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Characterization of the tobacco eIF-4A gene family

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Abstract

Characterization of cDNAs encoding eukaryotic translation initiation factor 4A (eIF-4A) indicates the expression of a minimum of ten related genes in tobacco leaf cells. The ten groups fall into two gene families, NeIF-4A2 and NeIF-4A3. The majority of the cDNAs exhibit significant sequence similarity to the NeIF-4A2 family at both the DNA and deduced amino acid levels. Northern analysis using specific probes indicates variable expression of four family members in various tobacco organs. Western analysis, using an anti-tobacco eIF-4A polyclonal antibody, reveals a complex pattern of immunologically related polypeptides of approximately 46 kDa. Subcellular fractionation suggests that at least one eIF-4A-related polypeptide is located in the chloroplast where it is ribosome-associated.

Introduction

Translation initiation factor 4A (eIF-4A) is a crucial element in the eukaryotic translation initiation pathway. eIF-4A is a ssRNA-dependent ATPase which functions as an RNA helicase when complexed with an accessory protein, eIF-4B. eIF-4A is also a subunit of the eIF-4F protein complex involved in the initial step of translation initiation, 5′mG cap recognition [14].

Only two expressed eIF-4A genes are present in mammalian and yeast systems. In the murine system the genes exhibit over 90% identity and are differentially expressed in various tissues [7], while in yeast the two genes code for identical proteins [6].

Tobacco eIF-4A gene organization and expression differs from the mammalian and yeast systems. Two eIF-4A genes have been isolated which exhibit 64% amino acid identity with one another at the protein level [9]. Furthermore, although expression levels of the tobacco genes, NeIF-4A2 and NeIF-4A3, varied between plant organs, the two genes were coordinately expressed in each organ. Finally, Southern analysis indicated that the eIF-4A genes actually fall into two multigene families in tobacco. These results suggested that a number of eIF-4A genes exist in tobacco and

The nucleotide sequence data reported will appear in the GenBank and EMBL Nucleotide Sequence Databases under the accession numbers X79135 (NeIF-4A9), X79136 (NeIF-4A11), X79137 (NeIF-4A7), X79138 (NeIF-4A15), X79139 (NeIF-4A6), X19140 (NeIF-4A13) and X79141 (NeIF-4A14).

raise the questions as to why tobacco requires the expression of multiple divergent eIF-4A genes. As an initial step to investigate these questions, we have extensively characterized the tobacco eIF-4A gene family.

Materials and methods

Plant material

Nicotiana tabacum cv. Samsun NN was grown as described [9].

cDNA clone isolation

A *Nicotiana tabacum* cv. SR1 leaf λZAP cDNA library purchased from Stratagene (catalogue number 936002) was screened at low stringency using standard protocols [1]. The tobacco NeIF-4A1 cDNA [9], labelled with ^{32}P-dCTP using a random primer labelling kit (Boehringer Mannheim), was used as the probe. All of the positive clones, 57 in total, were picked and rescreened. Phagemid particles were isolated from single hybridizing plaques by zapping as described by the manufacturer (Strategene).

DNA sequence determination and analysis

The cDNA clones were sequenced by dideoxy chain termination using a series of synthetic oligonucleotide primers. Sequence analysis was performed with the University of Wisconsin Genetics Computer (UWGCG) Sequence Analysis Software Package as described [9].

Northern analysis

Total RNA was isolated through hot phenol extraction and lithium chloride precipitation [1]. The quantity and quality of the RNA were determined before separation on glyoxal gels and blotting [1]. Northern blots were prewashed for 1 h at 65 °C in 1 × SSC, 0.1% SDS and then prehybridized in 50% deionized formamide, 5 × Denhardt's solution, 0.2% SDS, 5 × SSPE and 100 μg/ml ssDNA for 4 h at 65 °C. For hybridization, fresh buffer was added containing gene-specific anti-sense RNA probes, derived from 3′ UTR regions of the indicated cDNA clones. RNA probes were generated using T3 or T7 RNA polymerase and a Stratagene kit. Hybridization was carried out for a minimum of 24 h at 65 °C. The blots were washed twice in 1 × SSPE, 0.5% SDS for 10 min each at 65 °C and at least once in 0.1 × SSPE, 0.5% SDS for 10 min at 65 °C. Blots were exposed to Fuji XAR X-ray film at − 80 °C for 6–120 h.

Gene-specific probe production

Gene-specific probes for tobacco NeIF-4A gene family members, 1, 3, 5, 9, 11 and 15, were produced by using restriction enzyme cleavages sites within or close to the translation termination codon and including all or part of the 3′ UTR of each family member. Specifically, the NeIF-4A5 probe contained a 283 bp fragment extending from a *Taq* I site 35 bp 5′ of the translational stop codon and continuing to the end of the cDNA clone at an *Eco* RI site in the polylinker; the 272 bp NeIF-4A15 probe extended from a *Hin*d III site 2 bp 3′ of the translational stop codon and continued through the 3′ UTR to an *Eco* RI site in the polylinker; the NeIF-4A9 probe included a 153 bp *Eco* RV fragment within the 3′ UTR starting 84 bp 3′ of the translational stop codon and ending 20 bp 5′ of the poly(A) tail; the NeIF-4A11 probe consisted of a 227 bp *Bam* HI fragment starting 107 bp 3′ of the translational stop codon and continuing to the polylinker; the NeIF-4A3 probe contained a 174 bp *Alu* I-*Bam* HI fragment starting 229 bp 3′ of the translational stop codon and extending through the 3′ UTR to the polylinker; and a 191 bp *Eco* RV fragment starting 73 bp 3′ of the translational stop codon and continuing to the polylinker from the NeIF-4A1 cDNA 3′ UTR was utilized as a probe for the highly related NeIF-4A2 family members.

