNA left border were designed to amplify the T-DNA flanking region by using inverse polymerase chain reaction (PCR). Cloning and sequencing showed that the 1.6- and 1.0-kb EcoRI fragments were adjacent to one another and were included within the 7.2-kb XbaI fragment (Figure 4A). The 1.0-kb fragment contained a partial T-DNA left border, the end of the *MIP1* gene, and the *rbcS* E9 terminator sequence present in the original construct (Figure 4A). The 1.6-kb fragment contained a 0.2-kb T-DNA left border sequence and an unknown 1.4-kb sequence (P1400 in Figure 4A), presumably derived from the tobacco genome. DNA gel blotting of tobacco genomic DNA using this 1.4-kb sequence as the probe confirmed that the latter was included within the previously identified cosegregating 7.2-kb XbaI band and also within novel 5.2- and 3.0-kb XbaI (Figure 4B) fragments. Additional DNA gel blotting studies of heterozygous and homozygous *vdI* mutant and wild-type plants with *SpeI* and *HindIII* enzymes confirmed that wild-type tobacco, an amphitetrapioid species, contained two *VDL* copies (Figure 4B). The *VDL* locus was designated as that disrupted by T-DNA, and *VDL'* was the presumed locus from the other tobacco parental genome.

A series of overlapping genomic fragments that included the *VDL* and *VDL'* loci was cloned by successive inverse PCR amplifications and sequenced. Finally, the full genomic sequence was obtained by direct PCR for the *VDL* locus. The sequences of both loci were very similar, except for additional 1.3- and 2.6-kb fragments present in *VDL* introns 3 and 9, respectively, and complete divergence in the 5' region (shown as hatched for *VDL'* in Figure 5A). The T-DNA insert was incomplete as already suggested (Figure 4A).

A database search showed that two sequences had high homology with DEAD box RNA helicases (discussed in detail below). Corresponding cDNA clones were obtained from wild-type RNA by 5' and 3' rapid amplification of cDNA ends and direct reverse transcriptase (RT)-PCR with *VDL*- or *VDL'*-specific primers. cDNA clones corresponding to three partially overlapping transcripts were obtained for the *VDL* and *VDL'* loci (Figure 5B), all of which contained the same 5' region but differed in their 3' region as a result of alternate polyadenylation or intron splicing. *VDL*-1 and *VDL'*-1 represented the longest transcripts and were assembled from 10 exons. In *VDL*-2 and *VDL'*-2, an alternative polyadenylation site terminated the transcript after exon 2 (2b in Figure 5B). In *VDL*-3 and *VDL'*-3, alternative splicing placed exon 3 (which terminates with a polyadenyl-

ation site), rather than exon 4, next to exon 2. For the *VDL'* locus, cDNA clones for five additional transcripts (*VDL'*-4 to *VDL'*-8) were found. These corresponded to *VDL'*-1, except that some internal exons were absent, and in *VDL'*-6, *VDL'*-7, and *VDL'*-8, the splicing sites were slightly displaced for exons 5, 6, or 11, which were therefore shorter (Figure 5B). RT-PCR with primers corresponding to the untranslated 5' region of exon 1 and the last exon (11) confirmed that a single band (corresponding to *VDL*-1) was found for *VDL* and that at least five bands were found for *VDL'* (corresponding to *VDL'*-1 and *VDL'*-4 to *VDL'*-8; Figure 6A).

In the *vdI* Mutant, *VDL*-2 Transcript Accumulates, whereas *VDL*-1 and *VDL*-3 Transcripts Are Undetectable

In the *vdI* mutant, the T-DNA was inserted into intron 2. DNA gel blot analysis of genomic DNA cut independently with three restriction enzymes confirmed that the size of the restriction fragment corresponding to the *VDL* locus was increased by ~2 kb, the size of the truncated T-DNA (Figure 4C). As expected, the heterozygous plant contained both the wild-type and T-DNA-inserted forms. In addition, a common band (e.g., 3 kb with XbaI) was found for the three plants and corresponded to the *VDL'* locus.

Because the T-DNA sequence contains a copy of the *rbcS* E9 transcription terminator, the *vdI* transcript would be expected to terminate prematurely. To determine the effect of T-DNA insertion on the transcript profile, we performed RT-PCR analysis with a *VDL*-specific 5' primer localized within the untranslated region of exon 1 and with 3' primers corresponding to exons 11, 3, and 2b (*VDL*-1, *VDL*-3, and *VDL*-2, respectively). RT-PCR was chosen rather than RNA gel blotting because the strong similarity of the *VDL* and *VDL'* sequences and the short untranslated sequences made it difficult to design probes specific enough for RNA gel blotting.

Using leaf RNA and the 3' primer corresponding to exon 11, 3, or 2b, we obtained single bands for the wild type and the heterozygous *vdI* mutant (Figure 6B). These bands corresponded to *VDL*-1, *VDL*-3, and *VDL*-2, respectively. For the homozygous *vdI* mutant, no signal was found with the exon 11 (*VDL*-1) or exon 3 (*VDL*-3) primer, but a band was obtained with the exon 2b (*VDL*-2) primer. This band was stronger in the heterozygous and homozygous mutants but not for the *VDL'* locus (Figure 6B). These data are in agreement with transcript termination within the T-DNA and polyadenylation at the *VDL* site downstream of exon 2b, leading to accumulation of the *VDL*-2 transcript.

Because roots or flowers were also affected in *vdI* plants, we examined whether transcripts corresponding to *VDL*-1 and *VDL'*-1 were expressed in those organs. A band corresponding to the full-length *VDL* transcript was indeed seen in all three organs, whereas signals for *VDL'* were seen only in leaves (Figure 6C).

