The Plant Cell, Vol. 4, 1575–1588, December 1992 © 1992 American Society of Plant Physiologists

Expression of Antisense or Sense RNA of an Ankyrin Repeat-Containing Gene Blocks Chloroplast Differentiation in Arabidopsis

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The Arabidopsis *AKR* gene that encodes a protein with four ankyrin repeats (a 33-amino acid motif that appears in the 89K domain of the human protein ankyrin) was isolated and characterized. A short sequence outside the ankyrin repeats is similar to that of the protein of the Drosophila muscle segment homeobox (*msh*) gene. The expression of the *AKR* gene is light dependent, and transgenic Arabidopsis plants with two or more copies of an antisense or sense *AKR* construct became chlorotic in a developmentally regulated manner. The chlorotic phenotype was genetically transmitted to the next generation, although most chlorotic plants produced much less seed. Reduced presence of thylakoid membranes and loss of grana are found in the plastids of chlorotic leaves, indicating that antisense or sense *AKR* has blocked chloroplast differentiation. This study indicates the importance of ankyrin repeat-containing proteins, not only in yeast and animals, but in plants as well.

INTRODUCTION

An Arabidopsis gene that encodes a protein with four ankyrin repeats was isolated by using a chicken cytochrome b_5 gene as a probe under conditions of very low stringency (Zhang, 1989). The ankyrin repeat is a 33-amino acid motif that appears 22 times in tandem in the 89K domain of the human protein ankyrin (Lux et al., 1990a). Because this motif was initially found in the yeast cell-cycle genes *SWI6*, *SWI4*, and *CDC10* (Aves et al., 1985; Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989a), it has been called the CDC10/SWI6 repeat or ankyrin repeat. It has subsequently been found in Notch of Drosophila (Wharton et al., 1985); *Lin-12*, *Glp-1*, and *Fem-1* of *Caenorhabditis elegans* (Yochem et al., 1988; Yochem and Greenwald, 1989; Spence et al., 1990); and the proto-oncogene *bcl-3* (Ohno et al., 1990) and the gene coding for the p50 DNA binding subunit of NF-κB of animal cells (Bours et al., 1990; Ghosh et al., 1990; Kieran et al., 1990). The fact that proteins from such diverse sources contain these repeats strongly suggests that the ankyrin repeat plays an important role(s) in the function of these proteins. One proposal, based on studies with human ankyrin proteins (Lux et al., 1990a; Bennett, 1992), suggests that ankyrin repeats serve as domains where integral membrane proteins and cytoskeletal proteins interact. Another possibility based on studies of the SWI4 and SWI6 proteins of the cell-cycle box factor–DNA complex (Andrews and Herskowitz, 1989b; Taba et al., 1991) is that ankyrin repeat–containing proteins are involved in transcriptional complexes. Lamarco et al. (1991) and Thompson et al. (1991) showed that ankyrin repeats are involved in bringing together subunits of the GA binding protein (GABP), thereby allowing GABP to bind stably to its target DNA sequence. The ankyrin repeat–bearing subunit GABPβ together with another subunit GABPα were shown to be directly associated with DNA by cross-linking experiments. A possible role that the ankyrin repeat might play in these diverse proteins may be as an interface where the ankyrin repeats interact with other proteins (e.g., receptor proteins, cytoskeletal proteins, or signal proteins); consequently, the ankyrin repeat should be considered an important regulatory domain employed by numerous proteins (Thompson et al., 1991).

The Arabidopsis ankyrin repeat–containing gene has been named *AKR* (Arabidopsis anKyrin Repeat), and the protein it encodes has been designated AKRP (Arabidopsis anKyrin Repeat Protein). Besides ankyrin repeats, AKRP also contains sequences similar to the homeobox proteins of the Drosophila *Msh* family (Gehring, 1987a). Because of the involvement of *Msh* in lineage specification, and Notch, *Lin-12*, and *Glp-1* in the cell fate determination, as well as *SWI4* and *SWI6* in the control of mating-type switching, we postulate that AKRP might play a regulatory role in cell differentiation and development in higher plants. Therefore, we carried out an extensive study on the function, expression, and regulation of the *AKR* gene. Human hereditary spherocytosis (one of the most common

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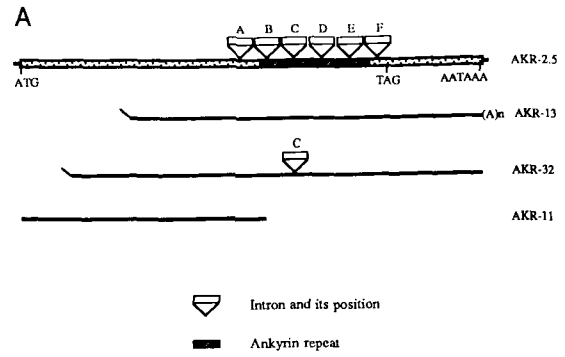

