Arabidopsis VARIEGATED 3 encodes a chloroplast-targeted, zinc-finger protein required for chloroplast and palisade cell development

Henrik Næsted[1], Agnethe Holm[1], Tom Jenkins[1], H. Bjørn Nielsen[2], Cassandra A. Harris[3], Michael H. Beale[3], Mathias Andersen[4], Alexandra Mant[5], Henrik Scheller[5], Bilal Camara[6], Ole Mattsson[1] and John Mundy[1,*]

[1]Institute of Molecular Biology, Copenhagen University, Øster Farimagsgade 2A, 1353 Copenhagen K, Denmark
[2]BioCentrum, Technical University of Denmark, Building 208, 2800 Lyngby, Denmark
[3]Long Ashton Research Station, University of Bristol, Weston Road, Bristol, BS41 9AF, UK
[4]Research Center Foulum, PO Box 50, 8839 Tjele, Denmark
[5]Institute of Plant Biology, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, 1871 Frederiksberg, Denmark
[6]CNRS, Institut de Biologie Moleculaire, Rue de General Zimmer 12, 67084 Strasbourg, France
*Author for correspondence (e-mail: mundy@biobase.dk)

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Summary

The stable, recessive *Arabidopsis variegated 3* (*var3*) mutant exhibits a variegated phenotype due to somatic areas lacking or containing developmentally retarded chloroplasts and greatly reduced numbers of palisade cells. The *VAR3* gene, isolated by transposon tagging, encodes the 85.9 kDa VAR3 protein containing novel repeats and zinc fingers described as protein interaction domains. VAR3 interacts specifically in yeast and in vitro with NCED4, a putative polyene chain or carotenoid dioxygenase, and both VAR3 and NCED4 accumulate in the chloroplast stroma. Metabolic profiling demonstrates that pigment profiles are qualitatively similar in wild type and *var3*, although *var3* accumulates lower levels of chlorophylls and carotenoids. These results indicate that VAR3 is a part of a protein complex required for normal chloroplast and palisade cell development.

Key words: VAR3, NCED4, Transposon tagging, Chloroplast development, Stromal subcellular localization

Introduction

Chloroplast functions are dependent upon proteins encoded both within the plastid and nuclear genomes. Nuclear genes whose mutations affect overall chloroplast function encode proteins involved in photosynthesis, plastid metabolism, chloroplast and host cell biogenesis, or in nuclear-chloroplast traffic and signaling. Signaling between the nucleus and chloroplasts regulates both plastid and nuclear gene transcription (reviewed by Mullet, 1993; Barkan et al., 1995). An example of chloroplast-to-nucleus signaling is the decreased transcription of nuclear *Lhcb (CAB)* and *RbcS* genes when plants are treated with the carotenoid biosynthetic inhibitor norflurazon that leads to photo-oxidized, nonfunctional plastids. However, *gun* (genome uncoupled) mutants constitutively express *Lhcb* genes under such conditions. Analysis of *gun* mutants indicates that the tetrapyrrole intermediate Mg-protoporphyrin (Mg-ProtoIX) acts as a chloroplast-to-nucleus signal that regulates expression of nuclear genes encoding chloroplast-targeted proteins (Strand et al., 2003).

Several classes of mutation result in variegated plants that have leaves consisting of normal green and also white or yellow sectors in which chloroplast development is retarded or disrupted. For example, variegation is seen in plants with unstable mutations that affect chloroplast function, such as those due to nuclear transposition events (Chatterjee et al., 1996; Keddie et al., 1996). In addition, variegation may be caused by the induction of defective plastids in some cells by stable, loss-of-function mutations in nuclear genes (reviewed by Rodermel, 2001; Sakamoto, 2003). Such mutant alleles that have been characterized in *Arabidopsis* include *chloroplast mutator* [*chm* (Abdelnoor et al., 2003)], *yellow variegated 1* [*var1*: Sakamoto et al., 2002) and *var2* (Takechi et al., 2000)] and *immutans* (*im*). *VAR1* and *VAR2* encode thylakoid proteins related to the bacterial chaperone metalloprotease FtsH. FtsH represents a family of 12 different nuclear genes, nine of which encode FtsH proteins located in the chloroplast. However, only mutations of *VAR1* or *VAR2* have been shown to cause typical leaf variegation and sensitivity to photoinhibition. This and other data indicate that VAR1 and VAR2 play important roles in the repair cycle of photosystem II thylakoid membranes (Sakamoto et al., 2003).

The formation of albino sectors in the *im* mutant is enhanced by increased light intensity. IM is a chloroplast-targeted, alternative oxidase involved in electron transfer associated with the desaturation of phytoene, a carotenoid intermediate, via phytoene desaturase (Carol et al., 1999; Wu et al., 1999). Carotenoids function both as accessory pigments in the photosynthetic apparatus, and in photo-protection by quenching reactive oxygen species. Thus, loss of *IM* function renders plastids susceptible to photo-oxidation. Interestingly, the promoter of nuclear-encoded phytoene desaturase is induced by

inhibitors of chlorophyll and carotenoid biosynthesis (Corona et al., 1996). This suggests that carotenoids serve as plastid signals regulating nuclear gene expression.

Genetic lesions affecting chloroplast development do not always alter leaf morphology. However, *Arabidopsis im* and several non-variegated recessive mutations [*pac1* (Reiter et al., 1994); *cla1* (Crowell et al., 2003); *cue1* (Streatfield et al., 1999)] exhibit perturbed chloroplast and leaf development, including failure in palisade cell expansion. As these genes encode chloroplast proteins, mutations in them may affect cell development because of incomplete chloroplast biogenesis. While most such mutations lead to decreased *Lhcb* and *RbcS* mRNA levels, *pac1* accumulates normal levels of *Lhcb* mRNA. This indicates that pathways mediating plastid developmental signals are separable from pathways that regulate expression of nuclear-encoded photosynthesis genes.

We describe a recessive, stably tagged *Arabidopsis* mutant, designated *variegated 3* (*var3*), that exhibits variegation and markedly reduced palisade cell numbers, but normal levels of *Lhcb* and *RbcS* mRNAs. The *VAR3* gene and corresponding full-length cDNA were isolated and shown to encode the novel VAR3 zinc-finger protein. Genetic, molecular and biochemical analyses indicate that VAR3 interacts with, and may thereby affect the activity of, chloroplast localized enzymes.

Materials and Methods

Isolation and characterization of *var3*

F3 progeny of transposants generated in ecotype L*er* (Sundaresan et al., 1995) were examined for visible mutant phenotypes by growing 12 plants per line in soil. Scanning electron microscopy (SEM) of leaves was performed using standard protocols. For transmission electron microscopy (TEM), leaves were fixed overnight at 5°C in 0.1 M phosphate-buffered saline (pH 7.0) 2.5% glutaraldehyde and 2% paraformaldehyde, postfixed in 1% OsO_4, dehydrated in acetone, followed by infiltration with epoxy resin (SPURR). Ultra thin sections were put on Formvar-covered whole grids and examined in a Jeol 100CX microscope.

Gene expression in *var3*

Total RNA from 2-week-old wild-type and *var3* plantlets exhibiting extensive variegation under constant illumination (435 μmol m^{-2} s^{-1}) was prepared for RNA blot hybridizations and reverse transcription (RT) using standard protocols (RNAgents total RNA; Promega). RT-PCR was performed with a kit (Perkin Elmer, N808-0069) using an RT primer (5′CAGTTCTGGTCGTTCACGAGCA) and forward primer (5′ATGAACAACTCCACCAGACTCATCTCCCTC). EF-1α control primers were 5′GTTTCACATCAACATTGTGTTCATTGG and 5′GAGTACTTGGGGGTAGTGGCATCC.

For photosynthetic gene expression analysis under photo-oxidative conditions, total RNA was prepared from *Arabidopsis* L*er* and *var3* seedlings according to the method of Logemann et al. (Logemann et al., 1987) and grown for 10 days in continuous white light (435 μmol m^{-2} s^{-1}) on 1× MS medium, 2% sucrose plates with or without 5 μM norflurazon. For RT-PCR, 1 μg of template total RNA was DNase treated according to manufacturer's protocols (Gibco-BRL). *Lhcb1* primers for RT-PCR were 5′GGTACGATGCTGGGAAAGAGC and 5′ACACTCACAGACAGACATGAA. RT-PCR was performed with 25 cycles to ensure that products would remain within the linear range. EF-1α control primers were again used for the control, and RT-PCR was performed with 27 cycles of amplification.