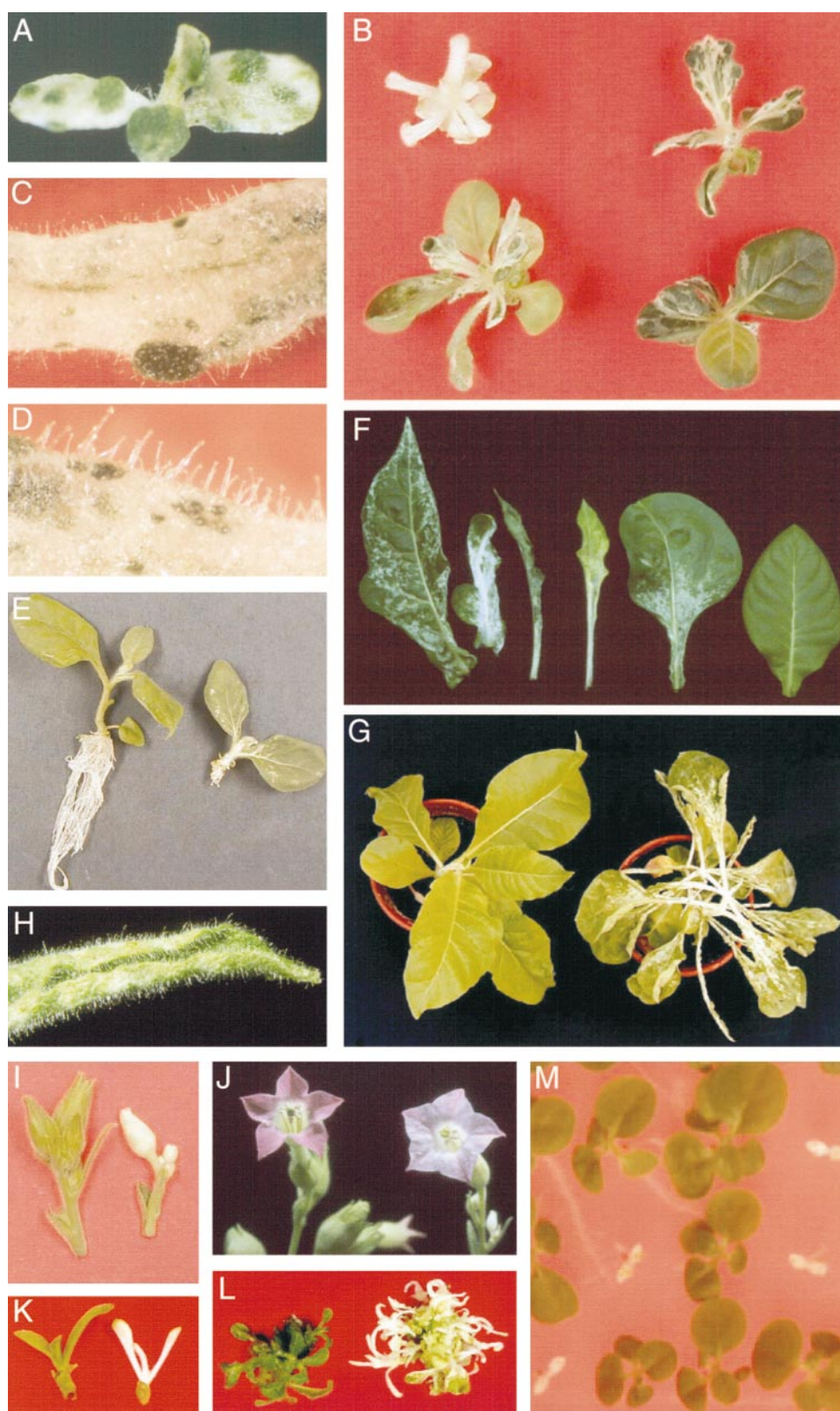


Figure 1. Phenotype of the *val* Mutant.

***VDL* and *VDL'* Loci Encode a Group of Putative Proteins with the Same N-Terminal Region, the Longest Product Being a Putative RNA Helicase**

The *VDL*-initiating translation codon was tentatively identified 86 (*VDL*) or 104 (*VDL'*) nucleotides downstream of the 5' end of the longest cDNA clone. It is in a good initiating context (CCCATGG), is the beginning of a reading frame of 466 codons, and is preceded by an in-frame stop codon in *VDL*. Comparing *VDL* and *VDL'* shows that the upstream sequence strongly diverges, whereas the downstream coding sequence is >98% identical.

The predicted *VDL*-1 or *VDL'*-1 protein (53 kD) is thought to be a putative DEAD box RNA helicase containing all of the conserved motifs (Figure 7). The proteins that show the greatest similarity with *VDL*-1 are DEAD box RNA helicases, which have been identified in several bacterial and animal species and are involved in various post-transcriptional steps in gene expression. The Arabidopsis genome project recently released a chromosome V contig that contains a sequence closely related to *vdL*-1 (67% identity between the predicted mature proteins).

VDL-2/*VDL'*-2 and *VDL*-3/*VDL'*-3 consisted of 132 and 168 amino acids, respectively, including the same 112 N-terminal residues seen in *VDL*-1/*VDL'*-1. No homology with the C-terminal sequences specific to *VDL*2 or *VDL*3 was found in the databases.

VDL'-4 contained the seven RNA helicase motifs but lacked a stretch of nonconserved sequence between motifs Ia and II. *VDL'*-5, *VDL'*-6, and *VDL'*-8 contained some of the RNA helicase motifs, whereas *VDL'*-7 contained none because of a frameshift (data not shown).

The *VDL* N-Terminal Region Contains a Chloroplast Transit Peptide

The strong similarity between *VDL*-1 or *VDL'*-1 and RNA helicases starts at residue 74. The more upstream coding re-

gion, common to all predicted *VDL* or *VDL'* products, has several of the features of plastid transit peptides (Von Heijne et al., 1989) because it is rich in Ser (13%) and Thr (16%) and has a very low content of Tyr, Asp, and Glu. This was further supported by a computer-based program (ChloroP; available at <http://www.cbs.dtu.dk/services/>), which predicted that the *VDL* N-terminal region contains a transit peptide with a cleavage site between residues 53 and 54 (with the typical Ala and Val at -1 and -3, respectively). To confirm this hypothesis experimentally, a translational fusion between the sequence encoding the first 73 N-terminal residues of *VDL*-1 and the green fluorescent protein (GFP) sequence was linked to the cauliflower mosaic virus (CaMV) 35S transcription promoter and electroporated into tobacco protoplasts for transient expression. The fusion protein clearly colocalized with the chloroplast chlorophyll, whereas a control GFP without transit peptide was detected in the cytosol (Figure 8).

Ectopic Expression of *VDL*-1 Restores a Wild-Type Phenotype

To prove that the defective phenotype is a direct consequence of the modified *VDL*-1 locus, we transformed homozygous *vdL* plant leaf discs with a cDNA clone corresponding to the *VDL*-1 transcript placed under the control of the CaMV 35S transcription promoter. A β -glucuronidase construct was used as a control. After transformation of the *vdL* mutant with the control construct and selection with hygromycin, only white and variegated calli and shoots developed, and those only slowly. However, in the *VDL*-1 construct, the majority of shoots were green and developed into normal plants (data not shown). When these plants were self-crossed, their progeny segregated into normal and variegated plants, showing Mendelian segregation (one, two, or more copies, depending on the transformant) of the

Figure 1. (continued).

- (A) A *vdL* seedling grown in vitro in Murashige and Skoog (MS) agar medium (see Methods).
- (B) Four *vdL* plants grown in vitro and showing various phenotypes. The seedlings (5 weeks old) were grown in MS agar medium.
- (C) and (D) Details of a *vdL* plant grown in vitro.
- (E) Wild-type (left) and *vdL* (right) plants grown in vitro (4 weeks old).
- (F) Leaves from various *vdL* plants grown in soil, showing various phenotypes.
- (G) Wild-type (1 month old, at left) and *vdL* (2 months old, at right) plants grown in soil.
- (H) Detail of a narrow leaf of a *vdL* plant similar to that shown in (G).
- (I) Flower bud from wild-type (left) and *vdL* (right) plants.
- (J) Flower from wild-type (left) and *vdL* (right) plants.
- (K) Development of an axillary bud on green stem sections derived from wild-type (left) and *vdL* (right) plants and transferred for 2 weeks to MS agar medium.
- (L) Leaf discs from wild-type (left) and *vdL* (right) green leaves transferred to an MS agar medium for shoot regeneration.
- (M) A transgenic line (T6-5-8) resulting from the transformation of a *vdL* homozygous plant with a wild-type *VDL*-1 cDNA clone was grown until the flowering stage and then self-pollinated; seed was then sown on a growth medium without antibiotics. The photograph was taken 5 weeks after sowing.

