Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco

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ABSTRACT

Three cDNA clones coding for eukaryotic translation initiation factor 4A, eIF-4A, were isolated from a Nicotiana plumbaginifolia root cDNA library by heterologous screening. The clones comprise two distinct gene classes as two clones are highly similar while the third is divergent. The genes belong to a highly conserved gene family, the DEAD box supergene family, although the divergent clone contains a DESD box rather than the characteristic DEAD box. The two clones are representatives of separate small multigene families in both N. plumbaginifolia and N. tabacum. Representatives of each family are coordinately expressed in all plant organs examined. The 47 kD polypeptide product of one clone, overexpressed in E. *coli*, crossreacts immunologically with a rabbit reticulocyte eIF-4A polyclonal antibody. Taken together the data suggest that the two Nicotiana eIF-4A genes encode translation initiation factors. The sequence divergence and the coordinate expression of the two Nicotiana eIF-4A families provide an excellent system to determine if functionally distinct eIF-4A polypeptides are required for translation initiation in plants.

INTRODUCTION

Eukaryotic translation initiation is a complex process requiring a large number of dedicated proteins. Eukaryotic initiation factor 4A (eIF-4A) performs an essential role in this process. It is thought that eIF-4A, in conjunction with eIF-4B, acts as an ATPdependent RNA-helicase, which removes secondary structure from the 5' untranslated region of the mRNA, thereby allowing 40S ribosome subunit binding and subsequent Kozak scanning to the first AUG codon (1-3).

Two highly conserved forms of eIF-4A, eIF-4AI and eIF-4AII, are present in mammalian systems (4,5). In rabbit reticulocytes, eIF-4AII appears to be preferentially associated with the eIF-4F cap-binding complex, while eIF-4AI is observed in both the free and complexed state. The two forms act synergistically, since both are required for maximum translation *in vitro* (5). The mouse eIF-4A genes are also differentially expressed with eIF-4AII to eIF-4AI transcript ratios varying from 0.03 in thymus to 1.4 in kidney (4). Yeast also possesses two eIF-4A genes, TIF1 and TIF2, which are identical on the amino acid level and are coordinately expressed (6). The two genes can functionally complement one another, as inactivation of one gene has no effect

on cell viability while inactivation of both genes results in the abolition of protein synthesis and is lethal (6,7). Therefore, so far, only two, interchangeable eIF-4A proteins appear to be involved in translation initiation in yeast, while two functionally divergent eIF-4A forms may exist in mammalian systems.

The mouse eIF-4A and yeast TIF genes are the prototypes of a rapidly growing supergene family, the DEAD box family, whose members exhibit extensive amino acid similarity (8). DEAD box family members have been identified in organisms ranging from *E. coli* to humans and are involved in RNA splicing, ribosome assembly, and cell differentiation and growth, as well as translation initiation (9). The majority of DEAD box family members, therefore, have a function unrelated to translation initiation. This is most clearly seen in yeast, in which at least 14 DEAD box family members have been identified, although only the two TIF genes are associated with translation initiation. Presumably all eukaryotes contain a number of DEAD box family members which perform a diverse range of functions.

The situation is less clear in plants where eIF-4A has exclusively been studied on the biochemical level and then only in wheat germ. Wheat germ eIF-4A purifies as a single polypeptide of approximately 50 kD which is not associated with the eIF-4F cap-binding complex (10). Wheat germ eIF-4A exhibits RNA-dependent ATPase activity and also functions as an RNA helicase however it is unable to substitute for mammalian eIF-4A in either a mammalian *in vitro* translation system or RNA helicase assay, while the mammalian protein efficiently substitutes for wheat germ eIF-4A in both assays (10-12). The functional differences observed between the wheat germ and mammalian systems may arise from their different metabolic states, wheat germ being a desiccated, metabolically dormant tissue.

We report here the first molecular analysis of plant eIF-4A, as an initial step towards elucidating the number of eIF-4A genes and the role(s) which they may perform during plant development.