Standard protocols for microarray analysis by the Arabidopsis Functional Genomics Consortium were followed (afgc.stanford.edu/afgc_html/AFGCProtocols-fev2001.pdf), and the *var3* hybridization data is available under proposal #944-DCA-505-GEJ-7. A data set (http://genome-www4.stanford.edu/MicroArray/SMD/) of intensity signals from 14 wild-type arrays (CH1D_MEAN values of ExptID 11595, 10658, 11601, 10651, 11592, 9755, 10653, and CH2D_MEAN values of ExptID 11598, 10659, 11602, 10652, 11594, 8031, 10654) was used to estimate gene-specific variation in wild-type hybridizations, and two arrays for comparing *var3* mutant and wild type (ExptID 10653 and 10654, CH1D_MEAN and CH2D_MEAN). All data are from array print batch CIW-2_05-00. This data set was normalized by fitting a smoothed spline to the correlation between the cumulative density distribution of a virtual average array (one channel) and a given array channel. The spline function was subsequently applied to the array data points (Workmann et al., 2002). The 14 wild-type arrays exhibited consistent intensity measurements and allowed estimates of gene-specific standard deviation (std_{14Xwt}). The geometric mean of the z-like score (int_{var3}-int_{wt})/std_{14xwt} from the two arrays was then used to rank the genes in Fig. 2C.

Pigment profiling

Wild-type and *var3* seeds were germinated on MS medium containing 3% sucrose, and incubated at 22°C under constant light (435 μmol m^{-2} s^{-1}). Approximately 140 seedlings were harvested at the 2-4 leaf stage, freeze-dried, and five replicate methanol extractions prepared for each type of seedling using the method of Fraser et al. (Fraser et al., 2001). Extracts were analyzed for carotenoids and chlorophylls by HPLC with a photo-diode array detector according to Bramley (Bramley, 1992). Peak assignments were made by co-injection with authentic standards. Unknown carotenoids and chlorophylls were suggested by the UV spectra of the peaks.

VAR3 gene, cDNA and *var3* complementation

DNA for inverse PCR and Southern blotting was isolated with the DNeasy kit (Qiagen). Genomic sequence flanking the *Ds* insertion was amplified by inverse PCR. 50 ng of *var3* DNA was digested with *Eco*RI (20 μl volume) and self-ligated with *E. coli* T4 ligase (New England Biolabs). Two μl of the ligation was used as template with transposon/GUS primers (5′CCAGACTGAATGCCCACAGGCCGT-CG and 5′CGGCGATTTGGAAGGCAGAGAAGG). The *VAR3* gene was annotated from sequences surrounding the *Ds* insertion site using BLAST (www.ncbi.nlm.nih.gov/BLAST), NetPlantGene (cbs.dtu.dk/services/NetPGene), Psort (psort.nibb.ac.jp), ChloroP (cbs.dtu.dk/services/ChloroP), PFSCAN (irec.isb.ch/software/PFSCAN), PFAM (sanger.ac.uk/Software/Pfam/search.shtml), and ClustalW with Boxshade (clustalw.genome.ad.jp). Primers including the predicted *VAR3* start and stop codons (5′TATTAAAGATCTATGAACAACTC-CACCAGACTCATCTCCCT and 5′TATTAAAGATCTTCATTTAT-CTCCTTTACCAGTGGGATCAG) were used to amplify a *VAR3* cDNA by PCR using λ-phage DNA isolated from a cDNA library (CD4-6, Arabidopsis Biological Resource Center, Ohio, USA) as template with annealing at 55°C for 5 minutes and elongation at 68°C for 5 minutes. A 2277 bp fragment was amplified and cloned into a pCR-blunt vector (Invitrogen, k2700-20) and sequenced using an ABI 310 sequencer. Complementation of *var3* was performed with a genomic fragment of 4,941 bp, including 1,541 bp 5′ to the *VAR3* start codon and 539 bp 3′ to the stop codon (nt. 56,462 to 51,521 of clone MVA3, AB006706) isolated by long range PCR using ecotype Landsberg DNA template and primers 5′GACCCAGAAAACAAAA-GGTGGCGAG and 5′CTTGCCTCTAACGACGTGTTTTCGG. The product was cloned in the *Sma*I site of pCAMBIA 3300 carrying a BASTA resistance marker (thaliana.botany.wisc.edu/cambia), and the resultant construct transferred via *Agrobacterium* into *var3* plants by vacuum infiltration (Bechtold et al., 1993). T1 BASTA resistant, wild-type plants were selected and shown to carry the *VAR3* transgene, and to be homozygous for the *var3* insertion allele by PCR. Segregation analysis of T2 progeny showed that all BASTA resistant T1 plants

were homozygous for kanamycin resistance carried on *Ds*, and that the *var3* phenotype segregated 1:3 wild type.

VAR3 localization

An *Nco*I-linkered VAR3 cDNA fragment containing the ORF without the stop codon was generated by PCR with *Pfu* (Promega) and VAR3 linker-primers (5′TATTAACCATGGCCAACTCCACCAGACTCATCTCCCTC; 5′TATTAACCATGGATTTATCTCCTTTACCAGTGGGATCA) and the VAR3 cDNA template. This fragment was cloned in *Nco*I of binary vector pCAMBIA 1303 carrying a hygromycin resistance marker, generating a single ORF encoding VAR3, GUS and GFP under control of the 35S promoter. This construct was transformed via *Agrobacterium tumefaciens* strain GV3101 into wild type and *var3*. T1 plants were selected on hygromycin and analyzed for phenotype, GUS activity and GFP fluorescence. Two wild-type lines were also shown by PCR to contain GUS carried on pCAMBIA 1303 and to be homozygous for *Ds* in *var3*. These lines were analyzed for GFP fluorescence using a Zeiss LSM510 microscope.

VAR3 interaction analyses

A cDNA library was used according to the manufacturer's instructions (Clontech, FL4000AB). The full-length *VAR3* cDNA bait was cloned as a *Bam*HI-linkered fragment with primers 5′TATTAAGGATCCCGATGAACAACTCCACCAGACTCATCTCCCTC and 5′TATTAAGGATCCTCATTTATCTCCTTTACCAGTGGGATCAGAGTC into vector pGBD-C1 that was introduced into strain PJ69A-4A (James et al., 1996). This strain was transformed with library cDNA and screened for prototrophic growth on medium lacking tryptophan, leucine, histidine and adenine for 4 days at 30°C. A total of 15×10^6 transformants were screened to yield 60 positive clones. These clones were assayed for β-galactosidase activity that eliminated nine clones. DNA was extracted from the remaining clones and used as templates with prey pGAD10 primers (Clontech, 9103-1) to size inserts and for sequencing. One clone of each of the three prey insert groups was rescued in *E. coli* and re-sequenced. To confirm interactions, bait and prey plasmids were individually co-transformed into PJ69A-4A and re-evaluated for prototrophic growth and β-galactosidase activity. Control *Arabidopsis* cDNAs included IM (AF098072), Ran1 (X97379), Ran2 (X97380), Ran3 (X97381), RanBP1a (X97377), Ranbp1b (X97378), RanGAP (AF214559), and Ran1-GTP and Ran1-GDP variants (Haizel et al., 1997). To further confirm VAR3 interaction, prey proteins were synthesized by in vitro transcription/translation (TnT, Promega) and co-immunoprecipitated according to Clontech (Co-IP, PT3323-1). NCED4 lacks introns and the full-length open reading frame was amplified with *Pfu* from genomic DNA with *Eco*RI and *Xho*I linker primers (5′TATTAAGAATTCGCAATGGACTCTGTTTCTTCTTCTTC; 5′TATTAACTCGAGTGCGATTTGTTGTACGGGACGTAATT) and cloned into pGADT7.

Chloroplast import assays

VAR3 cDNA encoding the full-length precursor of VAR3 (preVAR3) was amplified by PCR and cloned in pGEM-T (Promega). The plasmid pGADT7:NCED4 encoding the full-length precursor of NCED4 (preNCED4) was partially digested with *Nco*I, to remove the vector HA epitope tag, and then religated. *VAR3* and *NCED4* were transcribed with T7 RNA polymerase and translated in vitro using a coupled transcription-translation rabbit reticulocyte lysate kit, (Promega) in the presence of L-[^{35}S]methionine (Amersham Biosciences). Plasmid constructs encoding wheat pre33K [33 kDa subunit of the oxygen-evolving complex (Kirwin et al., 1989)] and pea *RbcS* precursor were transcribed in vitro using SP6 RNA polymerase, and translated using a wheat germ lysate system (Promega), in the presence of L-[^{35}S]methionine.