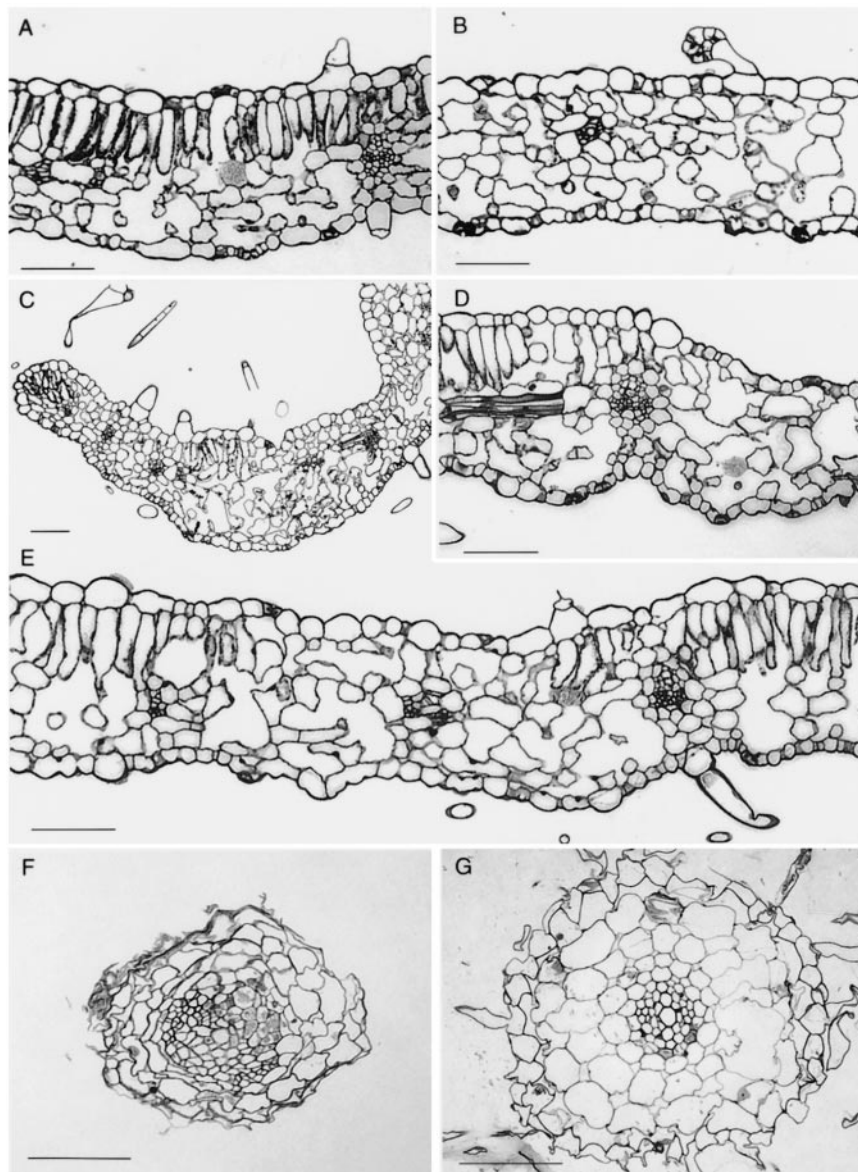


Figure 2. Light Microscopy Analysis of Wild-Type and Homozygous *vdI* Leaves and Roots.

(A) Cross-section from a green sector of a *vdI* leaf.

(B) Cross-section from a white sector of a *vdI* leaf.

(C) Cross-section from a curled *vdI* leaf similar to that shown in Figure 1H.

(D) Cross-section from a *vdI* leaf at a junction between a green (left) or white (right) sector.

(E) Cross-section from a white spot within a green sector of a *vdI* leaf.

(F) Cross-section from a *vdI* root. The epidermis and the endodermis are not well identified, and the vascular cylinder has a disorganized structure.

(G) Cross-section from a wild-type root.

Bars = 100 μ m.

complementing *VDL*-1 transgene. For example, plant T6-5-8 (Figure 1 M) gave 166 normal green plants and 65 small variegated plants, indicating a single locus for the ectopic *VDL*-1.

DISCUSSION

The tobacco *vdI* mutation has been identified within a gene that presumably encodes a chloroplast RNA helicase. The mutant leaves are variegated, the albino sectors are distorted and devoid of palisade parenchyma, and severely affected leaves are very narrow; however, the lamina is always present. The *vdI* mutant therefore differs from the *phantastica* mutant, which is characterized by a lack of lateral expansion (Waites and Hudson, 1995). Subcellular analysis showed that the plastids of albino cells do not develop stalked membranes. This correlation between defective chloroplast development and leaf morphogenesis supports a previous suggestion that a signal produced at the very

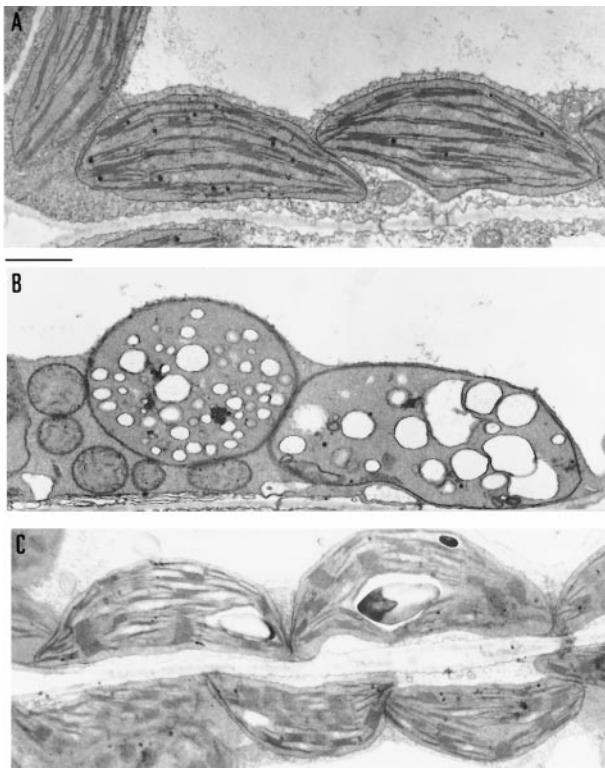


Figure 3. Transmission Electron Micrographs of *vdI* Leaf Cells.