EXPERIMENTAL PROCEDURES

Plant Material

Nicotiana plumbaginifolia and N. tabacum cv xanthi were grown from seed in either a growth chamber maintained at $27 + /-1^{\circ}$ C and 60% relative humidity with a 16:8 light:dark cycle or in a green house. Roots, stems and leaves were harvested from young plants containing 5–7 leaves. Flower buds were harvested when 1.0-2.0 cm in length, and fully expanded flowers were dissected into sepal, petal and reproductive organs (stamen and pistil) fractions. The shoot apex fraction included emerging leaves not longer than 0.5 cm. Harvested material was frozen immediately in liquid nitrogen and stored at -80° C.

cDNA Library Screening

Root and leaf lambda gt11 cDNA libraries produced from *N. plumbaginifolia* polyA⁺ RNA (kindly provided by G. Coruzzi) were screened at low stringency using an internal *Hind*III/*Hae*II fragment corresponding to bp 871 - 1708 of the yeast TIF1 gene (13). A total of 3×10^5 plaques from each library were screened by standard protocols (14) with TIF1 probe labelled with ³²P-dC-TP using a random primed labelling kit (Boehringer Mannheim). DNA inserts from twelve positive clones were then probed with the complete mouse eIF-4A cDNA (4).

DNA Sequence Determination and Analysis

Various restriction fragments were subcloned and sequenced using dideoxy sequencing. Amino acid sequence comparisons were performed using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package, version 6.2 (copyright 1990 by J.R. Devereux). Seven selected DEAD box DNA sequences were obtained from the EMBL DNA sequence data base (release 26 of March 1991) and their deduced amino acid sequences compared pairwise to NeIF-4A2 using the UWGCG program GAP. Multiple sequence alignment of the 'gapped' amino acid sequences was then performed manually using LINEUP, the results of which were printed using PRETTY.

Southern Blot Analysis

High molecular weight total genomic DNA was isolated from newly expanded leaves of *N. plumbaginifolia* and *N. tabacum* cv xanthi on caesium chloride gradients (15). DNA aliquots (5 μ g) were digested with the stated restriction endonucleases, electrophoresed in 0.8% agarose gels and transferred to nitrocellulose BA 85 filters (Schleicher & Schuell) by capillary blotting. The baked filters were prehybridized in 6×SSPE, 5×Denhardt's, 0.25% SDS for 4 h at 65°C and then hybridized in fresh solution containing 50 μ g/ml tRNA and ³²P-labelled DNA probes for 16 h at 65°C. Filters were washed in 2×SSPE, 0.1% SDS at room temperature and then in 0.2×SSPE, 0.1% SDS at 50°C. NeIF-4A2 and NeIF-4A3 do not cross hybridize under these conditions (data not shown).

Northern Blot Analysis

Total RNA was extracted from various *Nicotiana* organs by grinding in guanidinium thiocyanate and purification by centrifugation through a caesium chloride cushion (14). Equal aliquots of total RNA (5 μ g), as determined spectrophotometrically and by visualization in a formaldehyde gel, were denatured in glyoxal, electrophoresed in a 0.8% agarose gel and transferred to Nytran (Schleicher & Schuell) nylon membranes by capillary action. Blots were prehybridized, hybridized, and washed as described above for Southern blot analysis.

Quantitative S1 Nuclease Protection Analysis

Single stranded DNA probes specific for NeIF-4A2 and NeIF-4A3 were produced using oligonucleotide primers complimentary to sequence at the 5'ends of each mRNA: (3'-C-CGTGGTCTTCCAAGAGTTAAACTAC-5') corresponding to bp 12–37 of NeIF-4A2 and (3'-GCTCTGTAGTTTCCCGCA-

CCTTGG-5') corresponding to bp 24-47 for NeIF-4A3 as described (14). Quantitative S1 Nulease mapping was then performed using 20 µg of total RNA and 50 U S1 Nuclease (Boehringer Mannheim) as described (14). A number of controls were performed. DNA sequence ladders, using the NeIF-4Aspecific oligonucleotides as primers, were run on each gel. This verified that both of the S1 nuclease protected fragments were identical in size to those expected, 153 and 61 nucleotides for NeIF-4A2 and NeIF-4A3, respectively. A gene specific probe for the 3' end of atp1, a nuclear gene coding for the beta-subunit of the mitochondrial ATPase from N. plumbaginifolia (16), was included in each reaction to act as an internal control for equivalent total RNA addition. Although equal amounts of total RNA were utilized in each reaction, equal atp1 transcript levels were not observed in all organs (see Figure 6). This gene has traditionally been utilized as a control for equal RNA loads during Northern analysis of plant RNA, however other investigators have also observed non-constitutive expression of this gene between various plant organs (17). The two specific ssDNA probes added to each reaction, either NeIF-4A2 or NeIF-4A3 and atp1, were also incubated with tRNA to control for non-specific band formation. Probe excess over mRNA transcript levels was verified by titration of each probe with various amounts of total RNA.