The NeIF-4A1 probe would be expected to hybridize to all of the other NeIF-4A2 gene family members characterized in this paper as a result of the high DNA similarity within their 3′ UTR's.

Generation of NeIF-4A antiserum

The NeIF-4A1 cDNA [9], a member of the NeIF-4A2 gene family, was cloned into *Bam* HI-cleaved pET3C [15] as a *Bam* HI/*Bgl* II fragment. This construct allows the translation of a 33 kDa fusion protein consisting of 11 amino acids of the gene 10 protein, the major capsid protein of the phage T7, and the C-terminal 266 amino acids of the NeIF-4A1 polypeptide. NeIF-4A1 is a partial cDNA clone starting 41 amino acids 5′ of the DEAD box and continuing through the open reading frame to a long 3′ UTR. The fusion protein contains the DEAD, SAT and HRIGR amino acid motifs, which are highly conserved within the DEAD box gene family [12]. Inclusion bodies were purified and the 33 kDa polypeptide electroeluted after separation through a 15% SDS-polyacrylamide gel. The purified protein was used to raise antibodies in rat and rabbit.

Western analysis

Total soluble proteins were isolated from frozen plant material by grinding in liquid nitrogen and extracting in 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 M NaCl, 2% polyvinylpolypyrrolidone and 50 mM 2-mercaptoethanol. Proteins were quantified with the Bradford assay (BioRad) using BSA as the standard. SDS-PAGE electrophoresis and western analyses were performed as described previously [8].

Chloroplast and polysome isolation

Washed chloroplasts and chloroplast 30 K supernatants were isolated from pea plants according to the protocol described by Robinson and Barnett [10]. Crude and purified polysome fractions were prepared from purified chloroplasts on sucrose gradients [17]. For protease treatment, washed pea chloroplasts were resuspended in ice-cold thermolysin incubation buffer (0.35 M sucrose, 25 mM HEPES-KOH pH 7.6, 1 mM $CaCl_2$), thermolysin was added to the indicated concentration, and incubation took place on ice for 30 min. Protease activity was inhibited by the addition of EGTA (10 mM) and one volume of 2× Laemmli's SDS-PAGE loading buffer. eIF-4A polypeptides were detected by western blotting.

Wheat germ eIF-4A purification

Wheat germ eIF-4A was purified through the DEAE-cellulose step as described by Lax *et al.* [5]. eIF-4A containing fractions were pooled, desalted and applied to a Cibacron Blue 3GA column to which wheat germ eIF-4A did not bind. The flow through protein was used as the source of wheat germ eIF-4A.

Results

NeIF-4A gene family sequence analysis

We utilized one of our previously isolated eIF-4A cDNA's, NeIF-4A1 [9], to screen a *Nicotiana tabacum* SR1 leaf cDNA library at low stringency in order to obtain other members of the NeIF-4A gene family. Partial DNA sequence analysis of 34 of the 57 positive phage clones isolated in this screen indicated that they could be classified into ten groups. A comparison of the deduced amino acid sequences of a representative member of each of these groups is shown in Fig. 1. The DNA sequences of each of the clones belonging to one group were identical, with one exception: sequences of members of group NeIF-4A15 are highly related but not identical and the position of the poly(A) addition site was not conserved within any group (see below).