(A) Green sector of a *vdI* leaf. The plastid structure is similar to that found for the wild type shown in **(C)**.

(B) White sector of a *vdI* leaf. Plastids show a vesicular instead of lamellar structure.

(C) Wild-type leaf. Chloroplasts show the typical stacking thylakoids. Bar in **(A)** = 1 μ m for **(A)** to **(C)**.

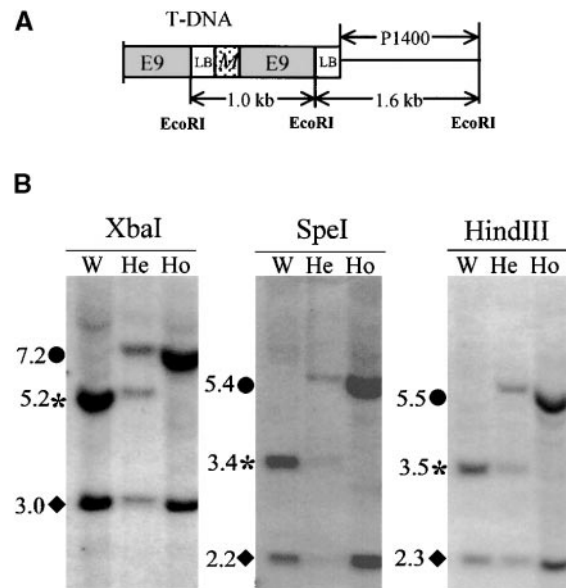


Figure 4. A T-DNA-Flanking Genomic Fragment Cosegregates with the *vdI* Mutation.

(A) Structure of the cosegregating T-DNA insert. The recombined T-DNA insert consisted of two copies of the T-DNA left border (LB), part of the *MIP* gene (*M*), and the E9 terminator. The cloned 1.6- and 1.0-kb *EcoRI* T-DNA fragments and the 1.4-kb genomic fragment (P1400) are indicated.

(B) Genomic DNA was extracted from wild-type (W), heterozygous (He), and homozygous (Ho) *vdI* plants and digested with the indicated restriction enzymes. After transfer, the DNA was hybridized with the primer-generated probe P1400. Circles indicate the *vdI* locus, asterisks indicate the wild-type *VDL* locus, and diamonds indicate the *VDL'* locus.

early developmental stage of chloroplasts stimulates leaf palisade division and expansion (Reiter et al., 1994; Wetzel et al., 1994; Chatterjee et al., 1996; Keddle et al., 1996).

VDL-1, the product encoded by the largest *VDL* transcript identified, belongs to the DEAD box RNA helicase family. These ATP-dependent enzymes unwind RNA:RNA or RNA:DNA duplexes and are involved in several important biological processes in the nucleus, cytosol, and mitochondria, including RNA stability, processing, editing, export from the nucleus, and translation. Some of these enzymes are involved in the regulation of cell growth and cell differentiation (reviewed in Luking et al., 1998; De la Cruz et al., 1999). Because we also demonstrated that the N-terminal region of *VDL*-1 contains a functional chloroplast transit peptide, this enzyme is predicted to be a chloroplast DEAD box RNA helicase.

The primary structure of DEAD box RNA helicases is well conserved, especially within the seven motifs (Figure 7). Of these, motifs I, II, and VI are the most conserved and have been identified as ATP binding and nucleic acid binding sites.

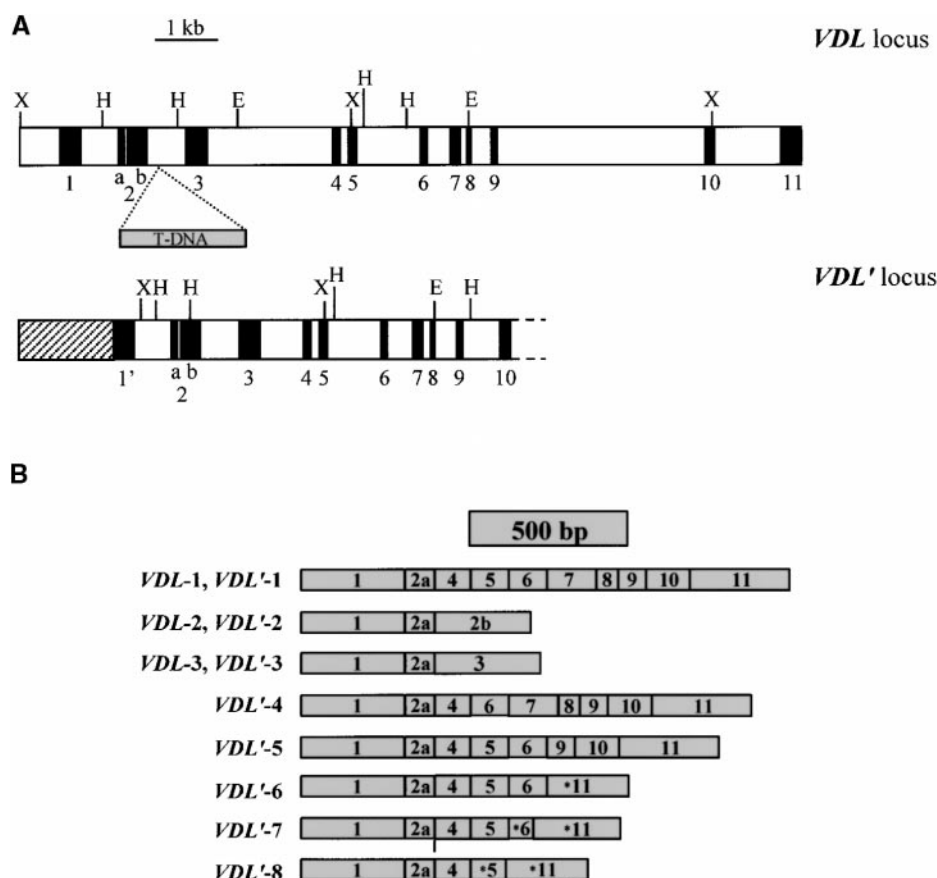


Figure 5. Genomic and cDNA Organization of the *VDL* and *VDL'* Loci.

(A) Genomic map of the *VDL* and *VDL'* loci showing exons (numbered black boxes), introns (open boxes), and the location of the T-DNA insert. EcoRI (E), HindIII (H), and XbaI (X) restriction sites are indicated. Because of the considerable difference in the 5' untranslated region, the first exons of the *VDL* and *VDL'* loci are named 1 and 1'. The hatched region in *VDL'* represents an upstream sequence that is completely different from that in *VDL*. The size of *VDL* introns 3 and 9 is based on restriction mapping (some gaps in the sequence remain). The sequence downstream of *VDL'* exon 10 has not been cloned.