Western Blot Analysis

PCR mutagenesis was utilized to create an *NdeI* restriction site which overlapped the ATG start codon of NeIF-4A2. The complete NeIF-4A2 coding sequence, as an *NdeI/Bam*HI fragment, was then cloned into pET3c (18), creating p4A2OE, which contains a direct in-frame fusion between the T7 RNA polymerase expression signals and the NeIF-4A2 coding sequence. NeIF-4A2 was then overexpressed in BL21(DE3)plysS cells containing p4A2OE and induced with IPTG (18).



Figure 1. Nicotiana eIF-4A 3' Untranslated RNA Structure. Possible stem-loop structures present in the 3' untranslated region of NeIF-4A1 and NeIF-4A3 are depicted. The structures have ΔG° values of -41 and -24 kcal/mol, respectively.

Polypeptides were separated in a 15% SDS-PAGE gel and transferred to nitrocellulose. The filter was incubated in the presence of polyclonal antibodies directed against either rabbit reticulocyte eIF-4A (diluted 1:1000; kindly provided by A.Thomas) or the yeast TIF protein (diluted 1:1000; kindly provided by H. Trachsel). Protein-antibody complexes were detected using 4-chloro-1-naphthol and H_2O_2 .

RESULTS

A root cDNA library from *N. plumbaginifolia* was probed, at low stringency, using an internal fragment of the yeast TIF1 genomic clone. A mixture of differentially hybridizing plaques