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1    catcatttttgcaacaaggatgagatccggccgagcgagattcgaaagtagcagagcagc
61   agcagcagcaggatcagaaagaaacaatggcgtttatttttcttcagattgactaatcaaa
121  ggacgaacgggaagtcaggcccatttgtttaagtcaggcccattAGAAAATGAGGCCCAT
                                                   M  R  P  I     4
181  TAAAATTGTCGGATACTACATCAGAGGCCGGCGAGGAGGAGAGGAGAAAGCTCCGTTATT
       K  I  V  G  Y  Y  I  R  G  R  R  G  G  E  E  K  A  P  L  F    24
241  CAAGATTATTCCGATAATGCAGTCACTCTCCACACCACACACCATATCTCTTCTTCTCCC
       K  I  I  P  I  M  Q  S  L  S  T  P  H  T  I  S  L  L  L  P    44
301  CGGAACCTCTCCGTCTCGTCTTTCTCCCTCTCTTCACTCCCTTGTTTTCCCCACTAGATT
       G  T  S  P  S  R  L  S  P  S  L  H  S  L  V  F  P  T  R  L    64
361  ACGCTCTCTTTCCTATTCTTCTCAAACGTCGATCCTCCCCGATGCCGGCGATGATTTCAT
       R  S  L  S  Y  S  S  Q  T  S  I  L  P  D  A  G  D  D  F  I    84
421  TGTCGGAGACTGTCTCGTCTACGAGGACGGCGTCTTCGAAGACCCTTACCTTGAGAAGGA
       V  G  D  C  L  V  Y  E  D  G  V  F  E  D  P  Y  L  E  K  E   104
481  GGTCACTCAGGTTGCGAAGCAGGAGCGCAAGAAGAATCGGCGTGGCGGGGCCAAGAGATT
       V  T  Q  V  A  K  Q  E  R  K  K  N  R  R  G  G  A  K  R  L   124
541  AGATGAATCCGAGATTGAGCCCGAGAACCTCGTGCCGGAGGAATGGAGGGATATTCAGGC
       D  E  S  E  I  E  P  E  N  L  V  P  E  E  W  R  D  I  Q  A   144
601  GGAGGTGAATCTGACGAAGAAGGACAAGCGCAAAATAGCGCAGGAGATGGAGTTCGGGGT
       E  V  N  L  T  K  K  D  K  R  K  I  A  Q  E  M  E  F  G  V   164
661  TCGGGTAGAGAAGAAGAGACAAGGGCTAATTCCGCTGAGGAAAGTTGACTTGAATGACTT
       R  V  E  K  K  R  Q  G  L  I  P  L  R  K  V  D  L  N  D  F   184
721  TCTCACGTACAAGGAAGCCAAGTTGGCTCAGTTGAGGCCTGTCATTCTCGATAAACCGGG
       L  T  Y  K  E  A  K  L  A  Q  L  R  P  V  I  L  D  K  P  G   204
781  CAATTTCTCCGACGACTTTGGAGCGTCAAGCGATGGAGAGACCGCTGTATCATCTCCCAG
       N  F  S  D  D  F  G  A  S  S  D  G  E  T  A  V  S  S  P  S   224
841  CGAGCGAGTGGCTCCTAAGAACCCTAGATGGGCAGTTTACGGAAAGGGATTCGACCACGT
       E  R  V  A  P  K  N  P  R  W  A  V  Y  G  K  G  F  D  H  V   244
901  TGCCAAGTTCTTCAATAGCGACAAGTACGATCCCAGCGACAAGAAATCCGACGGCCCTCG
       A  K  F  F  N  S  D  K  Y  D  P  S  D  K  K  S  D  G  P  R   264
961  AAAGCTGCTTTCAAAAGAAGAAAAGTTTATGCTCAATAGCCGGAATCCTGACCTAGCCGT
       K  L  L  S  K  E  E  K  F  M  L  N  S  R  N  P  D  L  A  V   284
1021 TGCCACATCAAAAAAATGGCTTCCTCTTCACACACTGGCAGCATGTGGAGAGTTTTATCT
       A  T  S  K  K  W  L  P  L  H  T  L  A  A  C  G  E  F  Y  L   304
1081 GGTTGATTCCTTGCTAAAGCACAATCTTGATATCAATGCAACCGATGTGGGCGGCTTGAC
       V  D  S  L  L  K  H  N  L  D  I  N  A  T  D  V  G  G  L  T   324
1141 AGCACTTCACCGAGCAATCATTGGTAAGAAGCAGGCTATTACTAACTACCTTCTGAGGGA
       A  L  H  R  A  I  I  G  K  K  Q  A  I  T  N  Y  L  L  R  E   344
1201 ATCGGCAAATCCATTTGTTCTTGATGACGAAGGTGCGACCTTGATGCACTATGCTGTGCA
       S  A  N  P  F  V  L  D  D  E  G  A  T  L  M  H  Y  A  V  Q   364
1261 AACAGCATCAGCTCCCACAATAAAACTTCTCCTACTGTATAACGCTGATATAAACGCTCA
       T  A  S  A  P  T  I  K  L  L  L  L  Y  N  A  D  I  N  A  Q   384
1321 GGACAGGGACGGGTGGACTCCACTGCACGTTGCAGTACAGGCCAGAAGAAGCGACATTGT
       D  R  D  G  W  T  P  L  H  V  A  V  Q  A  R  R  S  D  I  V   404
1381 AAAGCTTCTTTTGATAAAAGGGGCGGACATAGAAGTGAAGAACAAGGATGGGTTAACTCC
       K  L  L  L  I  K  G  A  D  I  E  V  K  N  K  D  G  L  T  P   424
1441 GCTTGGCTTTGCCTCTACCTTGGAAGAGAGACAAGGACGTATGAGGTGATGAAGCTGTTG
       L  G  F  A  S  T  L  E  E  R  Q  G  R  M  R                 439
1501 AAAGAGTTTCCACTTAGCAGACACAAGAAGAGATTGGTAACAACAGATGAAGATATTGAA
1561 TAGTCCTTTCAATTGCAGCTTGAAGTACATTCCCTTACGAGAACCTGAGAAAAGGAGATG
1621 GAGGTAAAGGTGATGATTAGGACATTAGAACCTCGGAGTCGGAGTGGGTCCACTGCCTCA
1681 ATTCCTTAAATTTGGTTTGCTGTTAATCTTATCCATCGATTTTGGATATTTATCACTTAT
1741 CCATTCTTAAAGAAAATATCTGAAAATAAAATAAAAAGTAATATATAT  1788
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Figure 1. Cloning and Sequencing of the Arabidopsis *AKR* Gene.

hereditary hemolytic anemias at the *SPH2* locus) is caused by defects or deficiency of erythrocyte ankyrin (Lux et al., 1990b), and in mouse, a recessive mutation, normoblastosis, which is linked to the ankyrin locus *Ank-1*, caused a 90% reduction in the expression of erythrocyte ankyrin in erythrocyte and brain tissues with a phenotype of severe hemolytic anemia (Peters et al., 1991). Because there is only one copy of the *AKR* gene in the Arabidopsis genome, we explored the physiological consequence of overexpressing antisense or sense RNA of the *AKR* gene in transgenic plants. Transgenic plants with two or more copies of the antisense or sense *AKR* construct exhibited a chlorotic phenotype in a developmentally regulated manner. The cellular defect of chlorosis is due to reduced plant pigments and the absence of mature chloroplasts in the affected tissues, an indication that AKRP is a component of a regulatory process of the temporal and spatial regulation of chloroplast differentiation in plant development.

RESULTS

Characteristics of the *AKR* Gene and Its Protein Product

One copy of the *AKR* gene is present in Arabidopsis as determined by genomic DNA gel blot analysis using a cDNA, AKR-13, as a probe under conditions of high stringency (data not shown). We haved mapped the *AKR* gene at 158 centimorgans of chromosome 5 of the revised restriction fragment length polymorphism map (Nam et al., 1989; S. Hanley and H. M. Goodman, unpublished data). The genomic sequences corresponding to AKR-13 and other cDNA clones shown in Figure 1A have been isolated. A sequence of the full-length transcript was derived from the three cDNA clones AKR-13, AKR-32, and AKR-11. A genomic clone, AKR-2.5, covering the full-length transcript was also sequenced, and the transcription initiation

(A) Schematic outline of the full-length transcript of the Arabidopsis *AKR* gene and the cDNA and genomic clones from which it was derived. AKR-13 and AKR-32 were from a Columbia leaf cDNA library, AKR-11 was a PCR–amplified cDNA product (race C24), and AKR-2.5 was from a genomic library constructed from the race Landsberg. The six introns (A to F) and their positions were determined by comparing the sequences of cDNA clones and the genomic clone. The first 49 bp of AKR-13 and the first 15 bp of AKR-32 are artifacts of cDNA cloning and are indicated in the figure by a narrow branched line. The figure is drawn roughly to scale.

(B) The sequence of the full-length transcript of *AKR* and the translated amino acid sequence of AKRP are shown. Nucleotides 1 to 164 were deduced from genomic sequencing, and the transcription start site was determined by a primer extension experiment. This region is indicated in lowercase letters. The start codon, stop codon, and the polyadenylation signal are shown in bold letters. The four complete ankyrin repeats are underlined. Introns occur after nucleotides 953, 1030, 1129, 1228, 1327, and 1426.

site was determined by a primer extension experiment (data not shown). The sequence of the predicted transcript is shown in Figure 1B. The first five nucleotides (CATCA) are consistent with the consensus sequence of the transcription initiation site of higher plant genes (Joshi, 1987). There are two ATGs at nucleotides 20 and 87 that could give rise to translation products of 31 and 21 amino acids, respectively. However, we have no evidence whether these peptides are translated or would be functional if translated in vivo, although there was evidence that a short peptide can serve as a hormone in the regulation of the systemic response to wounding in tomato (Pearce et al., 1991). The third ATG at nucleotide 170 precedes the longest open reading frame (ORF), and the ORF is interrupted by six introns somewhat clustered near the 3′ side of the gene (Figure 1A). Several ATGs are present within this ORF, but the first ATG is theoretically favored to serve as a start codon for translation, because it is in the context of a stronger ribosomal recognition site, 5′-AAAATGA-3′ (Kozak, 1987). There are no sequences similar to the consensus sequence for plant TATA boxes (Joshi, 1987) upstream of the transcriptional initiation site, and the message was polyadenylated 17 bp after the polyadenylation signal, AAATAAAA (Proudfoot, 1991).

