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Differential expression of genes encoding the hypusine-containing translation initiation factor, eIF-5A, in tobacco

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ABSTRACT

Two *Nicotiana plumbaginifolia* cDNA clones, NeIF-5A1 and NeIF-5A2, encoding eukaryotic translation initiation factor eIF-5A (formerly called eIF-4D) were cloned by heterologous screening with *Dictyostelium* and human eIF-5A probes. eIF-5A is the only protein known to contain a unique amino acid modification, hypusine. Comparison of the *Nicotiana* deduced amino acid sequences with those of other eIF-5A polypeptides reveals conservation throughout the coding sequence, especially in the region of the hypusine residue. Transcript analysis reveals that NeIF-5A1 is preferentially expressed in photosynthetic tissues, while NeIF-5A2 is constitutively expressed in all plant tissues examined. A polyclonal antibody was raised against NeIF-5A1 overexpressed in *E. coli*. NeIF-5A1 antiserum crossreacts with an 18 kDa polypeptide doublet in all tobacco tissues examined. At least one polypeptide of ca. 18 kDa from a diversity of higher and lower plants crossreacts with NeIF-5A1 antiserum.

INTRODUCTION

Eukaryotic translation initiation factor 5A (eIF-5A), formerly called eIF-4D, is a protein factor involved in the initiation of eukaryotic cellular protein synthesis. *In vitro*, eIF-5A stimulates the formation of the dipeptide analogue methionyl-puromycin, however, the precise role of eIF-5A *in vivo* is unknown (1). It has been suggested recently that eIF-5A stabilizes the charged initiator methionyl-tRNA at the peptidyltransferase centre of the 80 S initiation complex (2).

eIF-5A has been identified in all eukaryotes examined and is conserved as a 16–18 kDa protein with an acidic pI of 5–6 (3). It is characterized by the presence of hypusine, a unique modified amino acid. Hypusine is formed post-translationally via the transfer and hydroxylation of the butylamino group from the polyamine spermidine to the side chain amino group of a specific lysine residue (4). eIF-5A is the only hypusine-containing protein identified to date, as shown by *in vivo* spermidine labelling studies (5). The hypusine modification has recently been shown to be essential for eIF-5A activity *in vitro* (6,7) in the methionyl-puromycin assay. Furthermore, hypusinated eIF-5A has been shown to be necessary for cell viability in yeast (8).

Single cDNA clones encoding eIF-5A from both human liver (9) and the slime mould *Dictyostelium* (10), as well as, two genomic clones from yeast (8,11) have been characterized. Recently, an alfalfa cDNA clone was fortuitously isolated and sequenced (12). Interestingly, in yeast the two highly similar genes are differentially expressed in response to environmental anaerobic stress (8,11).

We are interested in the role(s) which plant eIF-5A may play as a link between protein synthesis initiation and developmental phenomena and stress responses in plants. Plant polyamines have been implicated in a wide variety of physiological effects including floral induction, embryogenesis, pathogen resistance, and cell growth, division, and differentiation (13–14). It is possible that eIF-5A is the intermediary through which polyamines could exert these effects. Although a single polypeptide which labels with spermidine has been identified in a number of plant systems (15–17), information on the identity or characterization of these proteins is lacking.

In this paper, we report the cloning and characterization of three genes encoding eIF-5A from *Nicotiana*. We show that while the genes are quite similar, they display differential patterns of expression in various tissues. We have generated a polyclonal antibody against plant eIF-5A overexpressed in *E. coli* and show that the eIF-5A polypeptide is conserved throughout the plant kingdom. Finally, sequence comparison reveals a strong conservation of amino acid sequence among eukaryotes, particularly in the vicinity of the hypusine domain, adding further evidence of the importance of this unique amino acid residue.

MATERIALS AND METHODS

Plant Material

Nicotiana tabacum cv Samsun NN was grown in a greenhouse. Vegetative tissues were harvested from 6 week old plants, containing 5–7 leaves. Green flower buds of 1–2 cm were collected intact, while expanded flowers were dissected, before complete dehiscence of anthers, into sepal, petal, ovary, stamen (anther plus filament) and stigma plus style fractions. Fruits were

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collected from fertilized flowers while still green. All plant material was frozen immediately in liquid nitrogen and stored at −80°C.