The deduced amino acid sequences of each of

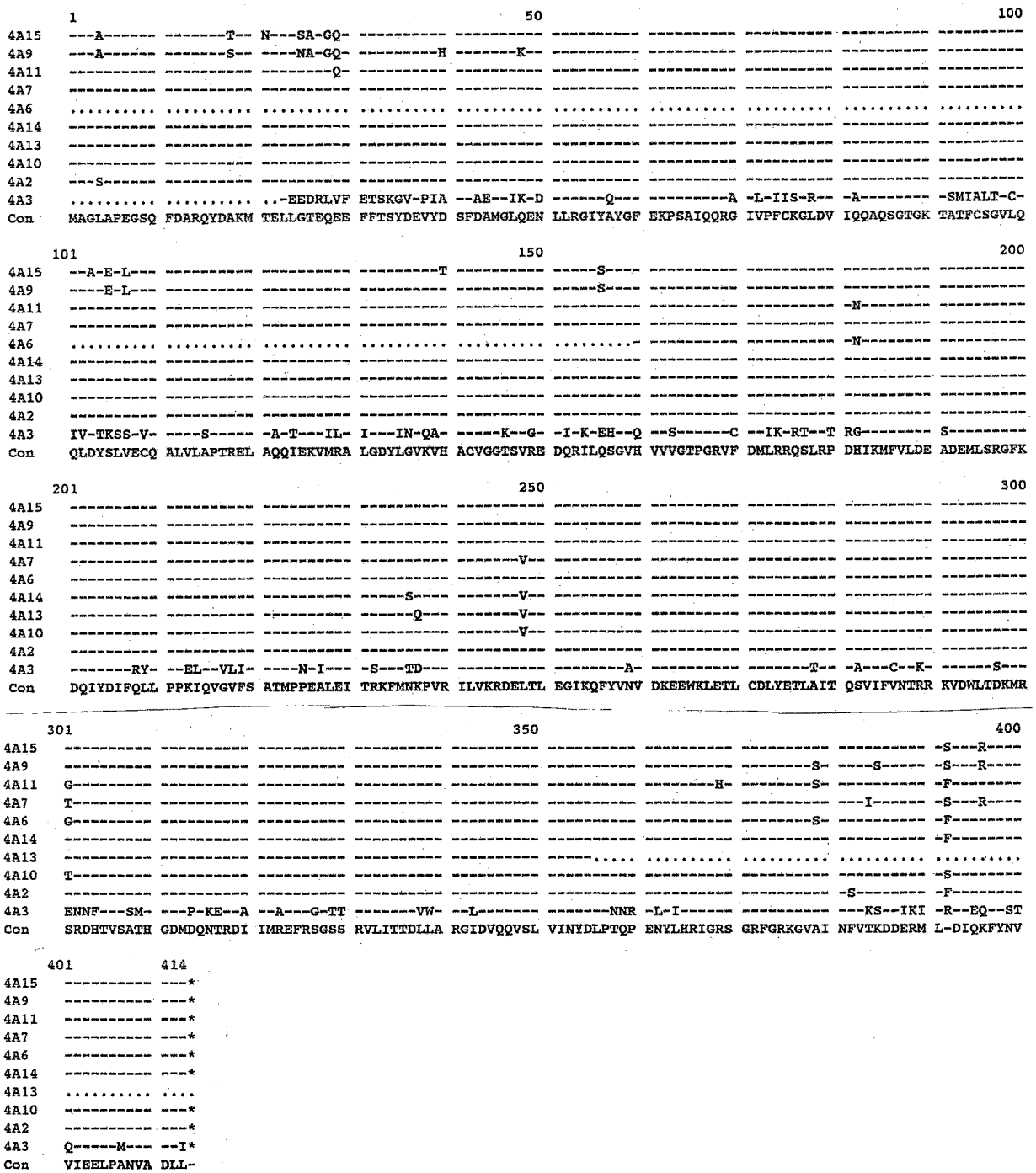

Fig. 1. Deduced amino acid sequence alignment of the nine NeIF-4A2 family members with the NeIF-4A2 and NeIF-4A3 sequence [8] for comparison. Dashes denote sequence identity, dots indicate the lack of coding region in this cDNA, capital letters indicate amino acid differences, and Con indicates the consensus sequence.

the groups are highly similar to NeIF-4A2, with identities ranging from 95 to 99% (Fig. 1). The coding region of each of the NeIF-4A family members described in this paper consists of a 1242 bp open reading frame specifying proteins containing 414 amino acids. Thus these groups

belong to the NeIF-4A2 gene family. The second eIF-4A gene family in tobacco, NeIF-4A3, exhibits only 64% amino acid sequence identity with NeIF-4A2 [9]. In fact, only one additional clone belonging to the NeIF-4A3 subfamily was isolated during the screening described here. The NeIF-4A6 group exhibited the highest amino acid identity to NeIF-4A3, 69%. None of the cDNA clones sequenced contained altered amino acid sequences in any of the conserved boxes which are characteristic of eIF-4A sequences, in contrast to the predicted amino acid sequences of some genomic eIF-4A genes which we have analysed (Brander *et al.*, in preparation).

Sequence alignment of the seven groups containing 5′-untranslated regions reveals significant sequence identity between all groups (Fig. 2A). The sequence identity was evident even though the lengths of this region were quite variable, ranging from 21 to 167 bp (Fig. 2A). The 5′ UTR sequences are CT-rich and while the 5′ UTR regions of groups 2 and 15 contain in-frame stop codons upstream of the presumed ATG the other 5′ UTR's do not. None of the 5′ UTR's contain either in-frame or out-of-frame ATG translation initiation codons. Most interestingly, alignment of the 5′ UTR's indicates that the NeIF-4A2 groups can be further classified into two distinct classes depending on the presence or absence of a 17 bp sequence (Fig. 2A). This sequence lies ca. 90 bp upstream of the presumed translation initiation codon. The 5′ UTR sequences can also be divided into two groups depending on the presence of secondary structure in this region. Extensive stem-loop structures can potentially be formed in the 5′ UTRs of NeIF-4A groups 2, 9, 13 and 14 while this region is structureless in groups 7, 10 and 15. Furthermore, these stem-loop structures all occur within 25 bp of the 5′ end of the UTR, in close proximity of the 5′ cap structure (data not shown).