The AKRP protein (encoded by the longest ORF) has one incomplete and four complete in-tandem ankyrin repeats located proximal to the C terminus (residues 282 to 428). The Arabidopsis ankyrin repeats are similar to the consensus derived from several well-studied proteins, as shown in Figure 2A. AKRP is 439 amino acids long with a corresponding M_r of 49,150. It is a basic protein (pI = 9.66) with lysine (37 residues) as the second most abundant amino acid (8.4%). Lysine plus arginine constitute 21% of the amino acids in the central part of the protein (residues 100 to 281), imparting to this region a hydrophilic character as visualized by hydropathy analysis (data not shown). The protein data bank PIR 29 was searched using the BLASTP program (Altschul et al., 1990) with amino acids 1 to 281 (outside the ankyrin repeats) as the query sequence; the best match scores were mainly DNA binding proteins. For example, residues 106 to 135 are similar to the nucleocapsid protein (Kapke and Brian, 1986) of the porcine transmissible gastroenteritis coronavirus (TGEV); within this sequence, amino acids 116 to 130 are also similar to the homeobox proteins encoded by the Drosophila *Msh* family, including H17 of honeybee (Walldorf et al., 1989), Quox-7 of quail (Takahashi and Douarin, 1990), and Hox-7, Hox-7.1, and Hox-8.1 of mouse (Hill et al., 1989; Robert et al., 1989; Monaghan et al., 1991), as shown in Figure 2B.

Light Dependence and Developmental Regulation of *AKR* Gene Expression

We carried out RNA gel blot analyses to determine the size, spatial distribution, and regulation of *AKR* message in Arabidopsis. Total RNA from 4-week-old plants was probed with the AKR-13 sequence. A 1.7- to 1.8-kb message, which was about the length expected from the cDNA clones and the primer extension results, was found. The *AKR* message was highest in leaves, less in flowers and subtending floral bracts, and least in inflorescence stems, as shown in Figure 3A. The chlorophyll *a/b* binding protein gene 4 (*Cab4*) message, used as control,

A

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AKRP                 INA-D--GWTPLH-AV------IV-LLL--NAD
CDC10; SWI4,6        LN-QD--G-TPLHWAA--AN--LV--L---GAN
Notch                -NA-D--G-TPLHLAAR---------LL---A-
Lin-12               ----D--GRTALH-AA-N-----V-YL----A-
Glp-1                ----D--GRTALHLAA-------V--L------
Bcl-3                --A-N--G-TPLH-AV----L--VRLLL--GA-
Ankyrin              --D----G-TPLH-AA--GH---V--LL--GA-
KBF1/p50             ----D--G-T-LHLA-------L---LL--GAD
GABPβ1              V-A-D-L--TPLH-AA---H---VEVLLK-GAD
Consensus            ----D--G-TPLH-AA-------V--LL--GA-
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B

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TGEV        333 SEVAKEQRKRKSRSKSAERSEQEVVPDALI 362
AKRP        106 TQVAKQERKKNRRGGAKRLDESEIEPENLV 135
MSH          51 NRRAKAKRLQEAEIE 65
H17          51 NRRAKAKRLQEAEIE 65
Quox-7       51 NRRAKARRLQEAELE 65
Hox-7, Hox-7.1, Hox-8.1  51 NRRAKARRLQEAELE 65
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: similar | identical

Figure 2. Sequence Similarites of AKRP to Other Ankyrin Repeat-Containing Proteins and to DNA Binding Proteins of the *Msh* Family and TGEV.

(A) Comparison of ankyrin repeats from different sources. The consensus for the Arabidopsis ankyrin repeats was derived using the following criteria: a specific amino acid is indicated if that position is occupied by a single amino acid in all four complete ankyrin repeats (4/4) or at least twice by a single amino acid (2/4) and the other two times by closely related amino acids. Other consensus sequences were derived from their corresponding sequences using the same criteria.

(B) Sequence similarity to homeobox proteins and the TGEV nucleocapsid protein. The numbering of the TGEV and AKRP sequences refers to their positions in the proteins, whereas the numbering of the homeobox proteins refers to their positions relative to the homeobox.

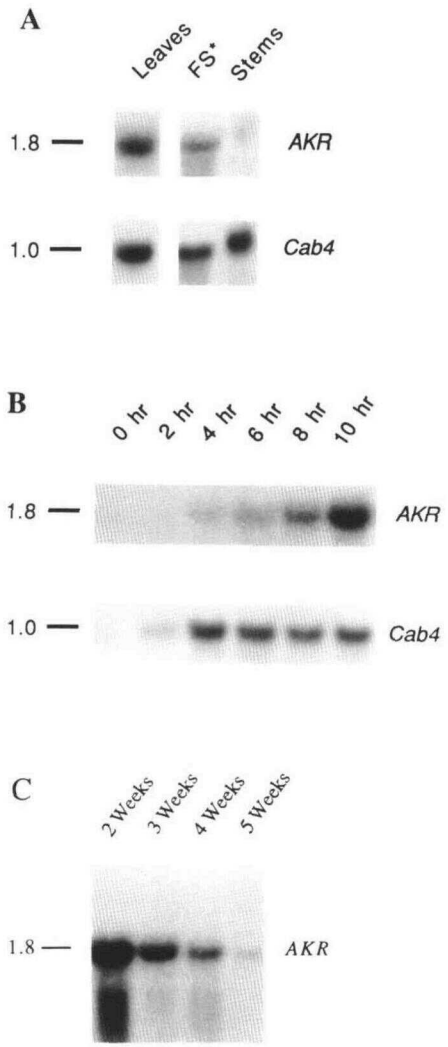

Figure 3. RNA Gel Blot Analysis of the Expression of the *AKR* Gene.

(A) *AKR* expression in different tissues.
(B) *AKR* expression in light-induced plants.
(C) *AKR* expression in different developmental stages.
The lengths of the RNA markers are given at left in kilobases. The same blots were used for the *AKR* and *Cab4* hybridizations in **(A)** and **(B)**, but the exposure time for *AKR* is about 20 times longer than that for *Cab4*. FS* (flower stalk), flower and the subtending floral bracts.

remained approximately the same in all three tissues. Interestingly, light had a dramatic effect on the steady state level of the *AKR* message. Plants were kept in the dark for 3 days, then shifted to light. At 0 hr (just before the shift to light), the message was barely detectable, but by 10 hr after the shift, the message reached its maximum level as shown in Figure 3B. Although the induction kinetics of the *AKR* message was not as fast as that of the *Cab4* control message (Figure 3B), there was clearly a relationship between the increase of the steady state level of the *AKR* message and light. The expression of the *AKR* gene appeared to be regulated developmentally. When RNAs isolated from the end of the second week up to the fifth week were probed with AKR-13, the highest expression level occurred in the second week and then declined as the plants developed further, as shown in Figure 3C.

Transgenic Plants with Antisense or Sense *AKR* Construct Turn Chlorotic

We used the cDNA sequence from nucleotides 1 to 1051 of AKR-13 for the antisense construct and the sequence from 457 to 1051 for the sense construct. Because AKR-13 was not full length (it was missing about 500 5′ nucleotides), the antisense construct was equivalent to using nucleotides 507 to 1508 of the full-length transcript. The sense construct was equivalent to nucleotides 914 to 1508 of the full-length transcript, and these nucleotides encode four ankyrin repeats with the putative ATG start codon at position 531. Both constructs were made in Agrobacterium vectors capable of transforming plants, as shown in Figure 4. The experiments were designed using AKR-13 because it was the first cDNA clone obtained. This did not present a problem even though AKR-13 was not full length, because it has been shown that partial sequences can be used as antisense and can interfere with the expression of the cognate endogenous genes (van der Krol et al., 1990a).