Pea (var. Kelvedon Wonder) chloroplasts were isolated and import assays performed according to the method of Robinson and Mant (Robinson and Mant, 2002). Briefly, intact chloroplasts equivalent to 50 μg chlorophyll were mixed with 8 mM MgATP, 5 mM unlabeled L-methionine, 5 mM unlabeled L-cysteine, 5 mM $MgCl_2$ and 12.5 μl translation mixture, all in HS buffer (330 mM sorbitol, 50 mM Hepes-KOH pH 8.0), final volume 150 μl. Assays were incubated for 60 minutes at 26°C in an illuminated water bath (150 μmol photon m^{-2} s^{-1}). Incubations were halted by the addition of 1 ml ice-cold HS, followed by centrifugation at 3000 ***g*** for 5 minutes at 4°C. Chloroplast pellets were resuspended in 120 μl HS and 30 μl removed, representing total chloroplasts. The remaining 90 μl chloroplasts were incubated for 40 minutes at 4°C with 0.2 mg ml^{-1} thermolysin (Protease type X; Sigma), 2.5 mM $CaCl_2$ in a final volume of 250 μl HS. The protease digestion was halted with 10 mM EDTA and one third of the sample removed (protease-treated chloroplasts sample). The remaining chloroplasts were centrifuged at 3000 ***g*** for 5 minutes at 4°C, and resuspended in lysis buffer (10 mM Hepes-KOH, pH 8.0, 5 mM $MgCl_2$, 10 mM EDTA). After 5 minutes incubation on ice, the lysate was centrifuged at 20,000 ***g*** for 5 minutes at 4°C. The supernatant was the stromal extract, and the pellet, thylakoid and envelope membranes. Membranes were resuspended in lysis buffer without EDTA and divided into equal portions. One portion was analyzed directly, the other after incubation with 0.2 mg ml^{-1} thermolysin, 2.5 mM $CaCl_2$ for 40 minutes on ice. Thermolysin incubation was stopped by addition of 10 mM EDTA and centrifugation at 20,000 ***g*** for 5 minutes at 4°C. All chloroplast fractions were mixed with protein sample buffer and heated to 95°C for 4 minutes before analysis by SDS-PAGE and fluorography.

Results

Characterization of the *var3* mutant

The *var3* phenotype was identified in a population of stable transposants generated with a *Ds* gene trap (Sundaresan et al., 1995). *var3* was inherited as a recessive allele that co-segregated with kanamycin resistance carried on the *Ds* element. *var3* displayed normal skotomorphogenesis, and the cotyledons developed normally in the light, but the first true leaves exhibited variegation seen as yellow areas (compare Fig. 1A and B). This variegation became indistinct in older leaves so that the only phenotypic difference compared with wild type (L*er*) at flowering was pale flower bud sepals in *var3* (Fig. 1D and E). Variegation in *var3* differed from that in *im* (Fig. 1B,E compared with C,F) which consisted of pronounced, light-dependent sectoring. Light microscopy of *var3* leaves showed that yellow areas contained few if any palisade mesophyll cells, which resulted in a rough leaf surface (compare Fig. 1G and H). Scanning electron microscopy confirmed that these areas collapsed during critical point drying (Fig. 1I,J).

To study formation of the yellow areas, L*er* and *var3* plants were grown under high or low light intensities (16 hours light, 435 μmol·photons $m^{-2}\cdot s^{-1}$ and 125 μmol·$m^{-2}\cdot s^{-1}$) and *im* plants were grown under low light as a reference. Pictures were taken at 2- or 3-day intervals and leaf size and the relative size of the yellow areas were estimated by image analysis. In some of the emergent first to fourth leaves, up to 80% of the leaf area could be yellow, but their relative area decreased at a rate comparable to that of the leaf growth rate. Subsequent leaves exhibited markedly less patchiness. A decrease in the relative area of white sectors was also seen in *im*, but both the change in their percentage area and rate of decrease were lower (not shown). Moreover, all *im* rosette leaves formed could be equally affected, and demarcations between their green and white sectors remained distinct.

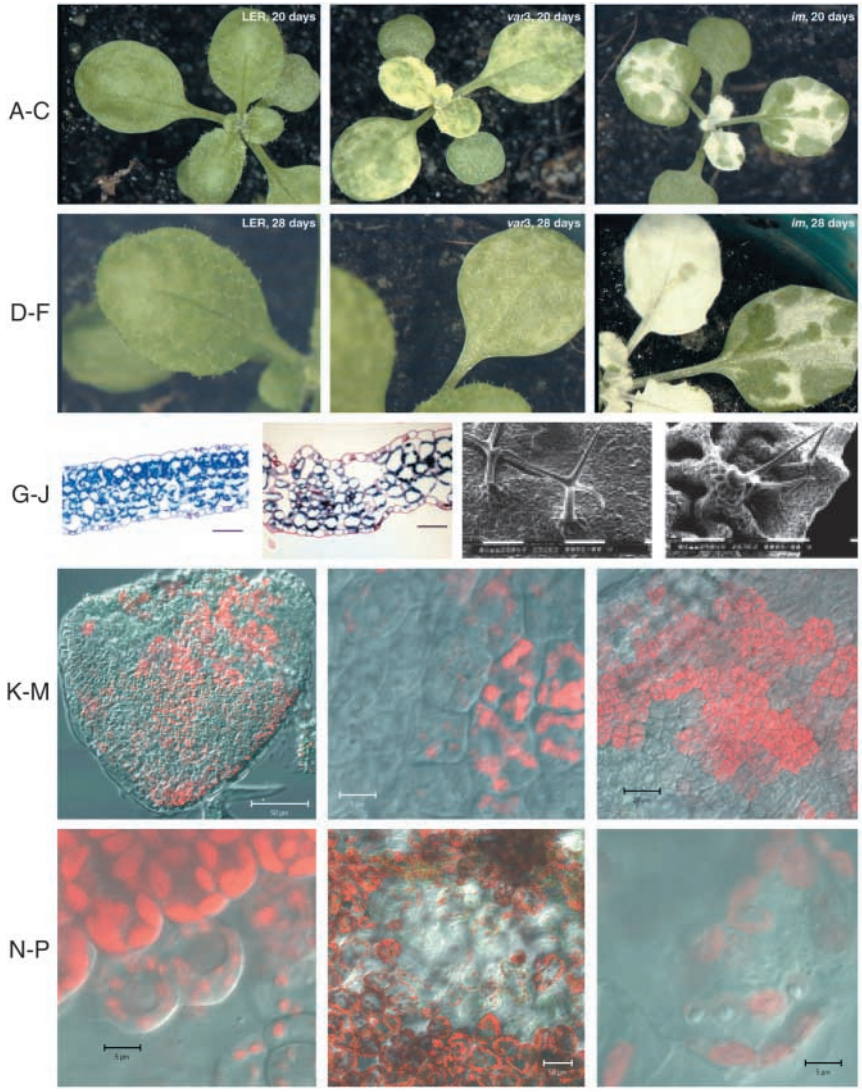


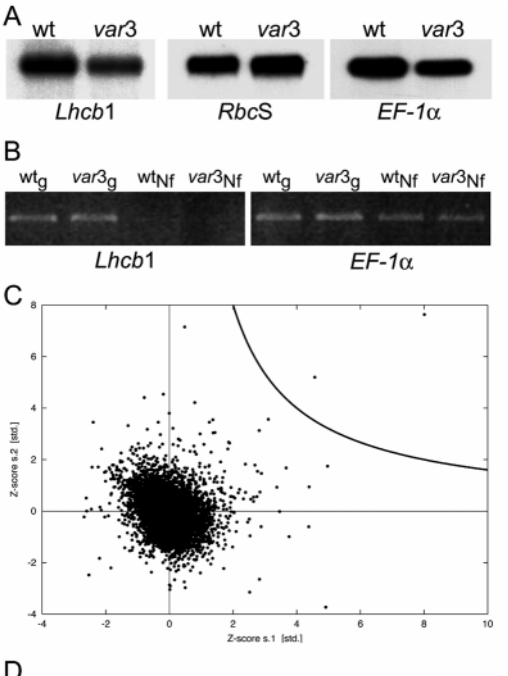
Fig. 1. The *var3* mutant. (A-C) Leaves of 20-day-old, wild type (L*er*), *var3* grown under high light and *immutans* (*im*) under low light. (D-F) Second or third true leaves at 28 days. (G-H) Light micrographs of cross sections of 12-day-old L*er* and *var3* leaves. Scale bars, 100 μm. (I-J) Scanning electron micrographs of first true leaves of 12-day-old L*er* and *var3*. Scale bars, 100 μm. (K-P) Confocal images with merged red and Nomarsky channels. Scale bars, 50 μm for K,O; 5 μm for L,N,P; 20 μm for M. K and N are of live material, other images are of formaldehyde fixated tissue. (K) Leaf initial before emergence at the shoot tip. (L) Detail from K showing cells with normal, reduced and undetectable chloroplast development. (M) Variegation in pre-emergent leaf. (N) Detail from M of chloroplasts with normal and arrested development. (O) Light patch with reduced or no chloroplast development, surrounded by normal mesophyll cells in a 16-day-old third leaf. (P) Plastids from the leaf in O with reduced development.