Alignment of the 3′ UTR regions of the seven subfamily members containing this region reveals a diversity of sequences (Fig. 2B). The 3′ UTR regions are quite variable in length (Fig. 2B) and exhibit identities to the NeIF-4A1 3′ UTR ranging from 37 to 92% (data not shown). The 3′ UTR regions of the NeIF-4A2 subfamily mem-

```
NeIF-4A2                                                TG CGGCGTAGAT CCGAGGAGTT TCAGACAAAA ..........
11
10                                                                    GGAGTT TCAGACAAAA ..........
13          CAATCTTTCT .....CTTTT .CCTCTTTCC TGAACTCCTG CGGCGTAGAT CCGAGGAGTT TCAGACAAAA ..........
14            ATCTTTCT ATTCTCTTTT TCCTCTGT.. . AACTCCTG CGGCGTAGAT CCGAGGAGTT TCAGACAAAA ACAGCAGTCT
7                                                                                    AAAA A..GCAGTCT
9                                                          CGTAGAT CCGAGAAGTT TGGGA.AAAA A.........
15                                                             AT CCGAGGAGCT TGCGT.AAAA ..........


NeIF-4A2    .......CCC TAA.TCCCCA ATCGCTATAA CCCACTTTTC AATACA.CTC TCTCTTTTCT AT.TCCTCAG ATCTTTTCCT
11                                                                                          CACTT
10          .......CCC TAACCCCCCA ATCGCTTTTA CCCACTTTTC ATTTCA.CTC TCTCTTTCCT AT.TCCTCAG ATCTTTTCCT
13          .......CCC TAACCCCCCA ATCGCTTTTA CCCACTTTTG CTTTCA.CTC TCTCTTTCAT AT.TCCTCAG ATCTTTTTCT
14          TTGCAAACCC TAACTTTCCA ATCGCTTTTA CCCACTTCTC CTTTCAACTC TCTCTTTGCT AT.TCCTCTC ATCTTTTCCT
7           TTGCAAACCC TAACTCTCCA ATCGCTTTTA CCCACTTTTC CTTTCAACTC TCTGTTTGCT AT.TCCTCAG ATCTTTTCCT
9           .......CCC TAACTTCTCT .....TATTA CTCACAACTG CAG.CATCTC ......TTCT CT.TCCTCTA ..........
15          .......CCC TAA.TTTTCA .......... .TCACTTTCT CTTT..TGTC ........AT ATCTCC.... ATCTTACTTT


NeIF-4A2    ..TATCTCTA TCAGTCATGG CTGGCTCGGC ACCAGAA...
11          CATATCTTTA TCAGTTATGG CAGGCTTGGC ACCAGAG...
01          CAT..CTCTA TCAGTCATGG CTGGCTTGGC ACCGGAA...
13          CAT..CTCTA TCAGTCATGG CTGGCTTGGC ACCGGAA...
14          CAT..CGCTA TCAGTCATGG CTGGCTTAGC ACCGGAA...
7           CGT..CTCTA TCAGTCATGG CTGGCTTGGC ACCGGAA...
9           CAT....CT. TCAGTCATGG CGGGTGCAGC TCCTGAA...
15          CAT....CT. TCAGTCATGG CGGGTGCAGC TCCAGAA...
                       Met
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Fig. 2A. Sequence comparison of the 5′ UTR of the NeIF-4A2 gene family cDNA members. Dots represent gaps introduced to maximize sequence homology. The putative ATG translation initiation codon is highlighted. The 17 bp sequence is underlined.

```
15          TGCTGATCTC CTGTAAAAGC TTTTTCCTTG CTTCAGTGGC AAAAATATAG AAGCTGGTAC TGTTTGGGCG GG.TTCTCTC
9           TGCTGATCTC CTGTAACCAG CTTGGACATT CAGAGGTTTT ACAATGTGGT GCTTTAGCTG TGTCGCGAAA CTTCCCCTCC
5           TGCTGATCTC CTCTGAAAAA CTTATGTTTG TGGCTGGAGC TTTAAGCAGA AAGAGTAACC ATGTTATAGT GTCCACACCA
11          TGCTGATCTC CTTTGATGCG ATTTTGGTTA TGGTGCGGGG AATTTTTCCT TACATGAAGT AGTTTAATAT ATTTATTCTT
7           GGCCGATCTC CTTTGATGTG GTTTTGGTTC TGTGAGGTGA ATTTCTTCTT TACATGAAGT AATTTAATAT CTT.ATTATT
NeIF-4A1    GGCCGATCTC CTTTGATGCG ATTTTGGTTC TGTGATCTAA ATTTGTTCTT TACATGAAGT AATTTAATAT TTT.ATTCTT

15          TCCACACATC TACCTCTTTT CTTCCCCGGC CCTCTCCTTT CATTTTCATA TTTTC.TTGT GTAATGAATG TCT.......
9           TTTCTTTTTC ATCTTCTTGA TATCCTTCTT ACCTTTTGTT TGTTTAGTAG TTTCTGTGAT TCTGAACTAA TGAGTAATGC
5           TTCCAGCTTA TAGCATAGAA TTAGTCTTTT AACTACCATT ATTATCTAGT GTCTAAGACA GGCCCGTGGC ATTAATGCTG
11          AGGCTTAGTT TCACTTTGCG CTGGACTTGG AATTGGGAAA TGCTGGATCC TTGATTTTGC CAAAGCACCA ......GCTG
7           AGGTTT.GTT AGATATCTA. TTGGTCTTAG AATTGGGAAA TGCAGGTGCC ATGATTCGCC ATGGAACCTG T.........
NeIF-4A1    AGG.TTAGTT AGATATCTA. CTGAACTAAG AATTGGGAAA TGCAGGTGCC ATGGTACGCC ATGGAACCTG T.........