One hundred thirty-six transgenic lines were produced with each of the two constructs. Of the regenerated plants, 19 antisense-transformed lines and 16 sense-transformed lines produced chlorotic progenies. Chlorotic lines showed the phenotype both on kanamycin plates and in soil without kanamycin,

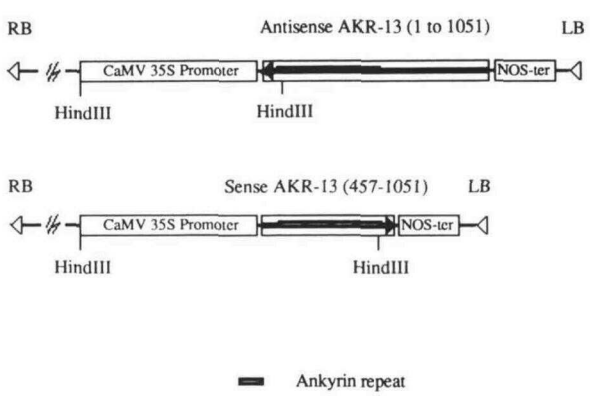

Figure 4. Construction of Antisense and Sense *AKR* Vectors Used to Transform Arabidopsis.

The drawing is not to scale. RB and LB are the right and left border sequences, respectively, of the Ti plasmid from Agrobacterium, and the NOS-ter is the terminator sequence from the nopaline synthase gene (Jefferson et al., 1987).

indicating that no direct relationship exists between the presence of kanamycin and chlorosis. Plants 3/3A, 3/8A, 3/50A, and 7/57A are examples of plants transformed with the antisense and sense constructs; they showed the chlorotic phenotype and were still fertile, as shown and described in Figure 5. The phenotype of some sense transformants (e.g., 7/57A) was very similar, if not identical, to the plants containing the antisense construct. The negative effects of introduced sense DNA on expression of endogenous genes in transgenic plants are well documented (Napoli et al., 1990; van der Krol et al., 1990b). In the experiments reported here, both sense and antisense constructs caused the chlorotic phenotype, whereas in more than 1000 transgenic control plants produced with different gene constructs, not a single similar chlorotic plant was found.

The frequencies with which the phenotype occurred (~16% in antisense-transformed plants and 13% in sense-transformed plants) and the appearance of the phenotype only with these constructs indicate that there was a direct causal relationship between the introduced sequences and the chlorotic phenotype. Some plants showed the chlorotic phenotype just before bolting (~4 weeks after planting). Some plants became yellowish at the basal part of the emerging fifth or sixth leaf, while distal leaf regions were green. The basal leaf regions maintained their chlorotic appearance even at maturity, whereas later emergent leaves and inflorescence shoots were yellowish or white throughout. Other plants turned chlorotic very late in development, usually starting from the tip of the growing stem. All plants were green at the cotyledon and two-leaf stage. Generally, the later the chlorosis occurred the bigger the plant grew and the more seeds it produced.

Correlation of Chlorosis with Reduced Steady State *AKR* Message Level in Antisense-Transformed Plants

We analyzed the DNAs and RNAs of the wild-type and antisense-transformed plants to determine if there were any correlations between chlorosis and the antisense message. The endogenous wild-type *AKR* gene was cut once by HindIII to produce bands of 2.6 and 0.9 kb on a genomic DNA gel blot, as shown in Figure 6A. In the antisense construction, HindIII cut once in the AKR-13 sequence to produce a 0.9-kb fragment corresponding in length to the shorter fragment of the endogenous gene and another fragment containing flanking host DNA sequences that could serve as an indication of the integration event. Transgenic line 3/2A had one T-DNA insertion, 3/58A had two T-DNA insertions, and 3/3A and 3/8AB had more than two T-DNA insertions. These numbers from gel blot data correlated well with the kanamycin segregation data, as given in Table 1. The line 3/50A segregated as a single insertion plant, but the genomic DNA gel blot data indicated two extra bands. The stronger intensity of the 4.3-kb band was probably due to the integration at one locus of several T-DNA copies in tandem, because the length of the band was exactly the size expected from the HindIII site of AKR-13 (through the T-DNA left border and right border) to the HindIII site just before the cauliflower mosaic virus (CaMV) 35S promoter (Figure 4). The weaker bands in Figure 6A above 2.6 kb in lanes designated 3/3A and 3/8AB were probably due to segregation of the multiple T-DNA insertions in plants pooled for DNA preparation. The data show that chlorosis occurred only in the lines having multiple copies of introduced antisense construct.

The RNA gel blot data, as shown in Figures 6B and 6C, indicate the presence of a very abundant antisense RNA in all the antisense-transformed plants, whereas the endogenous message of the wild-type gene was reduced severalfold only in chlorotic plants. Plant 3/8A that showed the most severe chlorosis also contained the lowest endogenous *AKR* message, and there was a direct correlation between the severity of chlorosis and the reduction of the endogenous *AKR* message, as shown in Table 2.

Endogenous *AKR* Message Varies among Plants Transformed with Sense Construct

For the same reason as above, we characterized plants transformed with the sense construct, and the results are shown in Figure 7. The genomic DNAs were digested with BamHI, which gave a single band in wild-type DNA when probed with AKR-13. BamHI cut once in the construct, but not within the *AKR* sequence, so that an extra band beside the endogenous *AKR* band would indicate an insertion event in the transgenic plants, as shown in Figure 7A. Plant 7/2A containing a single insertion produced an extra band that almost comigrated with the endogenous *AKR* band. However, the extra band could be shown to be a separate band by using other restriction enzymes, such as ClaI and BclI (data not shown). The chlorotic plants 7/25A and 7/62A contained only one copy of the sense *AKR* construct.

The RNA gel blot data, as shown in Figure 7B and Table 3, indicate that the endogenous *AKR* message level varied from 50% of plant 7/21A to the wild-type level of plant 7/57A. The most chlorotic plant was 7/57A, and it showed the highest expression of the introduced sense *AKR* gene; however, plant 7/21A with the lowest levels of the introduced sense *AKR* message showed chlorosis more severe than other plants, except for 7/57A. Therefore, there seems to be no simple relationship between the severity of chlorosis and the level of endogenous *AKR* message and the level of introduced sense *AKR* message. It should be noted that the extent of chlorosis in chlorotic plants transformed with the sense construct varied greatly among individual plants.