Confocal microscopy revealed that *var3* variegation started at early stages of chloroplast development shortly before the leaves emerged at the shoot apex (Fig. 1K). At this stage, areas of cells with normally developed chloroplasts were mingled with areas with partially developed and undeveloped plastids, as judged by the red fluorescence of chlorophyll and precursors and by plastid size (Fig. 1L-N). Since normal cells were found abutting cells in the yellow areas, the effect of *VAR3* appeared to be cell-autonomous. This pattern of yellow areas was retained in expanded leaves (Fig. 1O,P), and rudimentary lamellae could be seen in partially developed chloroplasts and 'vacuoles' of swollen proplastids within their cells. The presence of such small chloroplasts with reduced numbers of thylakoids was confirmed in these cells by transmission electron microscopy (not shown). In contrast, cells containing normal chloroplasts together with clearly undeveloped plastids were not observed. This indicates that *var3* cells are not heteroplastidic.

Gene expression in *var3*

The levels of expression of *Lhcb1* and other genes involved in photosynthetic reactions may be regulated by plastid-derived signals including chlorophyll precursors and carotenoids (reviewed by Rodermel, 2001). This initially suggested that the expression levels of nuclear genes like *Lhcb1* or *RbcS* might be reduced in *var3* because of its defective chloroplasts. However, northern blotting with total *var3* RNA revealed only a slightly less accumulation of *Lhcb1* and *RbcS* mRNAs than in wild type (Fig. 2A). In order to determine whether *var3* represented a *gun* mutant, expression of *Lhcb1* was investigated under photo-oxidative conditions in the presence of 5 μM norflurazon. Both L*er* and *var3* seedlings accumulated *Lhcb1* under nonphotobleaching conditions. However, *Lhcb1* mRNA was not detected under photo-oxidative conditions in the presence norflurazon (Fig. 2B). In contrast, control EF-1α (elongation factor 1 alpha) expression was detected in both the presence and absence of norflurazon. This indicates that VAR3 is not involved in the Mg-ProtoIX plastid-to-nucleus signaling pathway.

To gain insight into the effect of the *var3* mutation on global gene expression, expression profiling of wild-type versus *var3* was performed with microarrays of some 8,000 cDNAs. The dataset was analyzed using the standard deviation of signal



Acc#		Description	Mean Z	Fold change
T21734	C	*rpo*B	7.8	4.8
Z18079	NC	Lipase/Hydrolase-like	3.3	3.0
N95849	M	26S rRNA	3.0	2.0
T04321	NC	Protein Kinase APK1A	2.9	2.2
T43623	NC	HSP70-like	2.5	1.6
N95975	M	18S rRNA	2.5	1.7
R83940	C	50S Ribosomal Protein L16	2.2	1.6
T46363	C	rps7 ycf1	2.2	2.4
R65487	NC	RBCs Binding Protein a	2.2	1.7
T22251	NC	Elongation Factor	2.1	1.5
AA042388	C	AtClpC	2.1	1.8

Fig. 2. Gene expression in wild type and *var3*. (A) RNA blot of 20 μg total RNA from wild type (wt) and *var3* probed with labeled cDNAs of *CAB* (X56062), *RbcS* (X13611; *Lhcb1*) and *EF-1α* (16260) control. (B) RT-PCR detection of *Lhcb1* in wt and *var3* under non-photo-oxidative conditions (green, wt_g and $var3_g$) and photo-oxidative conditions (5 μM norflurazon, wt_{Nf} and $var3_{Nf}$) compared with *EF-1α* control. (C) Scatter plot of z-score of slide 1 versus z-score of slide 2 (z-score=(int_{var3}-int_{wt})/std_{14wt}). Dots in first and third quadrants represent consistent measurements of clones from both arrays from which the *var3* sample was measured higher or lower relative to wt, respectively. Dots in second and fourth quadrants represent inconsistent measurements. The line represents a geometric mean of 4 std., equal to the most extreme, inconsistent measurement (geometric mean of absolute values). (D) The table shows the top genes ranked after the geometric mean of z-scores. C, chloroplast encoded; M, mitochondrion encoded; N, nuclear encoded; NC, nuclear encoded chloroplast targeted. *The protein was formerly designated HSP70-7 but is now known as Hsc70-2 (Sung et al., 2001).

intensities of specific clones estimated from the values for 14 wild-type arrays in the database (Fig. 2C). This identified genes most likely to be differentially expressed in *var3* based on the geometric mean of the z-score from the two arrays (Fig. 2D). This confirmed that expression of *Lhcb1* and *RbcS* mRNAs monitored by the microarray was not significantly different in *var3* and L*er*. In addition, it indicated that the levels of mRNAs of genes encoding proteins involved in chloroplast transcription and translation, as well as rRNAs from both

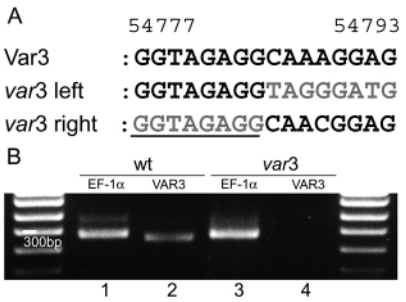


Fig. 3. *VAR3* locus and mRNA. (A) *VAR3* wild type, sequence of exon one (Var3), sequence of *var3* allele with *Ds* left border (in gray) (*var*3 left) and *var3* with *Ds* right border (gray) and the 8-bp repeat underlined (*var*3 right). Numbers give nucleotide position from the same sequence of wild type Col-O (complement of P1 clone MVA3, AB006706). (B) RT-PCR detection of *VAR3* mRNA in wild type (wt) and *var3* (lanes 2 and 4) compared to control *EF-1α* mRNA (lanes 1 and 3). Molecular markers are shown on both sides of the gel.

chloroplasts and mitochondria, were elevated in *var3* compared to wild type. This indicates that coordinated gene expression in the plastids, mitochondria and nucleus was perturbed in *var3* (Hedtke et al., 1999). For example, the most significantly elevated mRNA was *rpoB* or chloroplast-encoded RNA polymerase subunit 2. *rpoB* expression is dependent upon *DAG*, a nuclear gene whose unstable loss of function produces a phenotype similar to that of *var3* (Chatterjee et al., 1996). In addition, mRNA levels of a chloroplastic heat shock protein and a chaperone protein were elevated, and both of these types of proteins have been shown to associate with carotenoid metabolizing enzymes (Al-Babili et al., 1996; Bonk et al., 1997). This is of interest as VAR3 was shown to interact with homologs of 9-*cis*-expoxycarotenoid dioxygenase (NCED, below).

Isolation of *VAR3*

An *Eco*RI site approximately 200 bp from the *Ds* right border was used to isolate chromosomal sequence flanking *Ds* by inverse PCR. Product sequencing identified the site of *Ds* integration at nucleotide 54,785 of P1 clone MVA3 (AB006706) on chromosome V (Fig. 3A). The insertion site was confirmed by PCR using *var3* DNA as template with primers specific to each transposon end and to flanking genomic sequences. Sequencing revealed an 8 bp target site duplication typical of *Ds* insertions (Fig. 3A).

To identify the *VAR3* gene, 6 kb of clone MVA3 sequence (ecotype Columbia, Col) flanking the *Ds* insertion site was analyzed with NetPlantGene. This predicted four intron splice sites in five flanking regions with the codon capacity of exons. Exon assembly yielded a 2,274 bp open reading frame (ORF) encoding the VAR3 protein (Fig. 4A). The first predicted exon contained the *Ds* insertion after Ala46, indicating that *var3* does not produce functional VAR3 protein.