Isolation of DNA clones

The *Dictyostelium* cDNA for eIF-5A (10) was used as a heterologous probe to screen a lambda gt11 cDNA library produced from *Nicotiana plumbaginifolia* leaf polyA$^+$ RNA. Low stringency screening using ^{32}P-dCTP labelled probes (18,19) resulted in the isolation of clones of two different size classes which also cross-hybridized with the human eIF-5A cDNA (9). One of the *Nicotiana* cDNA clones was used to isolate its genomic homologue by high stringency screening of a *Nicotiana tabacum* cv Samsun NN genomic library produced in lambda EMBL3.

DNA Sequence Determination and Analysis

After restriction mapping of the two cDNA and one genomic DNA clones, various subclones were sequenced from double stranded templates using dideoxy sequencing with T7 Polymerase (Pharmacia). Analyses and comparisons of the determined sequences were performed as described (19).

Northern Blot Analysis

Total RNA was extracted from plant material in hot phenol after grinding in liquid nitrogen (20). Total RNA was quantified spectrophotometrically, visually on formaldehyde gels after staining with ethidium bromide and on Northern blots after staining with Methylene Blue (21). Equal aliquots of 10 μg were glyoxylated, electrophoresed on 1.2% agarose gels and transferred to Nytran (Schleicher & Schuell) (18). Filters were prehybridized at 65°C in 50% formamide, 5×Denhardt's, 0.2% SDS, 5×SSPE, 100 μg/ml yeast tRNA and hybridized at 65°C in the same solution containing ^{32}P-UTP labelled RNA probes. Gene specific antisense RNA probes derived from the 3′ untranslated regions (bp 441 to 702 for NeIF-5A1 and bp 684 to 1224 for NeIF-5A2) were generated from 1 μg of appropriately digested template cDNA cloned in pBluescript KS+ using a Stratagene kit. Blots were washed finally in 0.1×SSPE, 0.5% SDS at 65°C. Transcript size was determined by comparison with a BRL 1kb DNA ladder run on each gel.

Overexpression of NeIF-5A1 in *E. coli*

The NeIF-5A1 partial cDNA was cloned into the *Bam*H I site of pGEX-2T (Pharmacia) creating an in-frame fusion and the polypeptide was overexpressed in *E. coli* BB4 cells (22). The fusion polypeptide was cleaved with thrombin and NeIF-5A1 purified from a Glutathione-Sepharose 4B column (Pharmacia) (23). The cleaved 16 kDa partial NeIF-5A1 polypeptide was further purified on a polyacrylamide gel, electroeluted and injected into rats to generate polyclonal antiserum against tobacco NeIF-5A1.

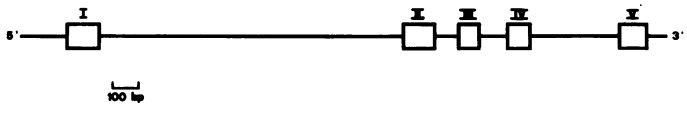

Figure 1. NeIF-5A3 Genomic Organization. DNA sequence analysis was used to determine the exon/intron boundaries within a 2.4 kb fragment of the original lambda clone. Exons (boxes) are numbered I−V and their respective sizes in amino acids are: 41, 41, 19, 25 and 33. A scale for 100 bp is indicated.

Western Analysis

Total soluble proteins were isolated from plant material by grinding in liquid nitrogen and extracting with 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 0.1 M NaCl, 2% polyvinyl-polypyrrolidone, 50 mM 2-mercaptoethanol. Proteins were quantified with the Bradford Assay (Biorad) using BSA as the standard and 30 μg total protein were electrophoresed on 15% SDS polyacrylamide gels and electroblotted to nitrocellulose (Schleicher & Schull). Western analysis was performed as described (24) with 1:1000 dilutions of antiserum and horseradish peroxidase conjugates. Polypeptide size was determined by comparison with Amersham Rainbow molecular weight markers run on each gel.