15          .......AAT TTAATGGTCC TAGTGTCTGA GATTATGAAC TTGTGAGTAA TTTACACTAG CGTTGTTTAA T.ATATCACT
9           ATATTAGCAT TTTGTAATCC ATCATCCTAT TTAGTGGTTT TCTTTTGCTA GTTTTTGACA .......... ..........
5           CT..CCAAAT TTTGCAGTAG GTAAG..... .......... .......... ......GTCT TTGTTTTTCA TTTCCCAGTT
11          CTGTCCCAAT TTAGTAGAAC TTTGAGTCGA CTATTG..CA AATTTTGTTT GGTTTTGTCA ..GTTT.GTA T.GTTTAAGC
7           .......AAT TTTCCTGTTT GGTTGGAGTA CCTACTATCA A.TTTTGT.C GGTTT.ATCT TTGTTTTGTA TTGT..AAGT
NeIF-4A1    .......AAT TTTCCAGTTT GGTTGGAGTA CCTACTATCA A.TTTTGT.C GGTTT.GTCT TTGTTTTGTA TTGT..AAGT

15          ATATTATA.C ATTGGTAGCT GCTGCTAG.T TTTTGACATC AAA..GGGTT GTATCTGAAG TACAA.TGTC AAAGTATGAA
9           .......... TCCA.AAGTT TTTGTA.TTT .GAAGAA.TG ATATCGAGTA CATTTTTAAT TTGCAAAAAA AAAAAAA
5           CTTAAACTTC TTTGGCATTT T..GTAGTTT ATTTGAGTTT TAAAAAAA
11          GTCTAATGAC TTAAAATTTT GTTGCTGTCT .GAATTGACT ATATTGAG.T TTAGCAGAGC TATTTTTCTC TGGAAGGAAA
7           CCAAAATAAC TCCA..AGTT TTGGATGC.T TTTTGAATTT TTATTGAGAT TTAACTCTTG GCCTTGTTAT TGTGTAAAAA
NeIF-4A1    CTAAAATAAC TTCA..AGTT TTGGATGTT. CTTTGAATTT TTATTGAGAT TTAA

15          ATCATTCACT TATGTCGAAG TATAATATCA AAAAAAAAAA
11          GCTTTTTGCT GGTGAAAAAA AA
7           AAAAAAAAAA
```

Fig. 2B. Sequence comparison of the 3′ UTR of the NeIF-4A2 gene family members. Dots represent gaps introduced to maximize sequence homology. The translational stop codon and the putative poly(A) adenylation signal are indicated in bold.

bers do not exhibit significant sequence identity with the NeIF-4A3 3′ UTR. The 3′ UTR's of NeIF-4A groups 1, 7 and 11 contain potential modified polyadenylation addition sequences, either AATAAC or AATGAC, at a variable distance from the poly(A) tail. The other NeIF-4A groups do not contain recognizable polyadenylation signals (Fig. 2B).

Despite the identity of the 5′ UTR, open reading frame and 3′ UTR DNA sequences of each of the clones belonging to one group, the position of the poly(A) addition site varied within each

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NeIF-4A10                                                                    POLY(A) ADDITION SIGNAL
CLONE

2           TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
47          TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
28          TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
4           TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
43          TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
29          TTTGGTTGGA CTATCTACTA TCAATTTTAT CGGTTTGCC. ...TTTTGTA TTGTAAGTCT ACAATAACTT C.AGTTTTTG GATGTTCTTT
NeIF-4A1    TTTGGTTGGA GTACCTACTA TCAATTTTGT CGGTTTGTCT TTGTTTTGTA TTGTAAGTCT AAAATAACTT CAAGTTTT.G GATGTTCTTT


2           GAAATTTTAT TGAGATTTAA CTAAAAAAAA A
47          GAAATTTTAT TGAGATTTAA CTCTTGACCT AAAAAAAAAA AAA
28          GAAATTTTAT TGAGATTTAA CTCTTGACCT TGAAAAAAAA AAAAAAAAAA AAAAAAA
4           GAAATTTTAT TGAGATTTAA CTCTTGACCT TGTTATTGTG TATGAGGAAG CTTTTAGAGG CAAAAAAAAA AAAAAAA
43          GAAATTTTAT TGAGATTTAA CTCTTGACCT TGTTATTGTG TATGAGGAAG CTTTTAGAGG CAAATTGTTT TAAAAAAAAA
29          GAAATTTTAT TGAGATTTAA CTCTTGACCT TGTTATTGTG TATGAGGAAG CTTTTAGAGG CAAATTGTTT TATTTGAAAA AAAAAAAAAA
NeIF-4A1    GAATTTTTAT TGAGATTTAA
```

Fig. 2C. Sequence comparison of the alternative polyadenylation sites in NeIF-4A2 subfamily 4A10 aligned with the NeIF-4A1 3′ UTR. Base pair variation between the NeIF-4A1 and NeIF-4A10 family members are underlined. The putative poly(A) addition signal sequence is indicated.

group. This variation is demonstrated in Fig. 2C using the NeIF-4A10 group as an example. The poly(A) addition site in this group occurs at a variable position ranging from 39 to 93 bp downstream of the presumed poly(A) addition signal. Even though six of the NeIF-4A10 clones isolated contained poly(A) tails, none of the clones contain identical poly(A) addition sites. Poly(A) site addition variability was also observed in each of the other NeIF-4A groups (data not shown). As previously observed with NeIF-4A1 and NeIF-4A3 [9], all of the NeIF-4A gene family members characterized in this paper have the potential to form significant stem-loop structures in their 3′ UTRs (data not shown).