(B) Alternative splicing products of the *VDL* and *VDL'* loci. The filled boxes indicate exons. The asterisks in front of exons 5, 6, and 11 in *VDL*-6, *VDL*-7, and *VDL*-8, indicate that the 5' or 3' borders differed from those in the other cDNA clones, resulting in shorter transcripts; the exact sequence can be found in the databases.

The function of the other four motifs remains unclear, although motif III might be involved in helicase activity. The lengths and sequences of the N- and C-terminal regions and the regions between the motifs differ between family members. These regions are believed either to have distinct biochemical activities that combine with the helicase activity to generate new enzyme activities or to be involved in RNA substrate specificity, which must be closely related to the biological function of the respective helicases (Bird et al., 1998; Eisen and Lucchesi, 1998). Thus, at this time, assigning a specific function to *VDL*-1 is difficult. An Arabidopsis open reading frame is very closely related to *VDL*-1. However, this putative gene was identified within sequences only

recently generated by the Arabidopsis genome project and therefore does not give any clue about *VDL*-1 function.

The proteins with the greatest identity to *VDL*-1 were DEAD box proteins involved in post-transcription and translation regulation in other organisms (Figure 7). In *Escherichia coli*, the DEAD box protein can suppress a defective mutant in ribosomal S2 protein (Toone et al., 1991), SRMB can suppress a mutant defective in 50S ribosomal subunit assembly and may interact with 23S RNA (Nishi et al., 1988), DBPA can unwind both 23S and 16S RNA and may play a role in the assembly of the active center of 50S ribosomal subunits (Boddeker et al., 1997), and RHLB plays a role in postreplication repair, RNA processing, and mRNA degradation in

the RNA degradosome (Py et al., 1996). In yeast, DED1 is involved in initiating translation (lost et al., 1999). The mouse translation initiation factor, eIF4A, is involved in protein synthesis initiation and is required for mRNA binding to ribosomes and for cap recognition (Nielsen et al., 1985). The *Drosophila* vasa protein is important in oocyte formation and in specification of the posterior structures of the embryo (Hay et al., 1988; Styhler et al., 1998). The human growth-regulated nuclear 68 protein is involved in organ differentiation/maturation in the fetus (Stevenson et al., 1998). This survey clearly shows that DEAD box proteins are involved in various post-transcriptional steps and that some of them govern developmental processes.

Given the prokaryotic origin of plastids, we were not surprised to find substantial identity between VDL-1 and bacterial DEAD box proteins. However, VDL-1 is not closely related to a recently identified mitochondrial DEAD box RNA helicase (Gagliardi et al., 1999). The observation that VDL is expressed in nonphotosynthetic tissues and that its disruption also affects roots and flowers indicates that the encoded product is involved in the differentiation of all plastids. Transcriptional and post-transcriptional regulation of these organelles is complex (Rochaix, 1996; Sugita and Sugiura, 1996; Stern et al., 1997). During the conversion of proplastids into chloroplasts and other plastids, the gene expression machinery undergoes an important change, notably an increased synthesis of proteins involved in the transcription/translation apparatus (discussed in Goldschmidt-Clermont, 1998). For instance, chloroplasts contain a multi-subunit complex that controls the amounts of specific RNAs at steady state (Hayes et al., 1999). A similar complex, called RNA degradosome, has been identified in *E. coli* and contains an RNA helicase presumably involved in unwinding structured RNA (Carpousis et al., 1999). We therefore propose that VDL-1 is involved in a general process of the plastid expression machinery. Clearly, a careful examination of the expression of several plastid genes and nuclear genes that encode plastid proteins in the *vdI* mutant is required to determine the stage at which VDL-1 plays a role.

The VDL gene is alternatively spliced. Two additional transcripts identified potentially encode proteins containing the same N-terminal chloroplast transit peptide as VDL-1. However, their predicted mature regions are short (13 and 8 kD, respectively), and the lack of homology with any database sequences makes it difficult to assign a possible function to these proteins. These same transcripts were found for VDL', the VDL homolog presumably belonging to the second genome of tobacco (an amphitetraploid), but five additional alternative transcripts were also identified. These are thought to encode polypeptides containing some or all of the seven RNA helicase motifs; VDL'-4 contains all seven motifs, whereas VDL'-5 and VDL'-6 contain the most conserved motifs (motifs I, Ia, II, and VI), which provide the ATP binding and nucleic acid binding sites. Whether these all function as RNA helicases is difficult to predict at this time. The sequence polymorphism created by the alternative splicing

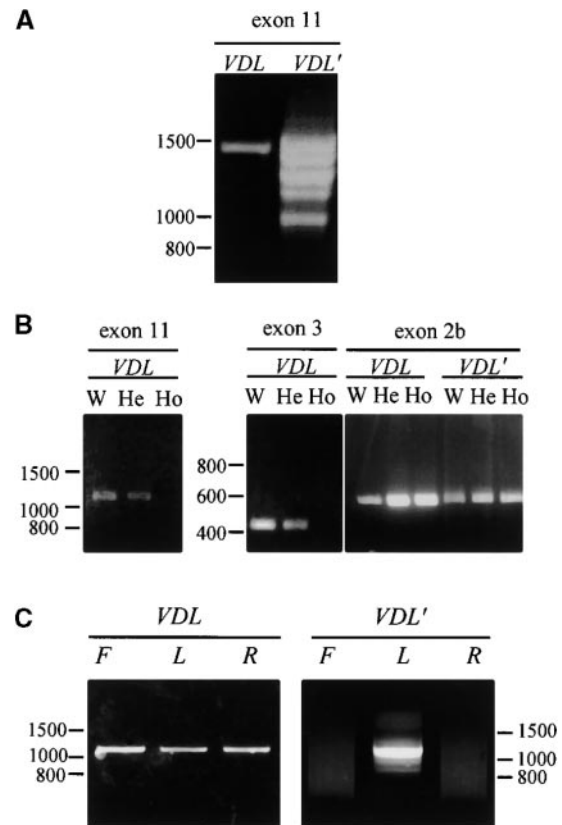


Figure 6. RT-PCR Transcript Analysis of the VDL and VDL' Loci.

(A) Poly(A)⁺ RNA from wild-type leaves was amplified by RT-PCR. The 5' primers were located in the 5' untranslated region and were specific to either VDL (pTA5'A) or VDL' (pTABis5'A); the 3' primers were common to VDL and VDL' and were located in exon 11 (pTFL65B).

(B) Poly(A)⁺ RNA from wild-type (W), heterozygous (He), and homozygous (Ho) (white sections) mutant leaves was amplified by RT-PCR. The 5' primers were as in **(A)**; the 3' primers were common to VDL and VDL' and corresponded to exon 11 (pTFL57), exon 2b (pRH14), or exon 3 (pRH11).

(C) Poly(A)⁺ RNA from wild-type flower (F), leaf (L), or root (R) was amplified by nested RT-PCR with, for VDL, primers pTA5A and pTFL50 (5') and pTFL57 (3'), and for VDL', primers pTABis5A (5') and pTFL65B and pTFL57 (3'). Note that, to simplify the pattern, the 3' primer used here for exon 11 was chosen to cover a region not present in VDL'-6, VDL'-7, and VDL'-8, which have a shorter exon 11 because of their alternative splicing.