RESULTS

Isolation of Clones

Two *N. plumbaginifolia* leaf cDNA clones, NeIF-5A1 and NeIF-5A2, which cross hybridized with both the *Dictyostelium* and human eIF-5A cDNA probes were selected for analysis. NeIF-5A1 was used to screen an *N. tabacum* genomic library and of the strongly hybridizing clones, NeIF-5A3, was chosen for further study.

Sequence Analysis

The DNA and deduced amino acid sequences for NeIF-5A1, NeIF-5A2 and NeIF-5A3 are available from the EMBL data bank. NeIF-5A1 is a partial cDNA of 702 bp containing an open reading frame (ORF) of 145 amino acids and 262 bp of 3′ untranslated region. A putative poly-adenylation signal, ATTAAA (25), is located 25 bp upstream of the terminal polyA

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          1                                                  50
NeIF-5A2  .......... MSDEEHQFES K...ADAGAS KTYPQQAGTI RKNGHIVIKG
NeIF-5A3  .......... MSDEEHHFES K...ADAGAS KTYPQQAGTI RKNGHIVIKN
 ALFALFA  .......... MSDEEHQFES K...ADAGAS KTYPQQAGTI RKNGYIVIKN
  TIF51A  .......... MSDEEHTFET ....ADAGSS ATYPMQCSAL RKNGFVVIKS
  TIF51B  .......... MSDEEHTFEN ....ADAGAS ATYPMQCSAL RKNGFVVIKG
   HUMAN  .......... MADDLDFETG .....DAGAS ATFPMQCSAL RKNGFVVLKG
  DICTYO  MKPLIMEYNK MSDNEALDVE DYAQAGSGAS LTFPIQCSAL RKNGFVVIKG

          51                                                 100
NeIF-5A2  RPCKVVEVST SKTGKHGHAK CHFVAIDIFT GKKLEDI.VPS SHNCDVPHV
NeIF-5A3  RPCKVVEVST SKTGKHGHAK CHFVAIDIFT GKKLEDI.VPS SHNCDVPHV
 ALFALFA  RPCKVVEVST SKTGKHGHAK CHFVAIDIFT SKKLEEVYVPS SHNCDVPHV
  TIF51A  RPCKIVDMST SKTGKHGHAK VHLVAIDIFT GKKLEDL.SPS THNMEVPVV
  TIF51B  RPCKIVDMST SKTGKHGHAK VHLVTLDIFT GKKLEDL.SPS THNLEVPFV
   HUMAN  RPCKIVEMST SKTGKHGHAK VHLVGIDIFT GKKYEDI.CPS THNMDVPNI
  DICTYO  FPCKIVDMST SKTGKHGHAK VNITAIDIFT GKKVEEI.CPS THNIDVPNV

          101                                                150
NeIF-5A2  NRTDYQLIDI SEDGFVSLLT ENGNTKDDLR LPTDDNLLTQI KDGFAEGKDL
NEIF-5A3  NRTDYQLIDI SEDGFVSLLT ENGNTKDDLR LPTDDNLLALI KDGFAEGKDL
 ALFALFA  NRTDYQLIDI SEDGFVSLLT ENGNTKDDLK LPTDDSLLTQI KDGFAEGKDL
  TIF51A  KRNEYQLLDI .DDGFLSLMN MDGDTKDDVK APEGE.LGDSL QTAFDEGKDL
  TIF51B  KRSEYQLLDI .DDGYLSLMT MDGETKDDVK APEGE.LGDSM QAAFDEGKDL
   HUMAN  KRNDFQLIGI .QDGYLSLLQ DSGEVREDLR LPEGD.LGKEI EQKYDCGEEI
  DICTYO  SRKEYTVMDV .QDGYLSLLD AGGEVKEDLA LPEDD.IGKEI TQMLKEGKEP