Expression of NeIF-4A gene family members

Gene-specific probes for NeIF-4A groups 1, 3, 5, 9, 11 and 15 were produced as described in Materials and methods. Gene-specific probes could not be generated for the other NeIF-4A groups as a result of the high sequence similarity between them (Fig. 2B). Northern blots containing total RNA isolated from six tobacco organs were probed with each NeIF-4A group-specific probe. Results of this analysis are shown in Fig. 3. As observed previously, NeIF-4A2 and NeIF-4A3 are expressed in all organs examined. The expression level of NeIF-4A2 exceeds that of NeIF-4A3 based on data obtained by northern blotting, nuclease S1 protection [9] and on the number of clones isolated from the cDNA library. The expression pattern of the four NeIF-4A2-related groups differed from that observed for either NeIF-4A2 or NeIF-4A3. Variability in the organ-specific expression of these four groups is also observed. For example, the NeIF-4A11 and NeIF-4A15 groups are expressed at relatively high levels in roots and fruit, while the NeIF-4A5 and NeIF-4A9 groups have relatively low expression in roots and fruit. In addition, NeIF-4A5 appears to be expressed at a relatively low level in all organs. Thus, both quantitative and qualitative differences in expression between NeIF-4A groups are observed.

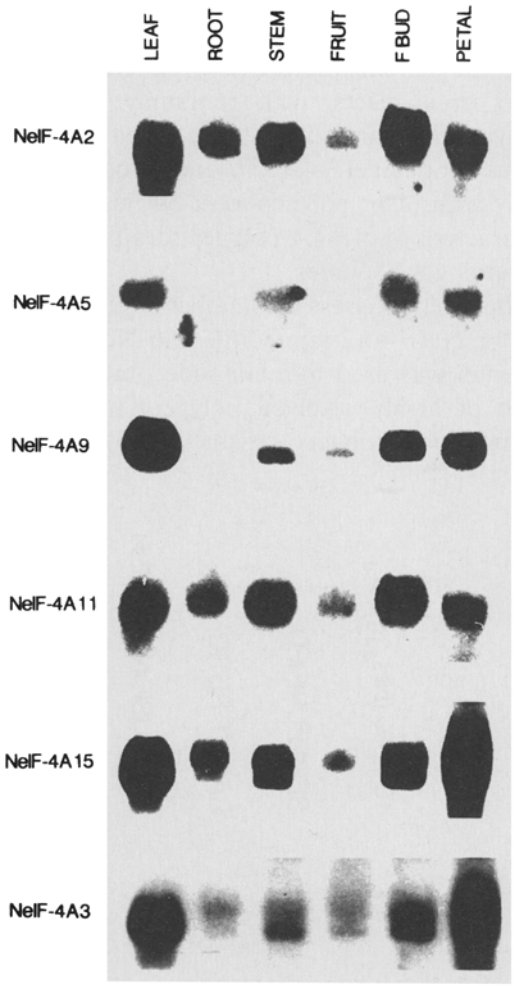

Fig. 3. Northern blot analysis of NeIF-4A family members with gene specific probes. Total RNA was isolated from the indicated tobacco organs and 20 μg was separated on a 1.3% glyoxal gel. The resulting northern blot was probed with gene-specific probes for each of the indicated NeIF-4A gene families. The NeIF-4A2 panel was probed with the 3′ end of the NeIF-4A1 cDNA, which cross-reacts with all members of the NeIF-4A2 group.

Western analysis of NeIF4A polypeptides

In order to characterize NeIF-4A gene expression at the protein level polyclonal antiserum, raised against NeIF-4A1 over-expressed in *Escherichia coli*, was generated in rats. This antiserum was tested against, and shown to cross-react with the original antigen used to produce the antiserum and well characterized eIF-4A proteins isolated from other sources including the 46–

50 kDa eIF-4A proteins purified from wheat germ and rabbit reticulocytes (Fig. 4). The antiserum also cross-reacts with a family of 46 kDa polypeptides present in tobacco leaves (Fig. 4). Thus our plant eIF-4A antiserum cross-reacts not only with plant polypeptides, but also with well characterized eIF-4A polypeptides isolated from heterologous sources.

In order to assess the number of gene products in the NeIF-4A2 family, the anti-NeIF-4A1 antiserum was used to immunodecorate a western blot of highly resolved polypeptides extracted from various tobacco organs (Fig. 5). All organs contain multiple polypeptides of ca. 46 kDa which cross-react with the NeIF-4A1 antiserum. Quantitatively, the number of discernible polypeptides varied, ranging from 3 in root to 7 in leaf. The qualitative distribution of NeIF-4A isoforms also varied between organs with the highest expression in root, stem, and apex and the lowest in flower organs and green tissue in general. Thus, a characteristic family of eIF-4A polypeptides exists in each tobacco organ.