The numbers at left and right denote molecular mass markers (kilobases).

might provide a means of altering substrate specificity or function. Oligomeric forms, generally dimers or hexamers, have been described for certain DNA helicases (Chao and Lohman, 1991; Amaratunga and Lohman, 1993) and are thought to provide the multiple nucleic acid binding sites necessary for helicase function. The crystal structure of RNA

helicase from genotype 1b hepatitis C virus also suggests a putative functional dimer model (Cho et al., 1998). Some DEAD box proteins require accessory proteins to function in vivo (Rozen et al., 1990; Gibson and Thompson, 1994; Sommerville, 1999). A more exhaustive search for all alternative *VDL'* transcripts at various developmental stages is required to gain more insight into the biological meaning of this finding.

Complementation of *vdI* disruption by ectopic expression of the longest *VDL* cDNA clearly indicated that the mutant phenotype results directly from the absence of the *VDL*-1 product rather than from the absence of *VDL*-3 or the increased expression of *VDL*-2. Although the *VDL'* locus is also transcribed into a *VDL*-1-like transcript, this cannot fully substitute for *VDL*-1. Two differences between *VDL* and *VDL'* might explain this observation. First, as mentioned above, perhaps the multiple *VDL'* alternative splicing events undergo spatial or temporal regulation; therefore, the concentration of the longest transcript may be too low to sustain synthesis of the required amount of RNA helicase. Second, the region that starts 67 nucleotides upstream of the presumed translation initiation codon shows complete divergence, probably as a result of a recombination event; thus the two loci are expected to be transcribed differently, resulting in no overlap or only a partial overlap of expression. This is clearly the case in roots and flowers, in which no transcript corresponding to *VDL'*-1 was found by RT-

PCR. In leaves, although *VDL'*-1 transcripts were found, expression of *VDL* and *VDL'* does not necessarily overlap in all cell types and at all developmental stages. To determine whether this is the case would require careful examination of the expression of the two loci at the cellular level during development.

Although the variegation of several mutants with defective chloroplasts has been linked to either cytoplasmic mutation or transposon excision from a nuclear gene (Reidi, 1973; Martinez-Zapater et al., 1992; Martienssen and Baron, 1994; Chatterjee and Martin, 1997), we were able to exclude these possibilities in the case of *vdI*. The *vdI* mutation showed clear Mendelian inheritance, and plants regenerated from phenotypically reverted sectors always showed a mutant phenotype and gave mutant progeny. From the pattern of variegation, the albino phenotype apparently can appear at any developmental stage and at various places within a leaf. It affects all the cells across a section or only cells within some layers. Phenotypic reversion can also occur, as shown by tiny green spots appearing in large white areas. Other cases of variegated mutants unrelated to transposon activity have been described. This is the case, for instance, for the tomato *ghost* mutant (Scolnik et al., 1987) and the *immutans* mutant, a yellow variegated mutant in Arabidopsis (Carol et al., 1999; Chen et al., 1999; Wu et al., 1999). In no case has the variegation been explained in molecular terms, but a threshold hypothesis is often suggested. In accor-

Accession number	Gene name	Species	Protein length	block A	block B	block C		
	VDL-1	Tobacco	466aa	86 GYVIFTEVQIALPFLYSGRDCVHAQTSGSKTLYAILLQIL	139 LIIVPTRELGMQV	197 IIVATMGLSQCMLEKHILKLDSCQVLADEVDFMNSK		
	VDL'-1	Tobacco	466aa	86 GYVIFTEVQIALPFLYSGRDCVHAQTSGSKTLYAILLQIL	139 LIIVPTRELGMQV	197 IIVATMGLSQCMLEKHILKLDSCQVLADEVDFMNSK		
AC069326		Arabidopsis	475aa	98 GFVFPTDIQREALPFLSTGRDCLHAQTSGSKTLYAILLQIL	151 VIIVPTRELGMQV	206 IIVATMGLSQCMLEKHILKLDSCQVLADEVDFMNSK		
BAA19295		Bacillus subtilis	511aa	39 GFEEATPIQAGTPIGLSHKDVGAQTGTGKTAAFGIPLV	92 IIVAPTRELAIQV	141 IIVGTGRLDLHINRRTIRANNTVVMDEADEMLMGF		
P23304	DEAD	E. coli	646aa	42 GYEKPSPIQACPIPHLLNGRDVLGMAQTSGSKTAAFLSPLL	95 IIVAPTRELAIQV	145 IIVGTGRLDLHILKRGTDLSKLGLVLEADEMLMGF		
P21507	SRMB	E. coli	444aa	23 GTRPTAIQAAALPAILAGDVRVGAQTGTGKTAAFLSPLL	80 IIVAPTRELAIQV	129 IIVATMGLSQCMLEKHILKLDSCQVLADEVDFMNSK		
P21693	DBPA	E. coli	457aa	22 GYLTMTPVQAAALPAILAGDVRVGAQTGTGKTAAFGIPLV	75 IIVAPTRELAIQV	125 IIVGTGRLDLHILKRGTDLSKLGLVLEADEMLMGF		
P24229	RHLB	E. coli	421aa	28 GFHNCTPIQALPLPILAGDVRGAQTGTGKTAAFLSTTF	88 IIVAPTRELAIQV	137 IIVGTGRLDLHILKRGTDLSKLGLVLEADEMLMGF		
P09052	VASA	Drosophila melanogaster	661aa	264 GKIKPTPIQALCSIPVIGSGRDLMACAQTGTGKTAAFLPIL	322 VIIVPTRELAIQV	371 VIIVGTGRLDLHIVDRTFTFVDFRVVLEADEMLMGF		
P06634	DED1	Saccharomyces cerevisiae	604aa	161 RETKPTPVQKYSYIVANGRDLMACAQTGTGKTGGFIPVL	229 VIIVAPTRELAIQV	278 LVIVGTGRLDLHILKRGTDLSKLGLVLEADEMLMGF		
FIMS4A	EIF4A	Mouse	390aa	35 GFEEKPSAIQRAILPCIKGYDVIAQAQSGTGKTATFAISIL	88 IIVAPTRELAIQV	138 IIVGTGRLDVPMLNRIKLSFYKMKVLEADEMLMGF		
1406327A	P68	Human	594aa	93 NFTEPTAIQAQGWPAVSLGMDVGAQTGTGKTLISYLPAL	151 IIVAPTRELAIQV	200 ICIATPGRILDFLEGKCTLNRRTYLVLEADEMLMGF		
				*****	*****	*****		
				Ia	Ib	Ic		
				g---t-#Q---p---g-D---AqG-GKT-----	##-PTRELQ-#	#-H-T-grl---#-----v#DEAd-m---g		
				Consensus				
				block D	block E	block F		
	VDL1	Tobacco	253	RQTIFASASIP	309	KNQVLLSLQVDSFESAIIFVSEQSEKSKAGNA	394	IAARGVDLPETHHINFDIPKDAVNYLHRAIGRTGRK
	VDL'1	Tobacco	253	RQTIFASASIP	309	KNQVLLSLQVDSFESAIIFVSEQSEKSKAGNA	394	IAARGVDLPETHHINFDIPKDAVNYLHRAIGRTGRK
AC069326		Arabidopsis	262	RQTIFASASIP	318	KNQVLLSLQVDSFESAIIFVSEQSEKSKAGND	403	IAARGVDLPETHHINFDIPKDAVNYLHRAIGRTGRK
BAA19295		Bacillus subtilis	194	RQTIFASATMP	245	KFDTITRLDLQSFELAVFGRTKRVDLEAAL	317	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P23304	DEAD	E. coli	198	RQTIFASATMP	249	KNEILVRFLEAEQDAALIFVTRKNATLEVAAL	321	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P21507	SRMB	E. coli	182	RQTIFASATMP	235	KTALLVHLKQPEATRSIVFVKRRVHLEAHL	307	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P21693	DBPA	E. coli	178	RQTIFASATMP	228	KIPLLQRLSLHQPSGVFCNTKKDCQAVCDAL	300	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P24229	RHLB	E. coli	192	RQNLFSATISL	246	KMRLLQRLILESEADGTIVFVETKRGADFLASFL	315	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P09052	VASA	Drosophila melanogaster	426	RQTIFASATMP	476	KMRLLQRLILESEADGTIVFVETKRGADFLASFL	548	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
P06634	DED1	Saccharomyces cerevisiae	335	RQTIFASATMP	386	KKSALLDLISASTDGLTLFVETKRGADFLASFL	458	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
FIMS4A	EIF4A	Mouse	191	RQTIFASATMP	243	KLDTICDLYETLITQAVIFVETKRGADFLASFL	315	VAARGLDISGVTHVNVNVDPPQESVYHRIIGRTGRA
1406327A	P68	Human	253	RQTIFASATMP	308	KLIRLMEIMESEKENTIVFVETKRRCDLTRKM	380	VASRGDLVDQKVFNVNVDPPNSSEDIYHRIIGRTGRA
				***	*****	*****	*****	
				III	IV	V	VI	
				-at##SAT-p	K---L-----IF--T-----	#aaRGd#d-----v-N-d-p-----yHRIIGRTgr		
				Consensus				