Chlorotic Tissues Do Not Contain Mature Chloroplasts

The chlorotic phenotype suggests that major pigments in the affected tissues have been reduced. We therefore measured the chlorophyll and carotenoid concentration in the chlorotic leaves of two representatives of antisense- and sense-

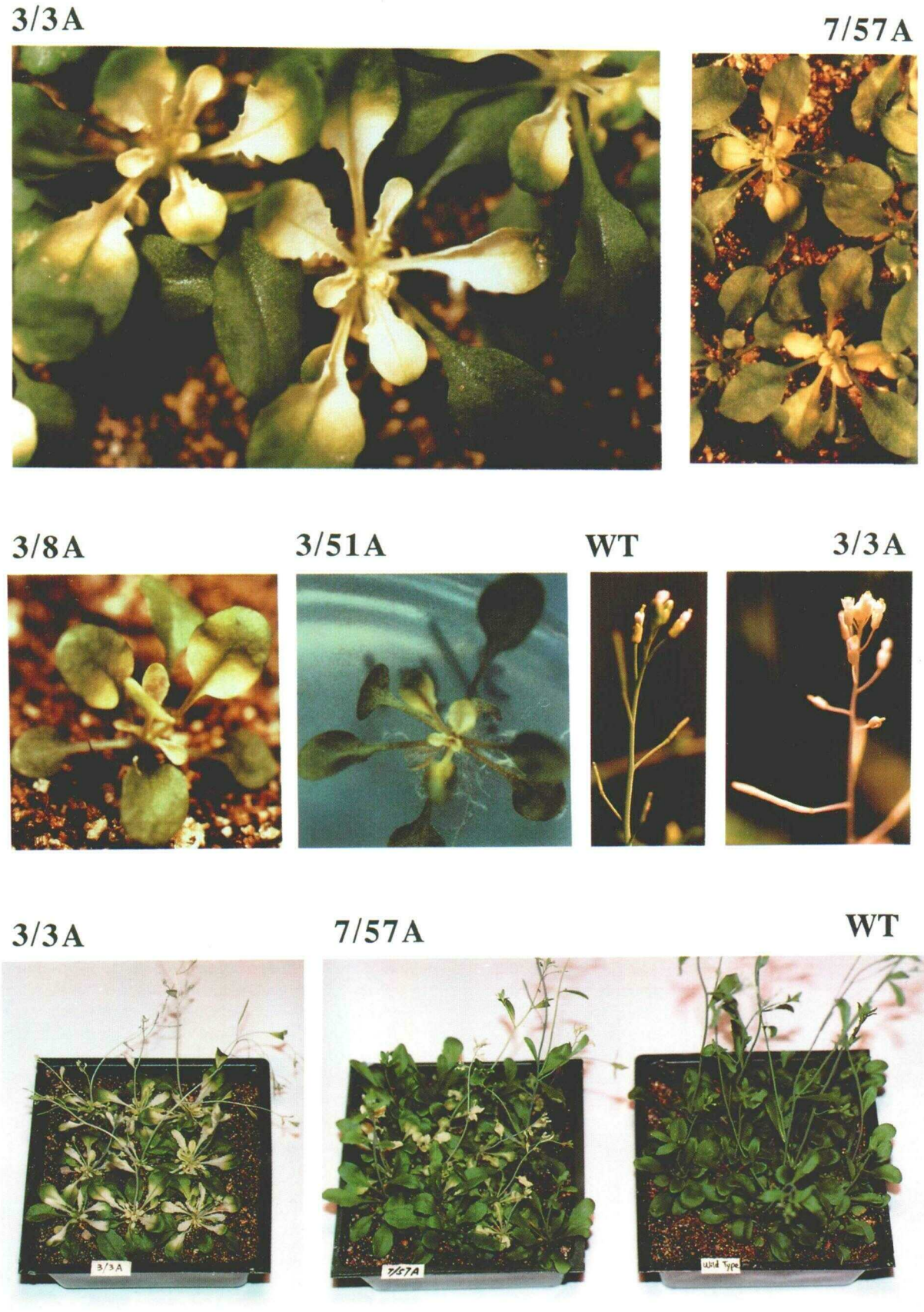

Figure 5. Examples of Chlorotic Plants and Wild-Type C24 Plants.

Table 1. Kanamycin Selection and T-DNA Copy Number of Selected Transgenic Plants

Plants	Kanamycin Resistant (No. of Plants)	Kanamycin Sensitive (No. of Plants)	T-DNA Insertion[a]
3/2	47	15	1 ($P > 0.7$)
3/3	58	0	>2 ($P > 0.1$)
3/8	47	0	>2 ($P > 0.1$)
3/50	15	8	1 ($P > 0.3$)
3/58	53	3	2 ($P > 0.5$)
7/2	94	30	1 ($P > 0.9$)
7/21	13	1	2 ($P > 0.5$)
7/25	18	5	1 ($P > 0.5$)
7/57	48	0	>2 ($P > 0.1$)
7/62	37	1	2 ($P > 0.2$)

[a] Number of independent insertion events and their probabilities from the Chi-square test.

transformed plants, 3/8A and 7/57A, respectively, as shown in Table 4. Plant 3/8A contained ~91 μg and plant 7/57A ~143 μg as compared to the wild-type plant that contained 2204 μg of total chlorophylls per gram of fresh tissue. The total carotenoid concentration also dropped from ~509 μg in the wild-type plant to ~40 μg and ~57 μg in 3/8A and 7/57A, respectively. In dark-grown plants where chlorophyll biosynthesis was blocked, the newly synthesized chlorophyll apoproteins were not stable, and were not generally detectable (Apel, 1979; Mullet, 1988). Without chloroplast and nuclear-encoded chlorophyll apoproteins, there would be no mature chloroplasts capable of photosynthesis.

Electron microscopic analysis of the chlorotic tissues of sense- and antisense-transformed plants revealed that most plastids in the chlorotic leaves are devoid of the distinctive grana thylakoid membranes, as shown in Figures 8A, 8B, and 8C. Instead, the few remaining thylakoid membranes became vesiculated and scattered throughout the stroma. Loss of stromal integrity is evident as electron-lucent areas that contain fibrillar and electron-dense material (Figures 8A, 8B, and 8C). These abnormal appearing plastids did not contain starch grains, but they did contain numerous plastoglobuli (lipids), which may be arranged in a clustered pattern (Figure 8C). In wild-type plants, chloroplasts contain starch grains and plastoglobuli as well as typical grana thylakoids (Figure 8D). The size and shape of plastids in chlorotic leaves varied from wild-type chloroplasts in that they were smaller and/or spherical in outline. However, the plastid envelope appeared to be intact, ensuring that these plastids are potentially still capable of interconverting themselves to other forms of plastids (Thomson and Whatley, 1980). The active biosynthetic activity of the plastids in the chlorotic tissues was indicated by the presence of approximately wild-type levels of *psb*A (encoding the D1 polypeptide of the photosystem II reaction center complex) and *rbc*L (encoding the large subunit of ribulose bisphosphate carboxylase) messages that are encoded by the chloroplast genome (data not shown).

DISCUSSION

We have characterized a novel higher plant gene, *AKR*, which might be involved in the regulatory process of chloroplast differentiation, because overexpressing the antisense RNA and sense RNA of the *AKR* gene in transgenic Arabidopsis plants caused a chlorotic phenotype. The chlorosis is the result of reduced plant chlorophylls and carotenoids and the absence of internal membrane development in the chloroplasts of the affected tissues. The expression of the *AKR* gene is light dependent and temporally and spatially regulated. For example, the steady state level of the *AKR* message is higher in leaves and flower stalks than in inflorescence stem; it also appears to be most abundant in the early stages of plant growth and then to decline with age. This may explain why the chlorotic plants are green when they are young and become chlorotic as the endogenous *AKR* message drops to some level that cannot overcome the effects of antisense or sense *AKR* messages. The heterogeneity observed at the onset of chlorosis could be due to the differences in expression level of the introduced sequence, which are, at least in part, determined by the sequence copy number, the chromosomal integration site, and de novo methylation of the introduced sequence itself (Ingelbrecht et al., 1991; Prols and Meyer, 1992).

Antisense Regulation

It is generally thought that the RNA duplex formed between endogenous mRNA and antisense RNA may impair mRNA processing, prevent further translation, or lead to rapid degradation (van der Krol et al., 1988). In our study, chlorosis is directly associated with the reduction of the endogenous *AKR* message. A large amount of antisense RNA found in a single

Figure 5. (continued).