          151                174
NeIF-5A2  VVSVMSAMGE EQICALKDIG PK*.
NeIF-5A3  VLSVMSAMGE EQICGIKDVG PK*.
 ALFALFA  VVSVMSAMGE EQICALKDIG GKN*
  TIF51A  MVTIISAMGE EAAISFKEAA RTD*
  TIF51B  MVTIISAMGE EAAISFKEAP RSD*
   HUMAN  LITVLSAMTE EAAVAIKAMA K*..
  DICTYO  LVSVISALGK EGVVSVKVSN N*..
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Figure 2. Comparison of eIF-5A Deduced Amino Acid Sequences. Computer analysis of the coding regions of the five reported eIF-5A genes from alfalfa (12), yeast (TIF51A and TIF51B) (8), human (9) and *Dictyostelium* (Dictyo) (10) was used to obtain an optimum sequence alignment with the two full length *Nicotiana* genes, NeIF-5A2 and NeIF-5A3. Amino acids which are identical for all sequences are underscored twice. The lysine residue in bold represents the putative hypusine residue.

tail. NeIF-5A2 is a cDNA of 1224 bp containing a complete ORF of 159 amino acids, 38 bp of 5′ and 709 bp of 3′ untranslated region. The deduced amino acid sequence for NeIF-5A2 encodes a polypeptide of 17,344 Da with a pI of 5.99. NeIF-5A3 is encoded on an 8 kb *N. tabacum* genomic fragment of which 2.4 kb was sequenced. The coding region comprises 5 exons and 4 introns (Figure 1). The exon splice sites are highly similar to those observed in other dicotyledenous plant sequences (26). The deduced amino acid sequence of the spliced exons encodes a 17,307 Da polypeptide with a pI of 6.14. There is a putative glycosylation site in all three polypeptides at residue 73 (NeIF-5A1) and 87 (NeIF-5A2 and NeIF-5A3).

Comparison of the three *Nicotiana* eIF-5A ORFs reveals high sequence similarity. At the DNA level, comparisons of NeIF-5A1 with NeIF-5A2, NeIF-5A1 with NeIF-5A3, and NeIF-5A2 with NeIF-5A3 indicates 83, 98, and 84% identity, respectively. NeIF-5A1 and NeIF-5A3 are identical at the amino acid level, while NeIF-5A2 and NeIF-5A3 share 95% identity. Figure 2 reveals the high amino acid similarity among all the eIF-5A genes cloned, especially in the region of the hypusine residue (lysine in bold lettering). NeIF-5A2 and NeIF-5A3 are most closely related to the alfalfa sequence (95% identity) and are least similar to *Dictyostelium* with which they share 50% identity.

Northern Analysis

Northern blots of total RNA from various vegetative and floral tissues of *N. tabacum* were probed with gene-specific RNA probes under high stringency. NeIF-5A1 hybridizes to a 1 kb transcript present in all tissues (Figure 3), however, the level of this transcript varies significantly between organs, being highest in leaf and sepal tissue, and lowest in root tissue. Additional, less intense hybridizing bands are also observed in leaf and sepal tissue. Conversely, NeIF-5A2 hybridizes to a 1.6 kb transcript whose expression pattern shows only relatively minor variations between organs (Figure 3). When either of the complete cDNA clones was used as probe (data not shown), a 1.6 kb and a 1 kb transcript were observed, however, the pattern of expression in both bands reflected that expected for the probe used (NeIF-5A1 highest in leaf and NeIF-5A2 constitutive).