To confirm the genomic annotation, we isolated a full-length cDNA from a L*er* inflorescence mRNA library using PCR primers including the 5′ start and 3′ stop codons. This sequence has been deposited in public databases with the accession number AY050223. The 3′ end of this *VAR3* cDNA was identical to the sequence of a subsequently deposited EST (AV539640) isolated from a cDNA library from root mRNAs.

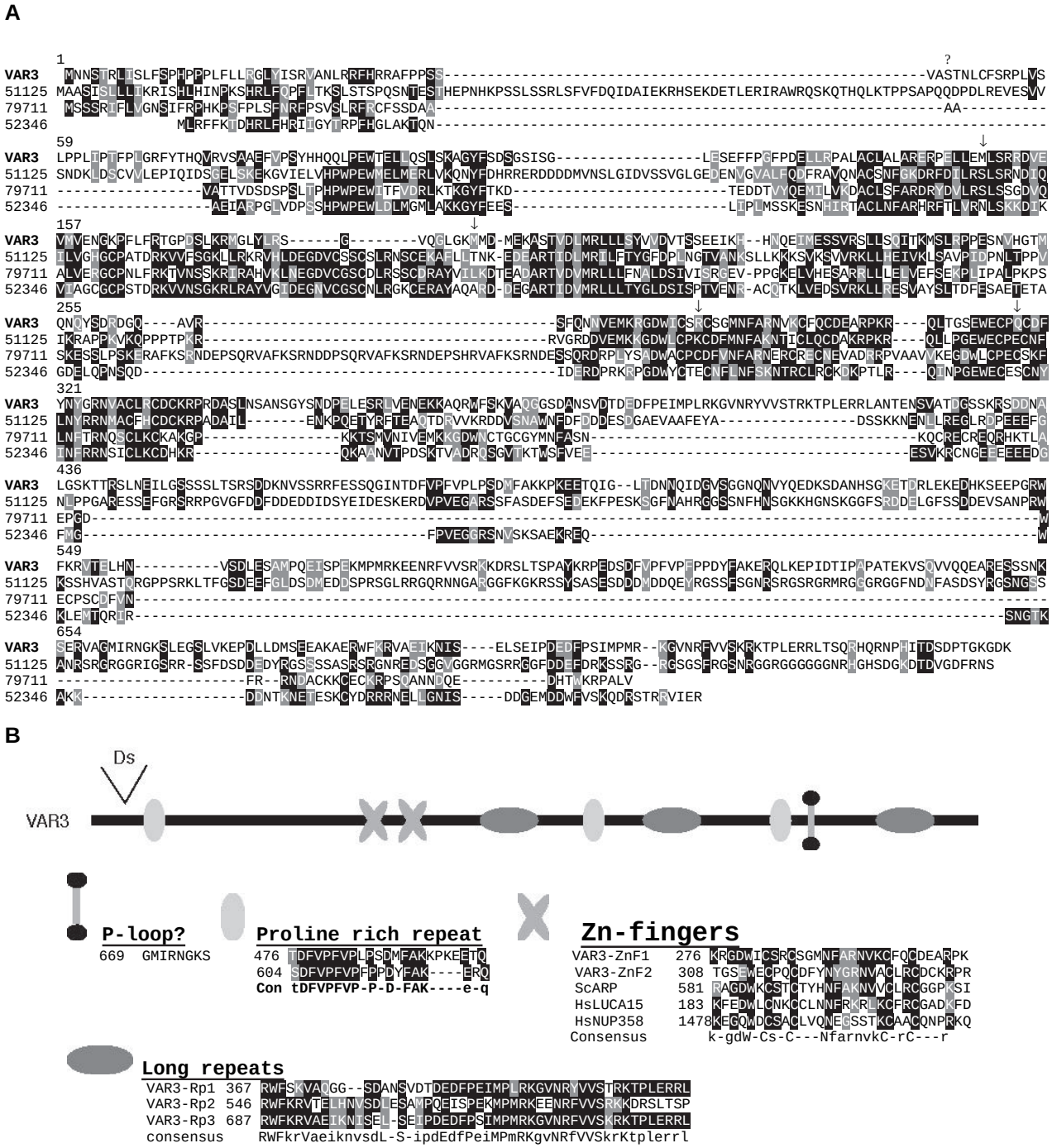


Fig. 4. VAR3 protein. (A) Alignment of VAR3 (accession number ATG517790) with three related *Arabidopsis* proteins (AAG51125, AAG52346 and AAF79711). Conserved residues are in black and similar residues in gray. Arrows indicate intron sites and the double arrow the *Ds* insertion site. (B) Top: similarity between VAR3 zinc-finger domains (ZnF1 and 2) with those from yeast (ScARP, P32770) and human nucleoporins (HsLUCA15, P52756; HsNUP358, P49792). Bottom: similarities defining the novel repeated regions of VAR3.

Completion of the *Arabidopsis* genome sequence produced an identical annotation of the *VAR3* gene (At5g17790).

The *var3* allele and *var3* complementation

Northern hybridization with the *VAR3* cDNA as probe failed to detect *VAR3* mRNAs in wild type and *var3*. In contrast, *VAR3* mRNA was detectable by reverse transcription PCR in wild type but not in *var3* (Fig. 3B). RT-PCR also detected low levels of *VAR3* mRNA in wild-type leaves and roots (not shown) from which the *VAR3* EST was isolated. These results indicate that *VAR3* mRNA is rare in wild type, and that the *Ds* insertion in *var3* produces a truncated mRNA that does not accumulate to detectable levels.

To confirm that the *Ds* insertion in *VAR3* was responsible for the *var3* phenotype, a wild-type *VAR3* genomic fragment was introduced into the *var3* mutant to examine complementation. The *VAR3* genomic fragment, including the transcribed region and 1500 bp of 5′ upstream and 500 bp of 3′ downstream sequence, was cloned into pCAMBIA3300 carrying BASTA resistance, and transformed via *A. tumefasciens* into homozygous kanamycin-resistant *var3* plants. Phenotypically wild-type, BASTA-resistant T1 progeny were shown by PCR to be homozygous for the *var3 Ds* allele, and to carry the complementing *VAR3* transgene. As expected, T2 progeny of these selfed T1 transformants were kanamycin resistant and segregated 3:1 for BASTA resistant, wild-type growth versus *var3* variegation. We conclude that loss of *VAR3* function produces the *var3* mutant phenotype.

Interestingly, one other T1 transformant showed somatic complementation such that about half of the plant appeared wild type, while the other half exhibited variegation as *var3*. PCR confirmed that the wild-type half tissue carried the *VAR3* transgene, while the variegated regions did not (data not shown). This confirmed that the effect of *VAR3* on plastid and palisade development is cell-autonomous.

VAR3 protein and homologs

The VAR3 protein contains 758 amino acids with a molecular mass of 85.9 kDa (Fig. 4A). Database homology searches revealed that VAR3 contains two tandem $C_2X_{10}C_2$ zinc-finger domains. These domains comprise a conserved 30 amino acid residue consensus (X_2GDWICX_2CX_3NFARRX_2CXRCX_2-PRPEX_2; pFAM00641) characterized in RAN binding protein 2 (RanBP2) and other nucleoporins (Fig. 4B). These proteins are involved in nuclear trafficking, and the zinc fingers of RanBP2 bind Ran-GDP (Nakielny et al., 1999). This suggests that the similar domains of VAR3 may be involved in protein-protein interactions. VAR3 also contains three longer 50 amino acid repeated sequences in the C-terminal region (Fig. 4B). Between these repeats are two shorter, proline-rich repeats that contain the minimal consensus signature of proteins interacting with WW (-P-P-X-Y-) or SH3 (-P-X-X-P-X-) domains (Pawson and Scott, 1997).

Proteins with overall sequence similarity to VAR3 are apparently only present in plant genomes including the monocot, rice (GI:13161434; BAB33004). *Arabidopsis* has three VAR3 homologs with similarities in their N-terminal regions, the zinc fingers, and some of the repeats in the C-terminal region (Fig. 4A,B). Two homologs (AAF79711 and AAG52346) are divergent only in the C-terminal half, while another (AAG51125) shares overall similarity to VAR3. VAR3 and these homologs may therefore have a modular structure composed of different protein-protein interaction domains.

The ChloroP algorithm predicted that VAR3 contains a 79 residue, N-terminal chloroplast targeting signal. ChloroP also predicted chloroplast target peptides for two of the *Arabidopsis* homologs (AAG51125 and AAF79711; Fig. 4B), but not for the third (AAG52346). Interestingly, the homolog (AAG51125) most similar to VAR3 also contains two putative, bipartite nuclear localization signals. It is therefore possible that VAR3 protein family members are differentially compartmentalized within cells.