Figure 7. Alignment of the *VDL*-1/*VDL'*-1 Protein Sequence with Sequences of Similar RNA Helicases.

Sequence alignment showing the conserved blocks and the consensus sequence of the aligned proteins. The seven motifs (I, Ia, II, III, IV, V, and VI) of DEAD box RNA helicases are indicated by asterisks. A conserved region of unknown function between motifs Ia and II is also indicated. Boldface letters indicate amino acids that are 100% conserved. Amino acid residues of the consensus sequence are in lowercase when conserved in at least 70% of the proteins displayed or in uppercase when they are 100% conserved. The number symbol (#) indicates the hydrophobic amino acid Met, Ile, Leu, or Val. aa, amino acids.

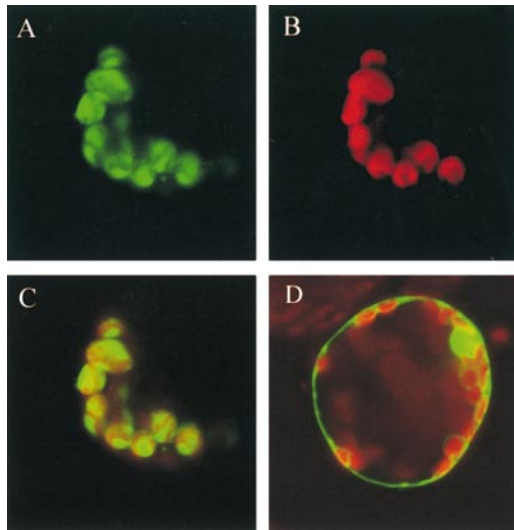


Figure 8. Predicted *VDL* Transit Peptide Imports the GFP into Chloroplasts.

The sequence coding for the first 73 amino acid residues of *VDL*-1 (*VDL*73) was inserted between the CaMV 35S promoter and the *GFP* sequence to give 35S-*VDL*73-*GFP* in a bacterial plasmid (see Methods). A 35S-*GFP* construct without the *VDL* sequence was used as a control. Both plasmids were electroporated into tobacco leaf protoplasts, and the fluorescence was analyzed after 24 hr with a confocal microscope.

- (A) GFP fluorescence in a protoplast transformed with 35S-*VDL*73-*GFP*.
 (B) Chlorophyll fluorescence of the protoplast shown in (A).
 (C) Merging of (A) and (B).
 (D) GFP fluorescence in a protoplast transformed with the control 35S-*GFP*.

dance with the discussion above of the differences between *VDL* and *VDL'*, we suggest that *VDL'* does not fully complement *VDL*, either because the exchanged transcription promoter region does not support correct expression or because possible regulation of the alternative splicing does not provide enough full-length transcripts. In those tissues or cells in which the required extent of *VDL* function is not reached, alteration of plastid gene expression might contribute, in a snowball effect, to further jamming of the expression machinery and finally lead to developmental arrest.

METHODS

Plant Materials

The *vdI* mutant was originally found in a population of transgenic tobacco (*Nicotiana tabacum*) plants expressing the yeast nuclear gene *MIP1* encoding a mitochondrial DNA polymerase linked to the cauliflower mosaic virus (CaMV) 35S transcription promoter (Grec et al.,

2000). The E6A transformant was chosen for this study because of the variegation phenotype in its F_1 progeny. The mutant was propagated at the heterozygous stage in the greenhouse (16 hr of light at 25°C). The self-fertilized progeny of heterozygous plants were maintained in vitro (16 hr of light at 25°C) in Murashige and Skoog (MS) agar (Murashige and Skoog, 1962): MS salts, 3% sucrose, and 0.7% agar.

T-DNA Cosegregation Analysis

Total DNA was extracted as described by Rogers and Bendich (1994). Restricted DNA (10 μ g) was electrophoresed on a 0.7% agarose gel and transferred to a nylon membrane (Hybond+; Amersham Corp.), which was prehybridized for 3 hr, then hybridized overnight at 42°C in $5 \times$ SSC (1 $\times$ SSC is 0.15 M NaCl and 0.015 M sodium citrate), $2 \times$ Denhardt's solution (1 $\times$ Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% BSA), 1% SDS, 330 μ g/mL salmon sperm DNA, 0.5 mM EDTA, pH 8.0, and 50% formamide. 32 P-labeled probes were prepared as follows. An 0.8-kb EcoRI/NruI fragment containing a 0.2-kb T-DNA left border region and a 0.6-kb flanking vector sequence was used for EcoRI-digested samples, whereas for XbaI-digested samples, a 1.4-kb XbaI/NruI fragment containing a 0.2-kb T-DNA left border region, a 0.6-kb E9 terminator, and a 0.6-kb flanking vector sequence was used. A 2.0-kb NheI fragment containing the *nptII* gene and the T-DNA right border was used for both EcoRI- and XbaI-digested samples. The blots were rinsed twice for 15 min at room temperature with $2 \times$ SSC and 0.1% SDS, then twice for 15 min at 65°C with $0.1 \times$ SSC and 0.1% SDS.