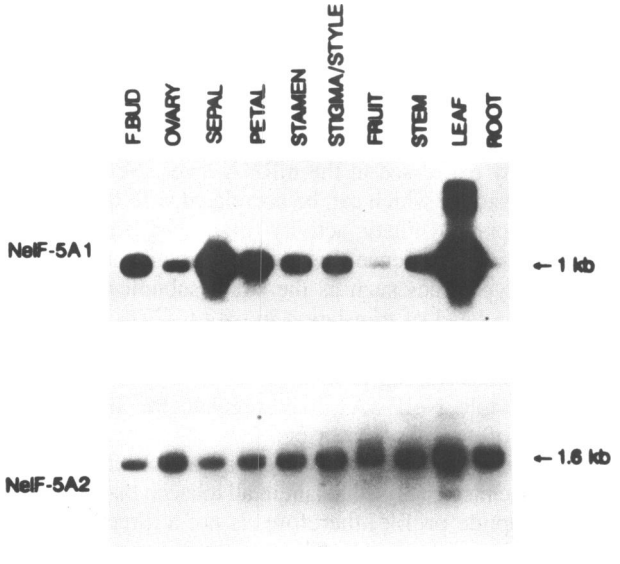

Figure 3. Northern Blot Analysis of Tobacco RNA. Replica Northern blots containing 10 μg total RNA per lane isolated from various tobacco tissues were probed with a 3′ gene-specific RNA probe for either NeIF-5A1 (top panel) or NeIF-5A2 (bottom panel). The autoradiograms were exposed for 3 days. Estimated transcript sizes are indicated on the right. F.BUD represents floral bud RNA.

Overexpression of NeIF-5A1

In order to generate a specific antibody against plant eIF-5A, the NeIF-5A1 partial cDNA was cloned into the *E. coli* expression vector, pGEX-2T, facilitating the translation of a 42 kDa glutathione-S-transferase(GST)-NeIF-5A1 fusion peptide (Figure 4A). Figure 4B shows the IPTG-induced expression of the GST-NeIF-5A1 fusion peptide and the absence of this polypeptide when either GST alone or NeIF-5A1 cloned in the reverse orientation are expressed in this system. Cells expressing the fusion peptide were lysed, centrifuged and passed over a Glutathione-Sepharose column. The absence of the GST-fusion

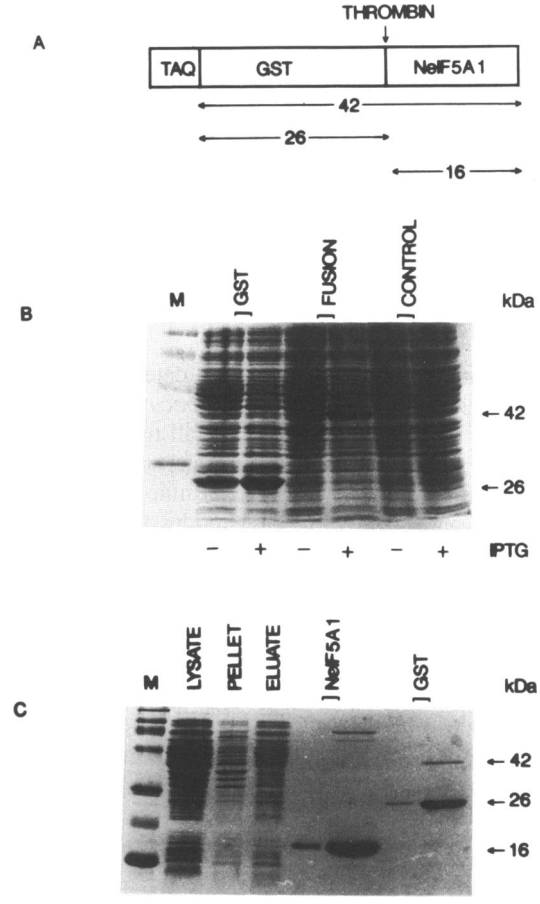

Figure 4. Purification of NeIF-5A1 Overexpressed in *E. coli*. **A.** Schematic gene construct used for GST-NeIF-5A1 overexpression. Arrows delineate the expected size (kDa) of polypeptides translated from the construction. **B.** SDS-PAGE polypeptide profiles of *E. coli* transformed with a GST construct alone (GST), the GST-NeIF-5A1 fusion construct (FUSION) or a control GST construct fused with NeIF-5A1 in the opposite orientation (CONTROL), in the absence (−) or presence (+) of IPTG. **C.** SDS-PAGE analysis of IPTG-induced *E. coli* containing the GST-NeIF-5A1 fusion construct. Cells were lysed (LYSATE) and pelleted (PELLET) and the supernatant passed over a Glutathione-Sepharose column and collected (ELUATE). After thrombin cleavage, the 16 kDa NeIF-5A1 polypeptide was eluted with buffer (NeIF-5A1; 1 μl and 10 μl) while the GST and residual fusion peptide required elution with free glutatione (GST; 1 μl and 10 μl).