The first number (3 or 7) indicates the plant series; numbering after the slash indicates a particular transgenic plant; WT designates wild-type plant. Plants in series 3 were transformed with the antisense construct and plants in series 7 with the sense construct. For example, 3/3 is the third transformed plant of the antisense construct series and 7/57 is the 57th in the sense construct series. The letter A indicates a particular progeny of the T_2 generation that corresponds to the original transformant with the same number and was subsequently maintained as an independent line.

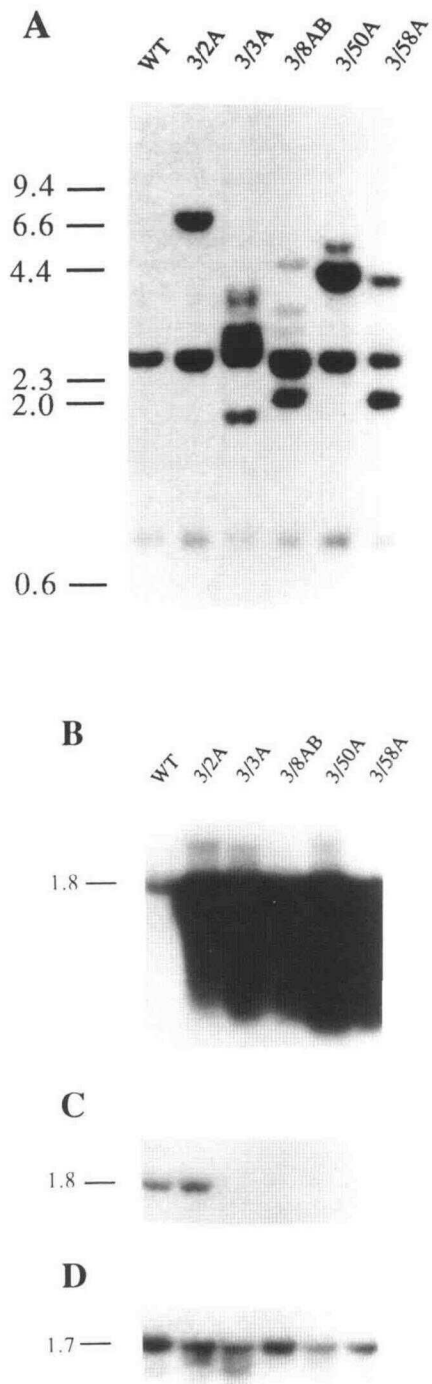

Figure 6. DNA and RNA Gel Blot Analyses of Plants Transformed with the Antisense Construct.

(A) Genomic DNA gel blot analysis of selected antisense-transformed plants. The DNAs were cut with HindIII and probed with AKR-13. HindIII-digested λ DNA length markers are given at left in kilobases. The lane designated 3/8AB was loaded with DNA from two chlorotic progeny of the original transgenic line 3/8.

(B) RNA gel blot analysis of the plants as given in **(A)**. The probe is the complete AKR-13 sequence that hybridizes to both antisense and endogenous messages.

(C) RNA gel blot analysis probed with the 3′ sequence (1137 to 1334) of AKR-13 that does not hybridize to antisense message.

(D) RNA gel blot probed with an NADPH-protochlorophyllide oxidoreductase gene probe.

The same blot was used in **(B)**, **(C)**, and **(D)**, and RNA markers are given at left in kilobases. In **(B)**, the lane designated 3/8AB was loaded with RNA from the same chlorotic progeny as given in **(A)**.

Table 2. Relative Intensities of the Endogenous and Antisense *AKR* Messages from RNA Gel Blot Analyses of Wild-Type and Selected Transgenic Plants[a]

Plant[b]	Chlorosis[c]	*AKR*[d]	anti-*AKR*
WT	–	5.9	
3/2A	–	7.9 (134%)	116.7
3/3A	+ + +	2.0 (34%)	152.0
3/8AB	+ + + +	1.1 (19%)	55.5
3/50A	+ +	2.5 (43%)	193.9
3/58A	+ +	2.3 (39%)	95.3

[a] The relative intensity data were collected from β-scanning of an RNA gel blot by a blot analyzer (Betascope 603, Betagene, Waltham, MA) and calibrated with the message of the protochlorophyllide oxidoreductase gene. Data are from the antisense-transformed plants shown in Figures 6B and 6C. *AKR*, endogenous *AKR* message; anti-*AKR*, antisense *AKR* message.

[b] WT, wild type.

[c] + indicates the severity of chlorosis; – indicates no chlorosis.

[d] The numbers within parentheses indicate the intensity in percent of the message as compared to the wild-type *AKR* message.

T-DNA insertion plant, 3/2A, did not reduce the steady state level of the endogenous *AKR* message, and so plant 3/2A did not become chlorotic. However, it is not clear why multiple copies of the antisense construct are needed to reduce the endogenous *AKR* message. Therefore, current hypotheses on antisense regulation might not explain this result.

Sense Regulation

The suppression by sense DNA is even more complicated, possibly involving interference of RNA strands during transcription or triggering a DNA modification process such as methylation of the homologous gene that may subsequently silence the gene permanently (Napoli et al., 1990; van der Krol et al., 1990b). The fact that the phenotype of some chlorotic plants transformed with sense construct is similar to that of antisense-transformed plants is different from the result with the petunia system where the phenotypes with the antisense chalcone synthase (CHS) gene were not the same as the sense phenotypes either in the degree of pigment reduction or in the

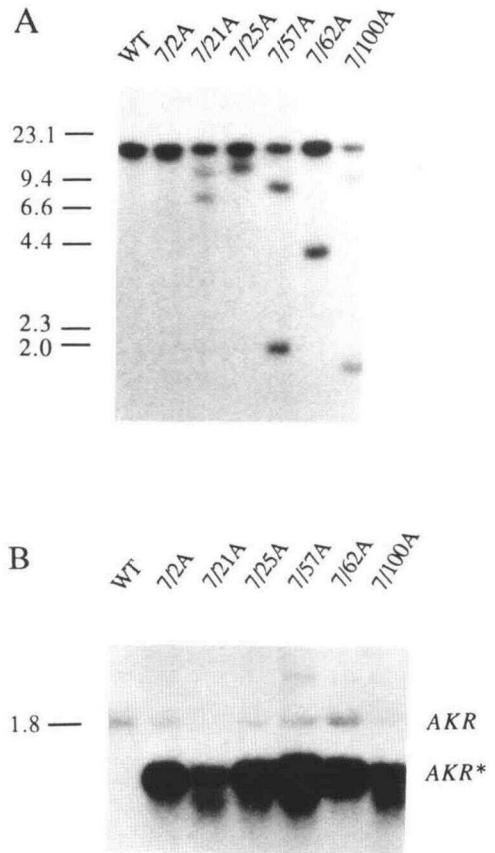

Figure 7. DNA and RNA Gel Blot Analyses of Plants Transformed with the Sense Construct.

(A) Genomic DNA gel blot analysis of selected plants with the sense construct. The DNAs were cut with BamHI and probed with AKR-13. HindIII-digested λ DNA length markers are given at left in kilobases. **(B)** RNA gel blot analysis of the same plants as given above. The probe is the complete AKR-13 sequence that hybridizes to both sense and endogenous messages. *AKR**, introduced sense *AKR* message. Length marker is given at left in kilobases.

nature of the patterns (Napoli et al., 1990). The differences between the Arabidopsis and petunia results are probably due not only to the rather complex mechanisms of gene silencing by antisense and sense DNAs, but also to the properties of the particular gene used in the experiments as well. In our study, the sense *AKR* construct can specify a truncated peptide with all four ankyrin repeats, and this peptide can presumably titrate (provided that it is translated and folded correctly) other protein(s) that otherwise would work together with AKRP through ankyrin repeats. The data shown in Table 3 suggest that both the reduction of endogenous *AKR* message and the high expression of the introduced sense *AKR* gene can contribute to the chlorotic phenotype, but the relationship between severity of chlorosis and endogenous *AKR* message or the introduced sense *AKR* message is not clear.