NE17-442	MARZALERZÓ	F DAKOF DACH	TELLS. TEQE	EFFISTOEVY	DSFDAMGLQE	WLLRGIYAY.	GFEKPSAIDO	RGIVPFCK.G	LOVIQUAQSE	TERTATFCSE
RE11-443		.MEEDRLVFE	TSK.6V	EPI	ASFAEMGIKD	DLLRGYYQY.	GFEKPSAIQO	RAVLP115.G	ROVIADADSG	TEKTSHIALT
elt-4Al		MEPE6		WWE1V	DSFOOMMELSE	SLLRGIYAY.	GFEKPSAIQO	RAILPCIK.G	YDVIAQAQSG	TEXTATFAIS
@11-4A11	MSGGSADYNR	EHGGPE6	HDPDGVIE.S	WWWEIV	ONFOOMMLKE	SLLRGIYAY.	GFEKPSAIQO	RAIIPCIK.G	YDYIAQAQSG	TEKTATFAIS
TIF1/TIF2	MSEGITD	• • • • • • • • • • •	IEES	OIOINADKAA	YKFODHELDE	MLLRGVFGY.	GFEEPSAIOO	RAIMP11.EG	HOVLAGAQSE	TEKTETFSIA
p68	EHPOLARR	TAQEVET. YR	RSKEITV.	.RGHNCPKPV	LNFYEANFPA	KVH. DV LARO	NFTEPTAIQA	QGV. PVALSG	LONVGVAQTE	SGKTLSYLLP
PL10	. SERL . EQEL	FSGGNT6.IN	FEKYDOIPVE	ATGKNCPPHI	ESFSOVENGE	.IIMGNIELT	RYTRPTPVQK	HAI.PIIKEK	RDLMACAQTG	SGKTAAFLLP
¥858	.SNDAIEI	SSGIASG.IH	FSKYNNIPVK	VTGSDVPOP1	QHFTSADLR.	DITIONVIKS	GYKIPTPIQK	CSI, PVISS6	ROLMACAQTE	SEKTANFLLP
PRPS	.SE.TVSSMS	E.MEVEE.LR	LS.LDWIKIK	GTGCPKPV	TKWSQLGLST	OTHVL ITEKL	HEGSL TP105	QAL . PAINSG	ROVIGISKTG	SGKTISYLLP
Consensus			1		F		-FPIQ-	B6	-0AQ-6	-GKT
No1F-4A2	VLOQLDYSLV	£	c	QALVLAPTRE	LAQQIEKVHR	ALGOYLGYKY	HACVGGTS	VRED.O.RIL	QS.GVHVVVG	TPGRYFDHL.
NeIF-4A3	VCOIVOTKSS	E		QALILSPTRE	LAAQTEKVIL	AIGOYINVQA	HAC166KS	VGEDIRKL	EH. GVQVVS6	TPGRYCDMIK
eIF-4Al	ILQQIELDLK	A	T	GALVLAPTRE	LAQQIQKVVH	ALGOYNGASC	HAC1GGTN	VRAEVQKL	QHEAPHIIVG	TPGRYFDMLN
e[F-4A][ILOOLEIEFK	E	T	GALVLAPTRE	LAQQIQKVIL	ALGOYNGATC	HAC1GGTN	VRKEHOKL	QAEAPHIYVG	TPGRYFDHLN
TIF1/TIF2	ALQRIDTSVK	A	P	GALMLAPTRE	LALQIOKVVH	ALAFHNDIKY	HAC1GGTS	FVED. AEGL	.RDA.01VVG	TPGRYFDHIO
p68	AIVHINHQ.P	FLERGOG	P	ICLVLAPTRE	LADOVODVAA	EYCRACRLK.	STC1.YGGAP	K6P01RDL	ER. GVEICIA	TPGRLIDFLE
PL10	ILSQITTOGP	GEALRANKEN	GKYGRRKQYP	ISLVLAPTRE	LAVOIYEEAR	KFSYRSRVR.	. PCVVYGGAD	16001RDL	ER. GCHLLYA	TPGRL VOINE
*434	ILSKLLEDPH	ELELGR		QVC1VSPTRE	LAIQIFNEAR	KFAFESYLKI	GIVYGGTS	FRH OKECT	TR. GCHYVIA	TPGRLLOFVD
PRP5	LLROVKAGRP	LSKHETG	P	HGLILAPTRE	LALOIHEEVT	KFTEADTSIR	SVCCT.GESE	HKK. OITDL	KR.GTELVVA	TPGRFIDILT
Consensus	4			L-LAPTRE	LA-01					TPG80
								•••		
NelF-4A2	RROSLAPD	HIKHF. VLDE	ADENL SRGFK	DOIYOIF.OL	LPPKID	GYFSATHPPE	ALELTRK	FHIR PVD TI V	KROFL TI FG	IKOFYVI
NeIF-4A3	RRTLRTRG	TKLL . ILDE	SDENL SRGFK	DOLYDVY	IPPELO. V	VI ISATI PRF	ELET	FHTDPV9 II V	KROFI TIFA	IKOFFYA
eIF-4AI	RRYLSPKY	IKNE, VLDE	ADENL SRGFK	DOLYDIF.OK	LUSUTO	VLL SATHPSO	VI.FV. TICK	FIRME	KKEFL TIFA	IROFYIN
elF-4AII	REYL SPICE	TICHE VIDE	ADEMI SRGEK	DOIYERY OK	INTSTO V	VERSATINGTO	VIEV THE	FINITE THE F	FFET TIES	TRACTIN
TIF1/TIF2	REPERTOR	TKHE TO DE	ADEN SSGER	FOLYOIF TH	I POTTO U	WEE CATHERIN	WER TTE	ENGLOUGIN	WYDEL TIES	TENERIN
p68	CONTINUER	TTYL. VLOF	ADRIAL DINGFE	POTRKINDO	IPPORG T	INSATUP I	FYRDI AFD	FLEDVININI	GALEL SANHO	IL OTYONCHO