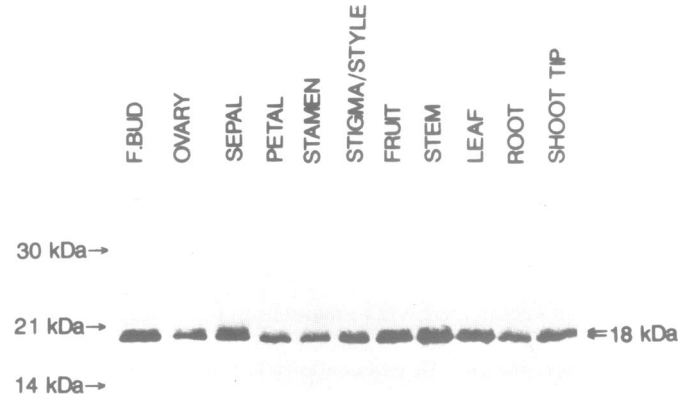

Figure 5. Western Blot Analysis of Tobacco Protein Extracts. A Western blot containing 30 μg soluble protein per lane from various tobacco tissues was immunodecorated with NeIF-5A1 antiserum. The approximate size of the immunoreactive doublet is indicated and molecular weight markers are indicated in kDa. F.BUD represents floral bud protein.

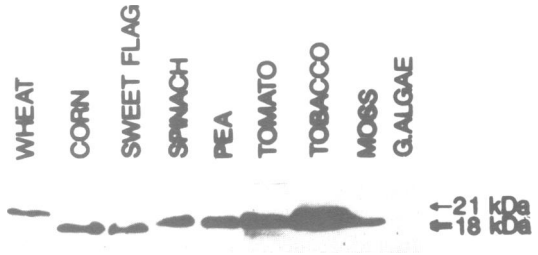

Figure 6. Garden Blot. A Western blot containing 30 μg per lane of soluble protein extracted from leaves of various plants and total moss and algal tissue was immunodecorated with NeIF-5A1 antiserum. The approximate sizes of the immunoreactive polypeptides are indicated. SWEET FLAG represents the monocotyledenous marsh plant *Acorus calamus*, moss represents *Physcomitrella patens* and G.ALGAE represents *Chlamydomonas reinhardtii*. There are weakly reactive polypeptides of 18 and 24 kDa in G.ALGAE which are not visible in the photograph.

peptide in the pre-column cell lysate pellet and the post-column eluate shows the polypeptide was soluble and bound to the column (Figure 4C). The fusion peptide was cleaved at the site of fusion by thrombin while still bound to the Glutathione-Sepharose column, resulting in the elution of a highly purified 16 kDa partial NeIF-5A1 polypeptide (Figure 4C). The NeIF-5A1 polypeptide was SDS gel purified before injection into rats to generate the NeIF-5A1 antiserum.

Western Analysis

NeIF-5A1 antiserum was used to detect immunoreactive polypeptides in extracts of various *N. tabacum* tissues (Figure 5). A doublet of 18 kDa was observed in all tissues with the same intensity and pattern (top band less intense than lower band). Western blots were also used to determine the cross-reactivity of NeIF-5A1 antiserum with polypeptides from the leaves of a variety of plants (Figure 6). At least one reactive polypeptide of 18−21 kDa was observed in all monocotyledenous and dicotyledenous plants tested. Furthermore, the moss *Physcomitrella patens* also showed a reaction at 18 kDa, while the green alga *Chlamydomonas reinhardtii* gave weak reactions at 18 and 24 kDa. When NeIF-5A1 antisera was tested against polypeptides from a diversity of organisms including yeast, *Dictyostelium*, *Drosophila* and human HeLa cells, a reaction with an 18−21 kDa polypeptide was only observed in yeast (data not shown).