VAR3 sub-cellular localization

To investigate VAR3 subcellular localization, *var3* was transformed with a construct carrying a hygromycin resistance marker and a translational fusion between VAR3, *E. coli* β-glucuronidase (GUS) and the *Aequorea victoria* green fluorescent protein (GFP) with 6× HIS tag (Var3-GUS-mGFP5-6×HIS) under control of the constitutive CaMV 35S promoter. Twelve wild-type looking, hygromycin-resistant T1 plants were selected that produced significant GUS reporter activity measured histochemically. T2 progeny of these lines proved to be kanamycin resistant and to segregate 3:1 hygromycin resistant wild type to hygromycin sensitive *var3*. This was confirmed for two lines by PCR for homozygosity of the *var3* insertion allele and for the VAR3 reporter transgene. Western blotting with an anti-GFP antibody detected the expected 182 kDa Var3-GUS-mGFP5-6×HIS fusion protein in Ni-NTA affinity purified proteins from total extracts of these plants (data not shown). These results indicate that expression of the triple fusion protein complemented *var3*.

Since VAR3 constitutes the N-terminal region of this functional triple fusion, this reporter is probably localized within cells similarly to wild-type VAR3 protein. Confocal microscopy was therefore used to examine the subcellular localization of GFP fluorescence in lines expressing GUS:GFP from the pCAMBIA 1303 control construct, and those expressing the complementing triple reporter. This showed that GFP fluorescence from the GUS:GFP control was localized throughout cells and not limited to the chloroplasts identified by their red autofluorescence (Fig. 5, panels 1-4 compared with 5-8). In contrast, GFP fluorescence from the triple VAR3:GUS:GFP reporter was clearly localized to chloroplasts (Fig. 5, panels 9-12). Closer inspection of this pattern in numerous cells revealed that although GFP fluorescence was visible throughout chloroplasts, it was concentrated in foci. These results indicate that the complementing VAR3 fusion accumulated in chloroplasts. The presence of GFP foci suggest it may accumulate in a suborganellar complex or compartment, although these foci may result from inappropriate aggregation of the fusion or GFP moieties because of their overexpression from the 35S promoter or to instability of the protein.

VAR3 interacts in yeast and in vitro with chloroplast-targeted proteins

VAR3 contains three different repeated domains, one of which is similar to the zinc-finger of RanBP2 that binds Ran-GDP (Nakielny et al., 1999). In the absence of biochemical data on VAR3 function, we used directed yeast two-hybrid assays to test whether VAR3 interacts with *Arabidopsis* Ran1, its GTP-binding form Ran-GTP, GDP-binding Ran-GDP, Ran2, as well as RanBP1a, RanBP1b and RanGAP1 controls (Haizel et al., 1997). None of these exhibited interaction with VAR3, although control transformations with RanBP1a interacted with Ran1 and Ran2, measured by adenine and histidine prototrophy and β-galactosidase assay (data not shown). This indicates that VAR3 zinc-fingers do not bind *Arabidopsis* Ran.

This prompted us to screen a yeast two-hybrid cDNA library for proteins that interact with VAR3. This screen of a library from mRNA of rosette plants identified 51 clones out of 1.5×10^7 transformants screened with full-length VAR3 as bait. These preys were sorted into three groups of plasmids by

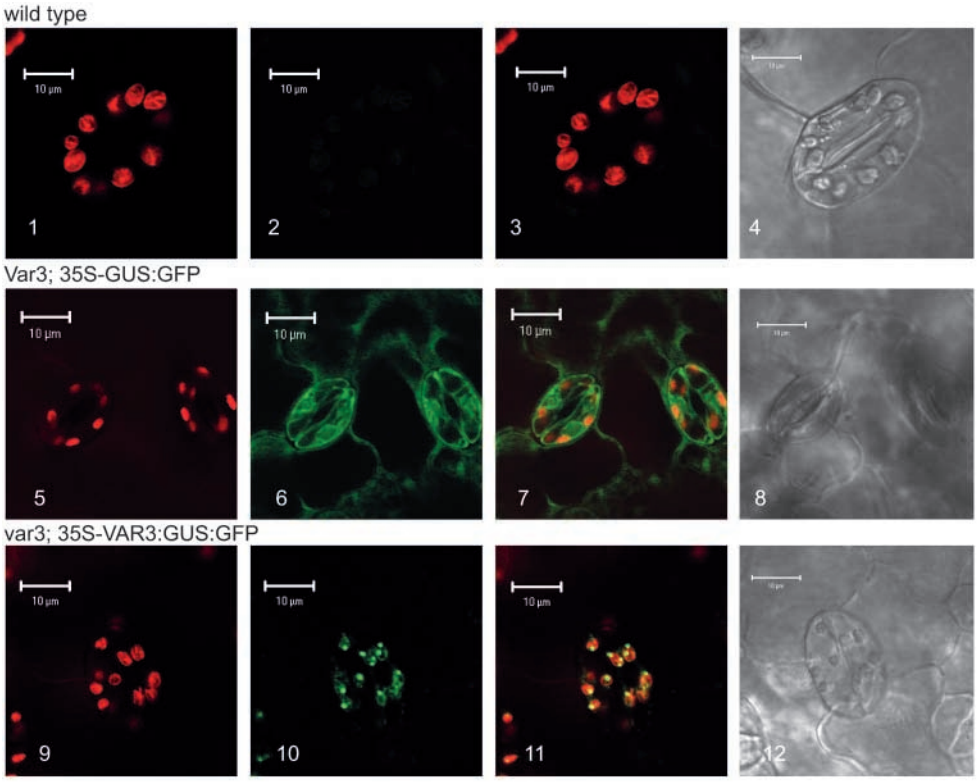


Fig. 5. VAR3 sub-cellular localization. Micrographs of stomata from wild type (top), *var3* expressing the 35S-GUS:GFP reporter from pCAMBIA 1303 (middle) and *var3* complemented to wild type by expression of the 35S-VAR3:GUS:GFP reporter (bottom). Panels 1, 5 and 9: confocal micrographs of chlorophyll red fluorescence. Panels 2, 6 and 10: fluorescence from GFP. Panels 3, 7 and 11: merged red and green images. Panels 4, 8 and 12: corresponding reference scanning Nomarsky micrographs.

hybridization and restriction mapping. Sequencing revealed that the two smallest groups of clones were out of frame fusions to unclassified proteins (At5g07890 and AAG52339), and were not examined further.

The largest group of clones encoded *At*NCED4 (At4g19170; AL021687), a chloroplast-targeted member of a family of enzymes similar to nine-*cis*-epoxycarotenoid dioxygenase (NCED) (Iuchi et al., 2001). Fig. 6A shows that the prey plasmid expressing 258 amino acids of the NCED4 C terminus fused to the Gal4 activation domain permitted growth of the yeast strain carrying the bait plasmid expressing the VAR3 Gal4 DNA-binding domain fusion, and that this was not due to either auto-activation of the nutritional markers by the NCED4-Gal4AD fusion, or to the VAR3-Gal4BD fusion. These results indicate that VAR3 interacts with NCED4 in yeast. In contrast, the strain carrying VAR3 and the IM alternative oxidase was incapable of prototrophic growth, indicating that VAR3 does not interact with IM.

To confirm these results, epitope-tagged, full-length VAR3 and the 259 amino acid N-terminal part of NCED4 from the cDNA library screen were synthesized by coupled in vitro transcription/translation and examined for interaction in vitro. This showed that VAR3, NCED4 (residues 336-595) and human lamin C control (LAM) were each of the expected size, and were immunoprecipitated with antibodies against the VAR3 N-terminal cMYC tag, or by the NCED4 or LAM N-terminal HA epitope tags (Fig. 6B, lanes 1-3). In addition, NCED4 was co-immunoprecipitated with c-Myc tagged VAR3 by anti-cMyc antibody, while LAM was not (Fig. 6B, lanes 4 and 5). This indicates that VAR3 is not a 'sticky' protein, and that VAR3 and NCED4 interact in vitro. The smaller NCED4 products, which probably result from premature termination as they are immunoprecipitated via the N-terminal HA epitope tag (lane 2), may bind to VAR3 or to each other (lane 4). The same results were obtained with the full-length NCED4 protein (not shown).