Inverse Polymerase Chain Reaction Amplification of T-DNA-Flanking Genomic Sequences

Genomic DNA was digested with the indicated restriction enzyme, then extracted with phenol/chloroform and precipitated with ethanol. Digested DNA (2 μ g) was self-circularized overnight at 16°C in 1 mL of ligation buffer containing 20 units of T4 ligase. After inactivation for 10 min at 75°C and ethanol precipitation, standard conditions were used for polymerase chain reaction (PCR) amplification. The primers for amplification of the 1.0-kb T-DNA-containing fragment were pTLB1 (5'-CATGGATCCATTAAGTTGTCTAAGCGTC-3') and pTLB2 (5'-GCCAAGCTTAACACATTGCGGACGTTTT-3'). The primers for amplification of the 1.6-kb T-DNA-containing fragment were pTLB3 (5'-CGCACCGATCGCCCTTCCCAACAG-3') and pTLB4 (5'-CGCTATTACGCCAGCTGGCGAAAG-3'). After cloning and sequencing, new pairs of primers were designed on the basis of the sequence information to obtain a series of overlapping genomic fragments.

3' and 5' Rapid Amplification of cDNA Ends

RNA was extracted from in vitro-grown wild-type seedlings by using the guanidinium method (Strommer et al., 1993) and poly(A)⁺ mRNA was purified with the PolyATtract mRNA Isolation System IV (Promega, Madison, WI). For 3' rapid amplification of cDNA ends (RACE), the first cDNA strand was synthesized by using an oligo(dT) primer. PCR was performed with an oligo(dT) primer and either primer pRH1 (5'-GGAAGAGGTTGGATATGTGAT-3') or primer pRH4 (5'-ACAGGT-TCTGGAACAAACC-3'). For a second amplification, the oligo(dT)

primer and either the nested primer pRH3 (5'-TCGGGACGTGAT-TGTGTGCTT-3') or pRH5 (5'-CTTGGCATACCTGCTACAAATA-3') were used.

For 5' RACE, double-stranded cDNA was synthesized from purified mRNA by using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) and ligated to adaptors. Specific 3' primers were designed on the basis of the sequence obtained from the 3' RACE clones: pRH8 (5'-AGTTCTGAAGGCTTTGCAGCGAGCA-3'), pRH11 (5'-GGTTTTGTGGAGGCATTGTCAG-3'), and pRH14 (5'-GAAACA-ACACTCCTTACAGAAACC-3'). PCR was then performed with 3'-specific and adaptor primers.

PCR products were cloned into pGEM-T Easy vector (Promega) and sequenced. Independent clones were sequenced to detect any mutations occurring during PCR.

The full-length cDNAs were obtained by PCR with specific 5' and 3' primers as follows: for *VDL*-1, pTA5*A (5'-GGATCCATCGTACTT-TTTCCTATTGTGT-3') and pTFL65B (5'-GGGGTACCCCTAAAGAAT-AGCTGTTCCACAACA-3'); for *VDL*'-1, *VDL*'-4, *VDL*'-5, *VDL*'-6, *VDL*'-7, and *VDL*'-8, pTABis5*A (5'-GGATCCCTCGAAACGGCCTCTACGC-3') and pTFL65B; for *VDL*-2, pTA5*A and pTFL60 (5'-GGGGTACCC-CGAATAATGTTCTCCCTACCTAGAG-3'); for *VDL*'-2, pTABis5*A and pTFL60; for *VDL*-3, pTA5*A and pTB3*B (5'-GGCAGATGG-AAAGTTAGTAATTC-3'); and for *VDL*'-3, pTABis5*A and pTB3*B.

The GenBank accession numbers for the cDNA and genomic sequences are AF261017 to AF261031.

RNA Extraction and Reverse Transcriptase-PCR

RNA was extracted from various tissues of wild-type and heterozygous and homozygous mutant plants by using the guanidinium method (defined above) and poly(A)+ mRNA was purified with the PolyATtract mRNA Isolation System IV (Promega). The first-strand cDNA was synthesized as for 3' RACE. PCR was performed with the primers defined above and mentioned in the legends. Other primers were as follows: pRH16 (5'-GCTGGGGAATTGAAGCACTAGCAAA-3'), pTFL50 (5'-AGTCCCTACTGGCTACTTCCCC-3'), and pTFL57 (5'-AGATAACCTCCTCCTCCTCGAAGT-3').

Transformation and Complementation of *vdI*

The plasmid pBinhygTX (Gatz et al., 1992), which contained a hygromycin resistance selectable marker, a modified 35S promoter (Triple-op), and the octopine synthase terminator, was used for expression of the *VDL*-1 cDNA. A 1.5-kb *Sall* fragment containing the *VDL*-1 cDNA was subcloned into the *Sall* site of pBinhygTX to generate p35Shyg*VDL*1. The plasmid BinhygTXGUS, containing the β -glucuronidase A (*gusA*) gene under the control of the Triple-op promoter (Gatz et al., 1992), was used as a control. Both plasmids were introduced into *Agrobacterium tumefaciens*. Leaf discs from a homozygous *vdI* plant were transformed as described by Rogers et al. (1986) and then selected in the presence of 50 μ g/mL hygromycin B. Regenerated shoots were transferred to MS agar containing 50 μ g/mL hygromycin B for root formation.

Expression of Green Fluorescent Protein Fusion Protein in Tobacco Protoplasts

We constructed a pUC-derived plasmid in which the green fluorescent protein gene (*GFP*) (Haseloff et al., 1997) was linked to the se-

quence encoding the 73 N-terminal amino acid residues of *VDL*-1. The junction read as follows: 5'- . . . CGGGAACCTCTGTCATGGT-CACCGGGGTACCATGAGTAAAGGAGAA . . . -3' (*VDL*-1 and *GFP* nucleotides are shown in roman and italic type, respectively; the *KpnI* site is underlined). The chimeric gene was placed under the control of the CaMV 35S transcription promoter. This construct and a control containing the *GFP* gene without any additional coding sequence were introduced into tobacco protoplasts by electroporation (Lukaszewicz et al., 1998). GFP expression was analyzed by confocal microscopy (MRC-1024 laser scanning confocal microscope; Bio-Rad) 24 hr after transformation.

Light and Transmission Electron Microscopy of Leaf or Root Sections

Tissue processing, embedding, and sectioning were performed as described by Harris et al. (1994).

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