PL 10	POK 1GL DE	CIEVE VEDE	ADDIE DINGET	POTRETYCON	THEREVENT	MARCATER C	E TONE APD	EL DEVIELAV	CR VESTER	TOWN
¥814	RTEITEED	TR EVALOR	ADDIE DIESES	FORESTATION	THERE IN T	PHESATER E	ELORMACE	CI CHANNENA!	CI VCCACCO	WATINE
P2P5	INDERLISTE	BIT EVYNDE	ADDI EDI GET	POITOIN P	TVRPNYA C	VI CEATERMY	PCCAVE	VINCOLUTI	MER CANVER	W/WED104
Consensus			ADall and a	-Olestere.				Farmer 19111	MSK. OKTACH	INDERICH.
	•			41 1						
NEIF-4AZ	VURLEWKLET	LCOLTEILAI	1	•••••	QSVIFVNI	RRKYDMLIDK	MICSROH IV	SATH, GOMOQ	NTROIEMREF	RSESSRVL
N81P-4A3	VEREEWOOD	LCOLYDILIS	1	•••••	QAVIFCHT	KRKYDWLISK	MKENNEIV	SSINH, GOMPQ	KERDAINAEF	REGT TRYL
41F-4A1	VEREEWLDT	LCOLVEILII	1	•••••	QAVIFINT	RRKYDWLIEK	HEALOF TV	SANH. GONDO	KERDVINREF	RSGS.SRVL
@1F-4411	VEREEWKLDT	LCOLYFILT	1	•••••	QAVIFLNT	ROKANDAL LEK	MQAINETV	SALH, GOHDQ	KERDVINREF	RSGS.SRVL
1111/1112	VELEETKTEC	LIDETOSIST	1		QAVIFCHT	RREVEELTTK	LRHOKFTV	SALY. SOLPO	QERDTINKEF	RS6SSRIL
po8	VERDE . KL IR	LNEEINSE	K	•••••	ENKTIVFVET	KRRCDEL TRK	HICEDGUPAHG	IH.GOKSQ	QERDAVLINEF	KHGKAPIL
PL10	VEEAD.KRSF	LLOLLIM. TG	ĸ	•••••	DSLILVFVET	KKGADSLEDF	LYHEGYACT.	S. [H. GORSQ	ROREEALHOF	RSGKSPIL
¥454	VIIKYA.KRSK	LIEILSE	•••••	· · · · · · · Q	ADGTIVEVET	KRGADFLASF	LSEKEFPTT.	S. IH. GORLQ	SOREGALROF	KINGSINKVL
PRP5	TENE VENN									WE EVHATE
Consensus	, HENE , NY DR	LVOL THERSE	FFDEVQSEND	GOSSOVEEVO	A.KATIFVSS	QNICOFISKK	LLNAGIV.TC	.AIHAGKPYQ	.ERLIMMLEKF	KK EKHSIL
	VE-K	LVOLINERSE	FFDEVQSEND	GQSSDVEEVO	A.KATIFYSS VIFT	0011CDF 15K0K -RD-LK	LLNAGIV.TC	.AIHAGKPYQ SH-GDQ	.ERLMWLEKF	•-6L
	VE-K	LVQL THERSE	FFDEVQSEND	GOSSOVEEVO	A.KATIFYSS	QN1CDF15KK -RD-LK	LL NAGI V. TC	.AIHAGKPYQ SH-GDQ	.ERLIMMLEKF RF	**6*****L
NeIF-4A2	VE-K	LVQLTHERSE LL	TOLPTOPENY	GOSSOVEEVO	A.KATIFVSS VIFT GRX6VAIN.S	QNICDFISKK -RD-LK VT.KDDERHL	LLNAGIV.TC	.AIHAGKPYQ SH-GDQ .NYVIEELPA	.ERLIMMLEKF	•-6L
Ne:F-4A2 Ne:F-4A3	VE-K	LYQLIHERSE LL DVQQVSLVIN DVQQVSLVIN	YDLPTOPENY YDLPTOPENY	GQSSDVEEVD	A.KAIIFVSS VIFT GRXGVAIN.S GRKGVAIN.F	QHICDFISKK +R++D+L++K VT.KDDERHL VK.SDDIKIL	LLNAGIV.TC T- F.DIQKFY R.DIEQYY	.AIHAGKPYQ SH-GDQ .NYVIEELPA .STQIDEMPH	.ERLIMMLEKF RF HVADLL* HVADLL*	**6*****L
Ne1F-4A2 Ne1F-4A3 e1F-4A1	ITTDLLARGI ITTDLLARGI ITTDLLARGI	LYQLIHERSE LL DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN	YOLPTOPENY YOLPTOPENY YOLPTNRELY YOLPTNRENY	GQSSDVEEVD LHRIGRSGRF IHRIGRSGRF IHRIGRSGRF	A.KAIIFVSS VIFT GRKGVAIN.S GRKGVAIN.F GRKGVAIN.H	QHICDFISKK -RD-LK VT.KDDERHL VK.SDDIKIL VT.EEDKRTL	LLNAGIV.TC F.DIQKFY R.DIEQYY R.DIETFY	.AIHAGKPYQ SH-GDQ .NYVIEELPA .STQIDENPM .NTSIEENPL	.ERLHWLEKF ••R••••F #VADLL* #VADL1* #VADL1*	₩EUISIL **6-****