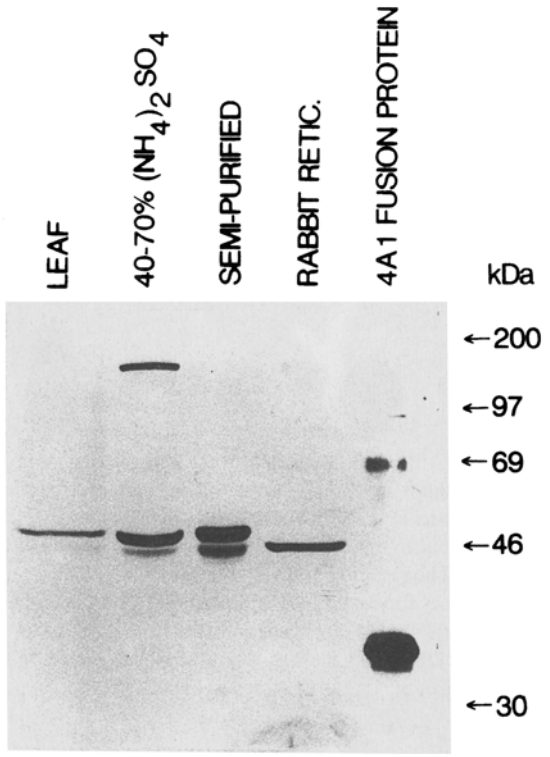

Fig. 4. Characterization of the anti-NeIF-4A1 antibody. Protein samples were separated on a 10% SDS-polyacrylamide gel and subjected to western analysis using the polyclonal rat anti-NeIF-4A1 antibody. From left to right: total wheat leaf lysate (30 μg); 40–70%, ammonium sulphate cut of wheat germ lysate (2.5 μg); semi-purified indicates wheat eIF-4A purified to the Cybacron 3GA stage (1.0 μg); 40–70% ammonium sulphate cut of a rabbit reticulocyte lysate (10 μg); and NeIF-4A1 fusion polypeptide purified from E. coli *inclusion bodies (1.0 μg). Molecular weight markers are indicated on the right.*

Subcellular location of NeIF-4A polypeptides

DEAD box proteins have been found not only in the cytoplasm but also in nuclei [4] and mitochondria [13]. In order to determine if distinct eIF-4A polypeptides are localized in different subcellular compartments, a western blot of soluble proteins extracted from purified chloroplasts was immunodecorated with anti-NeIF-4A antiserum (Fig. 6A). The antiserum detects a single 46 kDa polypeptide in chloroplast extracts. In order to ensure that this NeIF-4A immunoreactive polypeptide did not result from cytoplasmic contamination, purified chloroplasts were also treated with the protease thermolysin prior to electrophoresis and western blotting. Western analysis of chloroplasts which were lysed before protease treatment were also included as a control. The immunologically reactive polypeptides of 46 kDa observed in intact chloroplasts are thermolysin-resistant in contrast to those extracted from chloroplasts lysed before protease treatment which are not resistant. These results indicate that at least one eIF-4A-related polypeptide is subcellularly compartmentalized in the chloroplast.

Polysomes were purified from isolated chloroplasts in order to further clarify the possible role an NeIF-4A-related protein may perform in chloroplasts. Western analysis indicated that a 46 kDa polypeptide present in crude chloroplast polysome preparations cross-reacts with the NeIF-4A antiserum (Fig. 6B). Further purification of the chloroplast polysomes on sucrose gradients and subsequent western blotting indicated

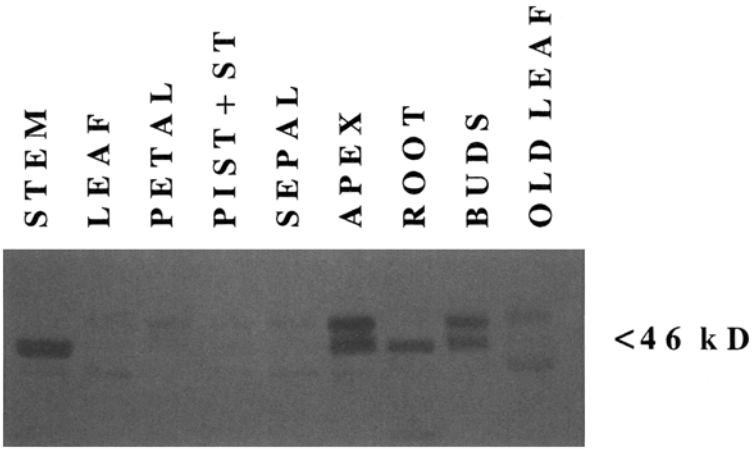

Fig. 5. Western analysis of NeIF-4A polypeptide profiles in various plant organs. Total soluble proteins were extracted from the indicated tobacco organs and separated on a 7–15% linear gradient polyacrylamide gel, 50 μg protein was loaded per lane. The resulting western blot was probed with the anti-NeIF-4A1 antibody.

that the NeIF-4A immunoreactive polypeptide was present in fractions 7–11 and 12–16 (Fig. 6B). These fractions are expected to contain polyribosomes [17]. These results clearly indicate that an NeIF-4A-related polypeptide is localized to the chloroplast and that it is ribosome-associated.

Discussion

Tobacco contains multiple gene copies coding for eIF-4A which comprise two distinct families [9]. In order to study the possible function of these genes it was necessary to determine the number of subfamily genes and their expression pattern. In this paper we address these questions by characterizing eIF-4A clones isolated from a tobacco leaf cDNA library. It is clear from our data that the NeIF-4A family, expressed in leaves, is composed of at least ten distinct genes which we have called groups. These groups fall into two distinct families, which we call NeIF-4A2 and NeIF-4A3 based on the genes we have previously described [9]. The nine new groups described in this paper clearly belong to the NeIF-4A2 family, based on the significant DNA sequence homology both in the coding and non-coding regions.