In vitro chloroplast import assays were used to confirm that VAR3 and NCED4 are targeted to chloroplasts (Fig. 6C). In vitro-translated, radiolabelled VAR3 and NCED4 were incubated with isolated, intact chloroplasts, and the chloroplasts fractionated post-import to determine the sub-plastid location of these proteins. For both VAR3 and NCED4, import was accompanied by processing to a smaller size, confirming that *VAR3* and *NCED4* encode precursor proteins. The smaller proteins were not a result of thermolysin processing of unimported VAR3 and NCED4, as demonstrated by control digestion of the in vitro translation mixture (lanes *Tr*+). Fractionation of the chloroplasts showed that VAR3 and NCED4 are located in the stroma, as is the case for the control stromal protein RbcS (SSU). A very small proportion of VAR3 and NCED4 co-localized with thylakoid membranes, but thermolysin digestion of the membranes demonstrated that this was surface associated, in contrast to the thylakoid lumen control 33 kDa subunit of the oxygen-evolving complex (33 k, Fig. 6C). These results confirm the results of the in vivo VAR3:GUS:GFP localization (Fig. 5). They also confirm that *At*NCED4, like the maize NCED VP14 (Tan et al., 2001), is chloroplast localized.

The *Arabidopsis* genome contains seven NCED-like genes, [*NCED1-6* and *9* (Iuchi et al., 2001)], and two more distantly related genes (*NCED7* and *8*). To examine whether VAR3 binding was specific to NCED4, directed two-hybrid assays were performed with the VAR3 bait and NCED3 (BAB01336) and NCED5 preys (At1g30100) This revealed that VAR3 interacted with NCED5, but not with NCED3 (data not shown). These results indicate that VAR3 specifically interacts with

certain NCED family members, including NCED4 and 5. NCED4 and 5 are not transcriptionally upregulated upon drought stress, and neither perform the cleavage of 9-cis-epoxycarotenoids (Iuchi et al., 2001). In contrast, NCED3 performs this cleavage reaction and is therefore involved in the biosynthesis of the plant hormone abscisic acid.

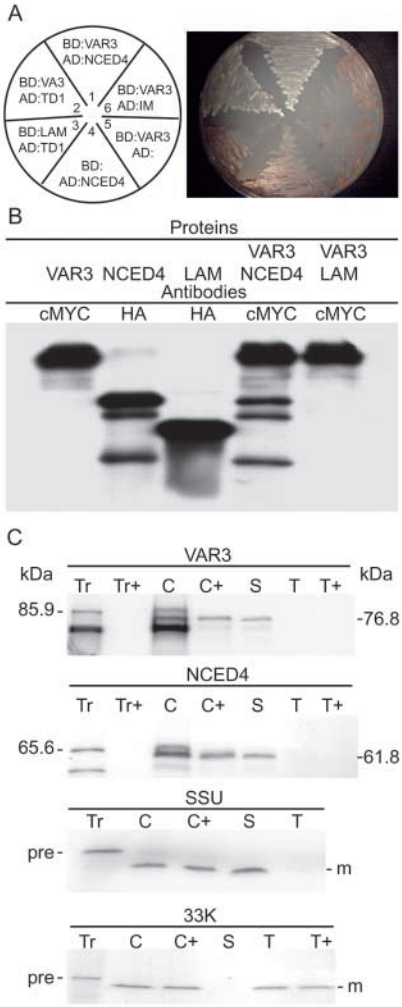


Fig. 6. VAR3 protein interaction and targeting. (A) Yeast two-hybrid interactions. Left panel shows growth of yeast strains carrying different plasmids on minimal medium lacking Trp, Leu, His and adenine. Right panel identifies fusion proteins expressed from the DNA-binding (BD bait) and activation domain (AD prey) plasmids in the yeast strains shown on the left. LAM is human lamin C, TD1 and VA3 (B) Co-immunoprecipitation (Co-IP) of VAR3 and NCED4. (Left to right) Lane 1, IP of cMyc-tagged VAR3; lane 2, IP of HA-tagged NCED4 (partial length open reading frame isolated from the cDNA library screen); lane 3, IP of HA-tagged LAM; lane 4, Co-IP of NCED4 with cMyc-tagged VAR3; lane 5, lack of Co-IP of control LAM with cMyc-tagged VAR3. Proteins and antibodies are noted above. (C) In vitro chloroplast import assay. Isolated, intact chloroplasts were incubated with in vitro translated, radiolabeled precursor proteins, and chloroplasts were then fractionated and samples analyzed by SDS-PAGE and fluorography. Gel lanes are: *Tr*, in vitro translation mixture; *Tr+*, translation mixture digested with thermolysin; *C*, total chloroplasts; *C+*, thermolysin-treated chloroplasts; *S*, stroma; *T*, thylakoid membranes; *T+*, thermolysin-treated thylakoids. *Pre-* and *m-* denote precursor and mature protein, respectively. SSU is a RbcS stromal marker and 33 K is the 33 kDa subunit of the oxygen-evolving complex, thylakoid lumen marker. The sizes (kDa) of precursor and mature forms of VAR3 and NCED4 are indicated.

var3 accumulates reduced levels of pigments

The variegated *im* mutant suffers from light dependent photo-oxidative damage because of a defect in carotenoid biosynthesis that leads to the accumulation of phytoene (Carol et al., 1999; Wu et al., 1999). Since VAR3 interacts with NCED homologs, *var3* variegation might also be due to an alteration in carotenoid biosynthesis or metabolism. To examine this, we performed comparative profiling, by HPLC-diode array, of carotenoids and chlorophylls in *var3* and wild-type plants. There was no qualitative difference in the pigment profiles of *var3* and wild-type when grown in 24-hour or 4-hours/day light regimes. However, under both light regimes there were significant reductions in the levels of all detected carotenoids, as well as a less severe reduction in chlorophylls a and b (Table 1). In both wild type and *var3* under the 4 hours/day light regime, one tetrapyrrole peak, tentatively assigned as the chlorophyll precursor protochlorophyllide, increased significantly compared to the levels in plants grown in continuous light. These results indicate that variegation in *var3* is unlike that in *im*, and is not due to loss of specific photo-protective pigments.

Discussion

The biogenesis of chloroplasts and the development of leaf cells containing them are regulated by intracellular signals between the plastid and the nucleus. Since most chloroplast protein functions are nuclear encoded, many nuclear mutations result in defective chloroplast biogenesis and lethal albinism (Barkan et al., 1995). In contrast, viable variegated mutants produced by stable nuclear mutations are rare. We identified the *variegated3* (*var3*) mutant in a screen of transposant lines generated with a modified maize *Ds* system (Sundaresan et al., 1995). Molecular analysis revealed that the element excised from the *DsG1* donor site on chromosome II and re-inserted with 8 bp flanking repeats into the *VAR3* gene on chromosome V. *Ds* insertion produced a truncated *VAR3* mRNA that was undetectable in *var3* by RT-PCR. Complementation of *var3* by transformation with a wild-type *VAR3* genomic fragment demonstrated that loss of *VAR3* expression is the cause of mutant variegation.

Chloroplasts in green areas of *var3* leaves were similar to those in wild type, while the yellow areas contained no, or only partially developed, chloroplasts. These characteristics are similar to those of other stable *Arabidopsis* mutants *chloroplast mutator*, *im*, *var1* and *var2*, as well as in unstable mutants such as *Arabidopsis albino 3* (Sundberg et al., 1997), *Antirrhinum olive* and maize *HCF 106* (Barkan et al., 1995). Since these mutants have partially developed but defective chloroplasts, the corresponding genes may be involved in the synthesis or assembly of the photosynthetic machinery, or in essential plastid metabolic functions.