DISCUSSION

We report here the characterization of plant homologues of the hypusine-containing protein, eIF-5A. We have isolated two different cDNA clones, NeIF-5A1 and NeIF-5A2, from the dicotyledenous plant *Nicotiana plumbaginifolia* which hybridize with the *Dictyostelium* and human eIF-5A genes. The similarity between the deduced amino acid sequence of the *Nicotiana* genes and the peptide sequences of the purified eIF-5A proteins from rabbit (9) and *Dictyostelium* (10) strongly suggests that NeIF-5A1 and NeIF-5A2 encode eIF-5A homologues.

There is considerable amino acid sequence similarity between the *Nicotiana* genes and all of the eIF-5A sequences reported to date, especially in the region of the hypusine residue where 12 surrounding amino acid residues are identical. In yeast a hypusine-containing eIF-5A is essential for growth (8). It would be interesting to determine if other mutations within this amino acid domain would also be lethal.

Conservation of the eIF-5A protein within the plant kingdom is shown by the cross reactivity of NeIF-5A1 antiserum with an 18−21 kDa polypeptide on Western blots of total protein extracts from various higher and lower plants. Interestingly, while the NeIF-5A1 polypeptide shares 50−60% identity with all non-plant eIF-5A deduced amino acid sequences, NeIF-5A1 antiserum did not react with polypeptides from most non-plant eukaryotic sources except yeast, which exhibits the highest similarity with the *Nicotiana* genes.

In yeast there are two highly similar eIF-5A genes whose expression is regulated by oxygen in a strictly complementary fashion. Furthermore, either yeast gene alone is sufficient for viability, suggesting that their products have similar roles (8). The presence of at least two expressed *Nicotiana* eIF-5A genes raises the question of whether their roles are specialized within the plant. Gene-specific probes indicate that although both genes are expressed in all tissues examined, each gene has a distinctive differential expression pattern. While NeIF-5A2 appears to be constitutively expressed at the mRNA level, NeIF-5A1 has an expression pattern which can be correlated with the presence or absence of photosynthetic activity (highest in leaves, lowest in roots). There is evidence that the light-regulated synthesis of specific polypeptides such as the small subunit of RUBISCO, occurs at the level of translation initiation (27). Assuming that mRNA expression is a reflection of protein levels, NeIF-5A2 may be a housekeeping protein involved in general translation initiation, while NeIF-5A1 may regulate the light-dependent translation of specific transcripts.

NeIF-5A1 antiserum crossreacts with an 18 kDa polypeptide doublet which is equally abundant in all tobacco tissues examined. The polypeptide profile, therefore, is not a direct reflection of the mRNA transcript levels. The constitutive expression of both members of the doublet makes it seem unlikely that the two polypeptides correspond to NeIF-5A1 and NeIF-5A2. The polypeptide doublet may be an indication that there are additional eIF-5A genes or post-translational modifications whose products contribute to the eIF-5A polypeptide pool. Other investigators have observed multiple forms of the hypusine-containing protein

using spermidine labelling of yeast (8). The constitutive protein profile can most simply be explained by a higher level of NeIF-5A2 (household) gene expression relative to NeIF-5A1, however, it is difficult to directly determine relative transcript and subsequent protein abundances.

NeIF-5A1 was used to isolate an *N. tabacum* genomic clone, NeIF-5A3. The NeIF-5A3 amino acid sequence is identical to NeIF-5A1, while the two genes are 98% similar at the DNA level. We do not know if these differences are merely the result of evolutionary changes based on the different genetic backgrounds of *N. plumbaginifolia* and *N. tabacum*, or if the two clones represent different genes. Interestingly, the coding sequence of NeIF-5A3 is composed of 5 exons with 4 introns. Using this information in conjunction with genomic Southern analysis (data not shown) we speculate that there is a small multi-gene family of eIF-5A genes in tobacco. The NeIF-5A3 clone contains 4 kb of upstream sequence which can be used in promoter studies to analyze the control of NeIF-5A1 expression with respect to tissue specificity and environmental factors. Information gained from expression studies at both the RNA and protein levels will provide further insight into the role(s) which eIF-5A performs in plants.

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