Possible Function of the *AKR* Gene

The reduction in the number of thylakoid membranes and the loss of organized grana coupled with the vesiculation of the few remaining thylakoid membranes from plastids of chlorotic leaves of both antisense- and sense-transformed plants appear to be the main cellular defect, while the developmental patterns of leaf, inflorescence stem, and flower are largely unaffected. This is similar to previous findings that inhibition of chloroplast development with herbicides (Reiss et al., 1983) or mutants that block chloroplast development and function (Mayfield and Taylor, 1987) have no direct effect on leaf morphogenesis. Many nuclear mutations that block specific processes in chloroplast biogenesis have been identified, including those which disrupt the structural organization of thylakoid membranes and the assembly of multiprotein complexes (Taylor, 1989). Plant organ development and chloroplast development are two separate processes, but the latter is coordinated and regulated by the former process. Based on the pleiotropic effects of antisense and sense *AKR* constructs on plants that lacked organized internal membranes and contained much reduced chlorophyll and carotenoid pigments, we propose that the developmentally regulated nuclear gene *AKR* might play a regulatory role in the process dealing with the temporal and spatial regulation of chloroplast development from proplastid. The residual pigments and internal membranes are probably due to the leaky nature of the antisense and sense technologies.

AKR might specify a transcription factor or a component of a transcription complex that controls genes required for chloroplast differentiation. AKRP is positively charged outside the ankyrin repeats and this region, amino acids 106 to 135, is similar to some well-studied DNA binding proteins (Figure 2B).

Table 3. Relative Intensities of the Endogenous and Sense *AKR* Messages from RNA Gel Blot Analyses of Wild-Type and Selected Transgenic Plants[a]

Plant[b]	Chlorosis[c]	*AKR*[d]	Sense *AKR*
WT	–	12.4	
7/2A	–	10.3 (83%)	35.3
7/21A	+ +	6.2 (50%)	18.4
7/25A	+	10.2 (82%)	44.9
7/57A	+ + +	12.3 (99%)	110.7
7/62A	+	15.4 (124%)	68.1
7/100A	+	10.2 (82%)	32.0

[a] The relative intensity data were collected from β-scanning of the RNA gel blot, as given in Table 2, and calibrated with the message of the *psb*A gene. Data are from the sense-transformed plants shown in Figure 7B. *AKR*, endogenous *AKR* message; sense *AKR*, sense *AKR* message.

[b] WT, wild type.

[c] +, severity of chlorosis; –, no chlorosis.

[d] Numbers within parentheses are as given in Table 2.

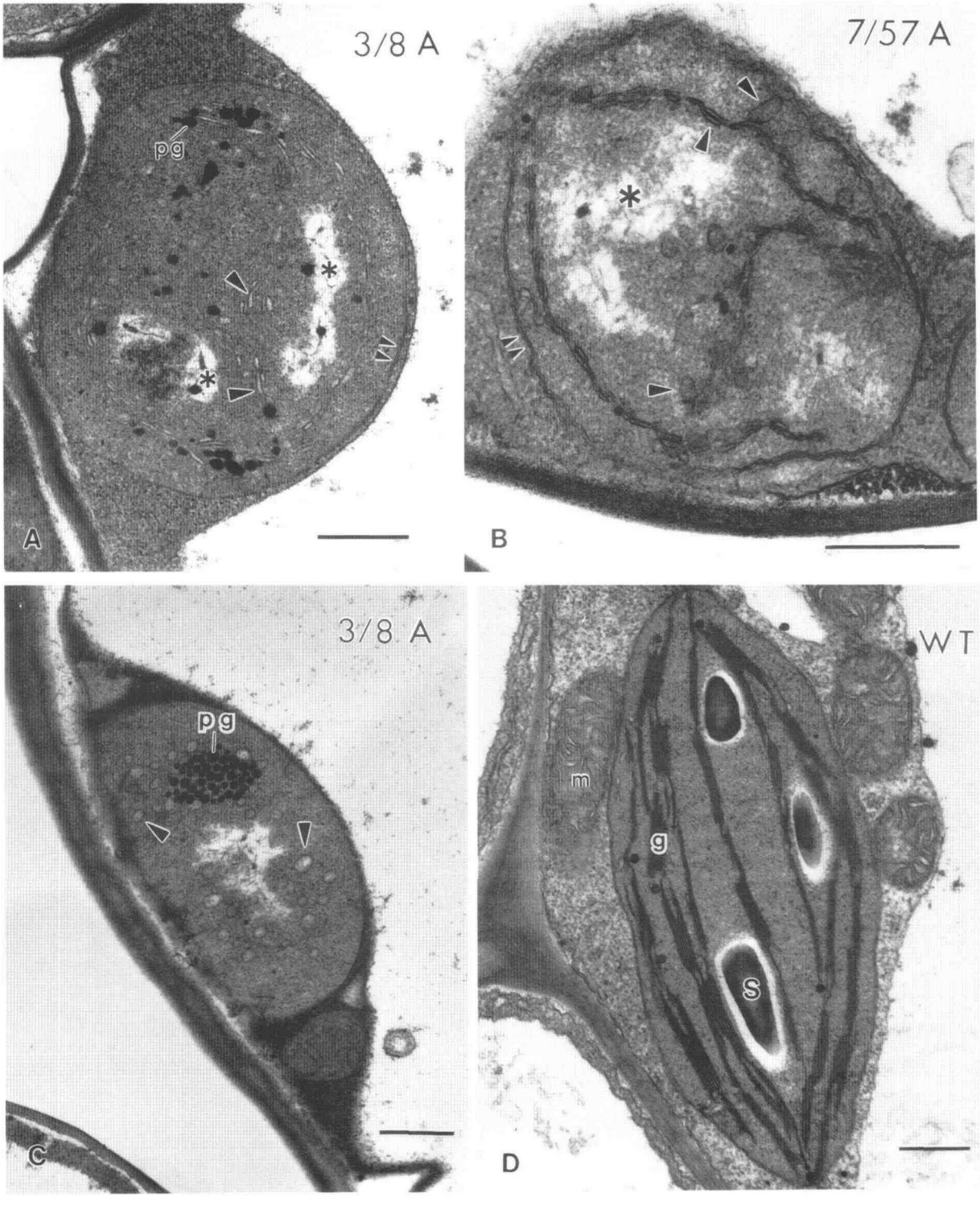

Figure 8. Plastids from Chlorotic Leaves of Transformants and from Wild-Type Arabidopsis.

Table 4. The Concentration of Carotenoids and Chlorophylls in Leaves of the Wild-Type Plant and in Chlorotic Leaves of 3/8A and 7/57A Plants[a]

Plant[b]	Chl *a*	Chl *b*	Chl *a/b*	Chl (Total)	Carotenoids
WT	1666 ± 72	538 ± 30	3.10 ± 0.09	2204 ± 98	509 ± 29
3/8A	52 ± 17	38 ± 15	1.39 ± 0.13	91 ± 32	40 ± 9
7/57A	103 ± 20	40 ± 7	2.54 ± 0.07	143 ± 27	57 ± 8

[a] Each data set are the mean of five plants ± SE. Chl, chlorophyll. The concentration is in micrograms per gram fresh weight of tissue.
[b] WT, wild-type plants; 3/8A, antisense transformant; 7/57A, sense transformant.