The eIF-4A clones characterized in this paper were isolated from a leaf cDNA library and consequently NeIF-4A genes not expressed in leaves would have escaped detection in our screen. Therefore, we believe that the set of genes described in this paper represent a specific leaf subset of the total eIF-4A gene family expressed in tobacco. This is reflected in our northern data in which various family members exhibit differences in the relative abundance in various plant organs as well as in our western data in which variations in the NeIF-4A polypeptide profile can also be observed. The level of expression of NeIF-4A2 family members is much higher than that for NeIF-4A3 members, as indicated by the number of clones obtained in our library screens and also by northern and nuclease S1 protection analysis ([9]; Fig. 3).

The presence of numerous distinct genes presents the intriguing question of differential function for closely related polypeptides. The lengths of the deduced amino acid sequence of all full length NeIF-4A2 family members characterized in this paper are identical. However, western analysis indicates the presence of a family of immunologically related 46 kDa polypeptides. This suggests either that minor sequence variants affect the mobility (see, for example, [3]) or that post-translational modification of some eIF-4A gene family members is involved in the produc-

Fig. 6A. Chloroplast localization of a NeIF-4A-related polypeptide. Intact chloroplasts were prepared by resuspending washed pea chloroplasts in thermolysin incubation buffer containing 0.35 M sucrose. Lysed chloroplasts were prepared by osmotic lysis of washed pea chloroplasts by resuspension in thermolysin incubation buffer without sucrose, incubation on ice for 5 min and centrifugation at 30 000 rpm for 20 min at 4 °C. The resulting pellet did not contain anti-NeIF-4A1 reactive material (lane P), therefore only the stromal supernatant was treated with thermolysin. Equal aliquots of both intact chloroplasts and lysed stromal supernatant (30 μg protein) were then incubated with thermolysin at the indicated concentrations for 30 min and analyzed by western blotting using the anti-NeIF-4A1 antibody.

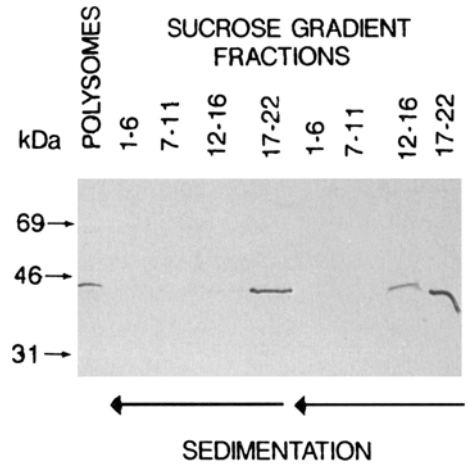

Fig. 6B. Association of the chloroplast NeIF-4A-related polypeptide with polysomes. Western analysis using the anti-NeIF-4A1 antibody was performed on crude and sucrose gradient-purified pea chloroplast polysomes. Proteins present in crude polysome preparations were loaded in the lane labelled 'polysomes'. Crude polysome preparations were further purified on two sucrose gradients and proteins present in fractions 1–6, 7–11, 12–16 and 17–22 were also subjected to western analysis. Three times as much crude polysome fraction was loaded on the sucrose gradient represented by the right-hand set of fractions.

tion of these polypeptides. Currently, the nature and possible functional significance of such modification(s) is unknown. However, there has been a single report of eIF-4A phosphorylation during anoxia in maize root tips [16].

Cell fractionation studies suggest a major reason for the tobacco requirement of multiple NeIF-4A-related proteins- localization to different cellular compartments. These studies clearly indicate that at least one NeIF-4A-related polypeptide is localized to the chloroplast. We do not know if one of the NeIF-4A family members described in this paper represents the gene encoding the chloroplast form. We tested three anti-eIF-4A antibody preparations for reactivity with chloroplast proteins. Two of them, the rat anti-NeIF-4A1 antibody described here and an antibody generated against yeast eIF-4A react with the same chloroplast polypeptide. However an antibody raised against the NeIF-4A1 polypeptide in rabbits did not react with organellar polypeptides (data not shown). Further work is required to establish the exact identity of the chloroplast polypeptide which cross-reacts with the anti-tobacco NeIF-4A antibody. Whatever its exact nature, this polypeptide is also associated with chloroplast ribosomes and therefore is presumably involved in the translation of at least some chloroplastically encoded transcripts. The chloroplastic eIF-4A polypeptide presumably functions as an RNA helicase, removing secondary structure from the 5′ UTR of chloroplastic transcripts allowing translation to occur. The translation of numerous chloroplast encoded transcripts is known to be tightly controlled, especially via light regulation. Genetic evidence from *Chlamydomonas* indicates that stable stem-loop structures in the 5′ UTR of these transcripts is involved in the mechanism controlling light-inducible expression [11]. Recently, a chloroplastic 46 kDa RNA binding protein has been shown to interact specifically with the psbA mRNA and involved in the light regulation of its translation [2]. It is possible that the RNA binding activity of this protein is 'light-activated', allowing it to

interact with the specific stem-loop structure present in the *psb*A 5′ UTR and with the chloroplastic eIF-4A. The eIF-4A-RNA-binding protein complex then generates the RNA helicase activity required to remove the secondary structure thereby allowing light-specific translation. This complex is therefore analogous to the eukaryotic eIF-4A/eIF-4B RNA helicase complex involved in translation initiation, in which eIF-4B provides the RNA-binding capability.

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