Measurements of photosynthetic pigments revealed no qualitative differences in pigments in *var3* compared to wild-type. However, carotenoid levels in *var3* were 35-57% of that

Table 1. Relative quantity of pigments in *var3* and l*er* plants grown under two different environmental conditions

Plant age (days)/light exposure (hours)		Neoxanthin	Violaxanthin	Unidentified carotenoid A	Unidentified carotenoid B	Lutein	Unidentified carotenoid C	Protochloro-phyllide	b-Carotene	Chlorophyll b	Chlorophyll a
12/24	**l*er* (wt)†**	1.27	1.28	0.78	0.64	5.22	0.38	0.56	3.06	3.46	8.98
	var 3†	0.53	0.73	0.28	0.31	2.18	0.20	0.14	1.08	1.41	6.19
	var 3* as % of l*er	41.91	57.31	35.67	47.57	41.83	53.42	24.52	35.11	40.79	68.93
18/4	**l*er* (wt)†**	0.52	0.42	0.52	0.54	2.66	0.16	2.41	1.33	1.54	7.06
	var 3†	0.29	0.07	0.18	0.29	1.61	0.10	1.48	0.51	0.73	2.35
	var 3* as % of l*er	56.90	15.81	34.81	54.01	60.44	59.74	61.32	37.92	47.69	33.28

†Mean of peak areas of replicates (mAU$\times 10^{-6}$), normalized to equivalent to 200 μg dry weight tissue.

in wild type, while chlorophyll a levels in *var3* were approximately 70% of wild type under high light regimes. This indicates that loss of *VAR3* function has a greater effect upon carotenoid levels than upon chlorophyll levels. Under low light regimes, total carotenoids and chlorophylls were significantly reduced in both *var3* and wild type grown in continuous light. An exception to this, in both lines, was levels of a tetrapyrrole, tentatively assigned as protochlorphyllide. This precursor accumulates in low light regimes when chlorophyll biosynthesis is down-regulated via attenuation of protochlorophyllide oxidoreductase. In this respect, *var3* retains the ability to modulate chlorophyll biosynthesis as does the wild type. Under low light regimes, the majority of carotenoid and chlorophyll (a+b) levels in *var3* were reduced in concert with those in wild type. A possible exception is the effect of low light on violaxanthin that was reduced by a factor of ten in *var3* compared to 33% in wild type. Although this may indicate a role for VAR3 in modulating lutein oxidation, no confirmatory accumulation of lutein, or decrease in neoxanthin, was observed. In summary, the profiling indicated that VAR3 does not affect production of individual pigments.

Reductions in *var3* pigment levels in whole leaves are not accompanied by relative decreases in mRNAs of major nuclear-encoded photosynthetic genes such as *Lhcb* (*CAB*) and *RbcS*. Therefore, mRNA accumulation patterns in *var3* appear similar to those of *pac1*, and of the *gun* mutants in which the expression of chloroplast-targeted, nuclear gene products is uncoupled from chloroplast development (Mochizuki et al., 2001; Strand et al., 2003; Møller et al., 2001). An explanation for this class of mutants is that they affect levels of Mg-ProtolX that represses *Lhcb* and *RbcS* expression in the absence of functional chloroplasts. Since the *var3* mutation primarily affects carotenoid levels, carotenogenic compounds may be involved in such signaling (Rodermel, 2001). However, the lack of *Lhcb* transcript accumulation in *var3* under photo-oxidative conditions indicates that *var3* is not a *gun* mutant. Thus, loss of *VAR3* function does not appear to affect the levels of tetrapyrrole or carotenogenic intermediates that regulate the expression of nuclear genes encoding chloroplast-targeted proteins.

Transcriptome analysis indicated that the levels of mRNAs encoding plastid and mitochondrial transcription and translation components are elevated in *var3*. The increase in *rpoB* mRNA levels is interesting because the *Antirrhinum* nuclear gene *DAG* is required for *rpoB* expression, such that unstable *dag* mutants exhibit variegation because of the resulting block in chloroplast differentiation (Chatterjee et al., 1996). A simple explanation for the increases in these mRNAs in *var3* is that they reflect a mechanism by which plastids attempt to increase overall protein synthesis to compensate for the failure of photosynthetic functions (Mullet, 1993). Alternatively, VAR3 might affect the production or transduction of signals regulating chloroplast gene expression, perhaps via its interaction with chloroplast localized enzymes (below). In any event, the interdependency of chloroplast and mitochondrial metabolic pathways may also explain why the expression of mitochondrial genes encoding translational components appear to be affected in *var3*. Such increases in mitochondrial gene expression have been described in albino barley leaves carrying the recessive nuclear *albostrians* mutation (Hedtke et al., 1999). Such effects are also opposite but analogous to that produced by mutation of the nuclear gene *CHM* causing mitochondrial genome rearrangements that affect chloroplast function (Abdelnoor et al., 2003). Taken together, these results suggest that signals involved in coordinating mitochondrial and plastid activities are perturbed in *var3*. The micro array results also revealed that the levels of mRNAs encoding a chloroplastic heat shock protein, cpHsc70-2, and a chaperone subunit, Cpn-60α, were elevated in *var3*. These proteins are likely orthologs of *Narcissus pseudonarcissus* chloroplastic HSP70 and Cpn60 chaperonin that are components of oligomeric assemblies with phytoene desaturase, and at least two other carotenoid biosynthetic enzymes (Bonk et al., 1997; Al-Babili et al., 1996). While circumstantial, these results suggest an additional link between VAR3 function and carotenoid metabolism.

VAR3 encodes a novel protein containing two zinc-finger domains conserved in nucleoporins and RNA binding proteins (pFAM00641). Such fingers in nucleoporins interact with RAN (Nakielny et al., 1999), although yeast two-hybrid assays did not reveal interactions between VAR3 and *Arabidopsis* nucleoporins or RAN. This suggests that this class of zinc fingers in different proteins mediate interactions to various proteins. VAR3 also contains long C-terminal repeats with proline rich regions reminiscent of motifs interacting with SH3 and WW domains, suggesting the existence of similarly folded protein sub-domains. Database searches indicate that VAR3 homologs are restricted to plants, suggesting they perform functions specific to plants. Their modular structure is evidenced in the two VAR3 homologs containing only some of the conserved domains. A third homolog is similar to VAR3 throughout its length, and may therefore perform VAR3 redundant functions.

In addition to the *var3* phenotype, two lines of evidence

indicate that VAR3 is required for normal chloroplast function. First, VAR3 has a chloroplast target peptide that enabled import to chloroplast stroma, and a VAR3 reporter fusion that complemented the *var3* phenotype was localized to chloroplasts. Second, yeast two-hybrid screening revealed that VAR3 specifically interacted with chloroplast localized NCED4 and 5, two of seven *Arabidopsis* proteins homologous to 9-*cis*-expoxycarotenoid dioxygenases (Schwartz et al., 1997; Iuchi et al., 2001; Tan et al., 2001). The interaction between VAR3 and NCED4 was confirmed in vitro, and both were shown to accumulate in stromal fractions by chloroplast import assays. These interactions are apparently specific, because VAR3 does not interact with the chloroplast-localized IM alternative oxidase involved in carotenoid biosynthesis, or with NCED3. NCED3 mRNA, and mRNAs of the maize *VP14*, avocado PaNCED1 and bean *PvNCED1* orthologs, are induced by water stress (Schwartz et al., 1997; Qin and Zeevaart, 1999; Iuchi et al., 2001). These enzymes catalyze cleavage of 9-*cis*-epoxycarotenoids to generate 2-*cis*,4-*trans*-xanthoxin, the major precursor of abscisic acid (ABA). Neither *At*NCED4 or 5 are directly involved in ABA biosynthesis since their mRNAs do not accumulate during desiccation, and recombinant NCED4 and 5 proteins do not cleave 9-*cis*-epoxycarotenoids in vitro (Iuchi et al., 2001). Interestingly, phylogenetic sequence analysis indicated that NCED4 is more closely related to *Crocus* CsZCD than to other *Arabidopsis* members of the NCED family. CsZCD catalyzes the 7,8(7′,8′) cleavage of zeaxanthin leading to the formation of chromoplast-derived apocarotenoids including crocin and safranal (Bouvier et al., 2003). Nonetheless, cleavage products of zeaxanthin, trans-lutein or trans-violaxanthin were not detected in in vitro or cellular assays with recombinant NCED expressed in *E. coli*. The enzymatic activity and physiological substrates or products of NCED 4 therefore remained to be determined.

In summary, our data are consistent with a role for VAR3 as a component of a protein complex that functions in a metabolic pathway required for chloroplast development. By analogy to *var1* and *var2* (Sakamoto, 2003; Sakamoto et al., 2003), the mechanism of *var3* variegation, including effects specific to cell-type and developmental stages, may also involve a redundant activity provided by the VAR3 homologs identified here. The striking effect of the *var3* mutation on the development of palisade cells in true leaves, but not in cotyledons, is similar to that seen in tomato *dcl* (Keddie et al., 1996) and *Arabidopsis var2* and *cue1* (Chen et al., 2000; Streatfield et al., 1999). VAR3 may therefore be required for both chloroplast development and palisade cell morphogenesis, although its stromal localization suggests that defects in palisade cell elongation are a secondary effect of incomplete chloroplast development. Nonetheless, these secondary effects may include the disruption of specific signals, including carotenogenic products, required for palisade morphogenesis (Rodermel, 2001). Such products may signal diverse developmental processes, as posited for those of the *Arabidopsis MAX4* dioxygenase-like gene (Sorefan et al., 2003) that may control synthesis of a metabolite(s) that acts downstream of auxin to inhibit shoot branching.

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