The last 10 nucleotides of the homeobox (residues 51 to 60) are highly conserved in homeobox proteins encoded by the *Msh* family, and they lie adjacent to the putative recognition helix of the DNA binding domain of the homeobox proteins (Gehring, 1987b). This suggests that they might play a role in the proteins' ability to bind to DNA. In AKRP, residues 106 to 130 can form a helix-turn-helix structure according to secondary structure predictions (data not shown; method of Garnier et al., 1978) with the amino acid residues NRRGG (numbers 116 to 120) forming the turn in the middle. It is not known whether this helix-turn-helix structure exists in vivo, and if it does, whether it can bind to DNA. On the other hand, *AKR* could also specify a structural protein that is responsible for the formation of the thylakoid membranes, because the intrinsic nature of ankyrin repeats in binding to some membranes and proteins (Bennett, 1992) might play a prominent role in chloroplast maturation during plastid inner membrane proliferation.

Recently, an Arabidopsis K^+ transport gene, *AKT1*, was cloned (Sentenac et al., 1992) and shown to encode a protein with three domains: (1) the expected channel-forming domain, (2) a cyclic nucleotide binding site, and (3) the C-terminal domain of six ankyrin repeats. The *AKT1* gene has no similarity to *AKR* at the DNA level, but the fact that ankyrin repeats have now been found in two Arabidopsis proteins indicates the possibility of a much larger family of proteins sharing the same relatively conserved amino acid motifs. The location of ankyrin repeats in the K^+ transporter of *AKT1* (cytosolic side of membrane) is reminiscent of the proteins of Notch, *Lin-12*, and *Glp-1*, a result that supports the proposed roles of ankyrin repeats as protein–protein interaction domains in transducing signals or in maintaining certain membrane proteins in cellular locations which optimize their function in tissues (Thompson et al., 1991; Bennett, 1992). However, the precise role of the *AKR* gene in chloroplast differentiation remains to be shown.

METHODS

Arabidopsis and Growth Conditions

Two wild ecotypes of *Arabidopsis thaliana* were used in these experiments, C24 and WS. Plants were normally grown in the greenhouse (22°C) for 4 weeks until harvested for RNA preparation. The 4-week-old plants (WS) used for the light induction experiment were kept in darkness for 3 days, then shifted to light for various times before the leaves were harvested for RNA preparation. The chlorotic leaves were harvested ~4 weeks after planting and were assayed for chlorophyll and carotenoid concentrations according to the method of Lichtenthaler and Wellburn (1955).

Libraries and cDNA Clones

The λgt10 cDNA library made from leaf mRNA of Arabidopsis (ecotype Columbia) was obtained from Dr. N. Crawford (University of California at San Diego) and the λfix genomic library made from Arabidopsis ecotype Landsberg from Dr. D. Voytas (Iowa State University, Ames).

Figure 8. (continued).

(A) Plastid from a chlorotic leaf of the antisense transformant 3/8A. Thylakoid membranes are dilated or vesiculated (arrowheads). Typical grana organization is absent (compare with **[D]**). The dense stroma is interrupted by electron-lucent areas (asterisks) that contain fibrillar material and electron-dense material which may represent plastoglobuli (pg). Numerous plastoglobuli are found throughout the stroma. A double plastid envelope is evident (double arrowheads).
(B) Plastid from a chlorotic leaf of the sense transformant 7/57A. Thylakoid membranes lack distinctive ordering into grana (compare with **[D]**). Vesiculation of thylakoid membranes is also evident (arrowheads). The asterisk indicates an electron-lucent area of stroma; double arrowheads show the plastid envelope.
(C) Plastid from a chlorotic leaf of the antisense transformant 3/8A. The plastoglobuli (pg) are clustered in this plastid. Vesiculation of thylakoid membranes (arrowheads) is also evident.
(D) Mature chloroplast from a wild-type (WT) Arabidopsis leaf. Grana thylakoids (g) are present as well as starch grains (S). The stroma is uniformly dense. m, mitochondrion.
Bars = 0.5 mm.

The cDNA clone for the NADPH-protochlorophyllide oxidoreductase gene (Benli et al., 1991) was obtained from Drs. K. Apel and C. Forreiter (Institut für Pflanzenwissenschaften der ETH, Zürich, Switzerland), and the cDNA clone for *Cab4* was isolated in this laboratory (Zhang et al., 1991).

DNA and RNA Manipulations

The AKR-32 and AKR-2.5 clones were isolated using AKR-13 as a probe. AKR-11 was cloned by synthesizing single-stranded cDNAs (reverse transcribed from mRNA templates), amplifying the AKR cDNA with synthetic primers designed from the genomic sequence (5′ side) and other cDNA sequences (3′ side) by polymerase chain reaction (PCR), and subcloning the amplified cDNA into the pBluescript KS+ plasmid for sequencing. The conditions for reverse transcription and PCR followed the suggestion by Domec et al. (1990). The primers for AKR-11 amplification are:

oAKR-1, 5′-AGGTCAGGATTCCGGCTATTGAG-3′
oAKR-3, 5′-AGAAAATGAGGCCCATTAAAATT-3′.

The primers oAKR-3 and oAKR-1 are identical to nucleotides 165 to 187 and 992 to 1014, respectively, of the full-length transcript (Figure 1B).

Genomic DNA was prepared according to the method of Dellaporta et al. (1983), digested with restriction enzymes, electrophoresed (5 μg per lane), blotted to a Biotrans nylon membrane, and hybridized with the AKR-13 probe. Total RNA was isolated according to the method of Logemann et al. (1987), separated by electrophoresis (15 μg per lane), blotted to a Biotrans nylon membrane, and hybridized to various probes. Hybridizations were carried out according to the method of Church and Gilbert (1984) using probes labeled by random priming. The washing conditions were as follows: two times (10 min each) in 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, and 5% SDS at 63°C; then four times (5 min each) in 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, and 1% SDS at 63°C.

Electron Microscopic Analysis

Three- to 4-week-old Arabidopsis plants were harvested and immediately fixed in 3% glutaraldehyde in 0.1 M phosphate, pH 7.2, for several hours at room temperature and postfixed overnight at 4°C with 1% osmium tetroxide in the same buffer. Tissues were dehydrated with an ethanol series and embedded in an epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 10A electron microscope (Carl Zeiss, Inc., Thornwood, NY) at 60 kV.

Arabidopsis Transformation

The binary vector pBIN121 (Jefferson et al., 1987) was digested with BamHI and SstI, and the β-glucuronidase gene sequence was replaced with antisense or sense AKR-13 sequences to which BamHI and SstI linkers were previously added; C24 Arabidopsis was then transformed with the antisense and sense constructs according to the protocol of Valvekens et al. (1988). The sequences contained in nucleotides 1 to 49 of AKR-13 were a result of a cDNA cloning artifact because they occurred again at nucleotides 855 to 903 and were not found in the corresponding place in other cDNA clones or in the genomic sequence. The presence of this 49-nucleotide sequence, a part of the ankyrin repeat–encoding sequence, should not alter the conclusions drawn from the antisense experiment described in this paper.

GenBank Accession Number

The accession number for the sequence reported in this paper is M82883.

ACKNOWLEDGMENTS

We thank James Haseloff for critically reading the manuscript; Susan Hanley for restriction fragment length polymorphism mapping of the *AKR* gene; Inhwan Hwang and Gabor Lazar for help with the transformation experiments; Klaus Apel, Christoph Forreiter, Daniel Voytas, and Nigel Crawford for the DNA probe and libraries used in this experiment. H.Z. would like to thank Chris Somerville of Michigan State University for his advice and support. This research is supported by a grant from Hoechst AG (Frankfurt, Germany).

Received September 11, 1992; accepted October 21, 1992.

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