NeIF-4A2 NeIF-4A3 eIF-4A1 eIF-4A11	VE-K ITTDLLARSI ITTDLLARSI ITTDLLARSI ITTDLLARSI	LYQLIHERSE LL DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN	YOLPTOPENY YOLPTOPENY YOLPTNRELY YOLPTNRENY	GQSSDVEEVD LHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF	A.KAIIFYSS YIFT GRX5VAIN.S GRX5VAIN.F GRX6VAIN.H GRX6VAIN.F	QHICDFISKK -RO-LK VT.KDDERHL VK.SDDIKIL VT.EEDKRIL VT.EEDKRIL	LLRAGIV.TC T- F.DIQKFY R.DIEQYY R.DIETFY RH.JETFY	AIHAGKPYQ SH-GDQ .NYVIEELPA .STQIDENPM .NTSIEENPL .NTTVEENPM	.ERLHMLEKF ••R•••••F #VADLL* #VADL1* #VADL1* #GADL1*	••6•••••[
NeIF-4A2 NeIF-4A3 eIF-4A1 eIF-4A11 TIF1/TIF2	VE-K ITTDLLARSI ITTDLLARSI ITTDLLARSI ITTDLLARSI ISTDLLARSI	LVQLIHERSE LL DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN	YOLPTOPENY YOLPTOPENY YOLPTNRELY YOLPTNRENY YOLPANKENY	GQSSDVEEVD LHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF	A.KAIIFVSS YIFT GRKGVAIN.S GRKGVAIN.F GRKGVAIN.F GRKGVAIN.F	QHICDFISKK +R++D+L+-K VT.KDDERHL VK.SDDIKIL VT.EEDKRIL VT.EEDKRIL VT.KEDVGAH	LLMAGIV.TC F.DIQKFY R.DIEQYY R.DIETFY RH.IETFY RE.LEKFY	AIHAGKPYQ SH-GDQ .NYVIEELPA .STQIDENPM .NTSIEENPL .NTTVEENPM .STQIEELPS	.ERLHWLEKF ••R•••••F HVADLL* HVADL1* HVADL1* NGADL1* DIATLLN*	••6•••••[
Ne1F-4A2 Ne1F-4A3 e1F-4A1 e1F-4A11 T1F1/T1F2 p68	VE-K ITTDLLARGI ITTDLLARGI ITTDLLARGI ITTDLLARGI ISTDLLARGI IATDVASRGL	LYQLIHERSE LL DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN	YOLPTOPENY YOLPTOPENY YOLPTNRELY YOLPTNRENY YOLPTNRENY YOLPANKENY YOTPNSSEDY	GQSSDVEEVO LHRIGRSGRF IHRIGRSGRF IHRIGRGGRF IHRIGRGGRF IHRIGRGGRF IHRIGRGRF	A.KAIIFVSS YIFT GRKGVAIN.S GRKGVAIN.F GRKGVAIN.F GRKGVAIN.F GRKGVAIN.F TKTGTAYT.F	QNICDFISKK +RO-LK VT.KDDERHL VK.SDOIKIL VT.EEDKRIL VT.EEDKRIL VT.KEDYGAN FTPH.,NIKQ	LLRAGIV.TC F.DIQKFY R.DIEQYY R.DIETFY RH.IETFY VSOLISVLRE	.AIHAGKPYQ SH-GDQ .NYYIEELPA .STQIDEHPM .RTSIEENPL .RTTVEEHPM .STQIEELPS AH.Q.AIHP.	.ERLHWLEKF RF HVADL1* HVADL1* HVADL1* HGADL1* DIATLLN* KLLQ	••6•••••L
NeIF-4A2 NeIF-4A3 eIF-4A1 eIF-4A11 TIF1/TIF2 p68 PL10	VE-K ITTDLLARGI ITTDLLARGI ITTDLLARGI ITTDLLARGI ISTDLLARGI IATDVASRGL VATAVAARGL	LYQLIHERSE LL DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DVQQVSLVIN DISKVKVIN	YDLPTOPENY YDLPTOPENY YDLPTNRELY YDLPTNRENY YDLPTNRENY YDLPANKENY YDYPHSSEOY FOLPSDIEEY	GQSSDVEEVO LHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRGRF IHRIGRGRF VHRIGRTGRV	A.KAIIFYSS YIFT GRKEVAIN.S GRKEVAIN.F GRKEVAIN.F GRKEVAIN.F TKTGTAYT.F GHLGLA.TSF	QNICDFISK +RO-LK VT.KDDERHL VK.SDDIKIL VT.EEDKRIL VT.EEDKRIL VT.KEDVGAM FTPHNIKQ FNERNINI	LLRAGIV.TC F.DIQKFY R.DIEQYY R.DIETFY RH.IETFY RE.LEKFY VSOLISVLRE TKOLLOLLVE	.AIHAGKPYQ SH-GDQ .NYYIEELPA .STQIDENPM .NTSIEENPL .NTTVEENPM .STQIEELPS AN.Q.AINP. AK.Q.,EVPS	ERLIMMLEKF **R*****F NVADLL* NVADL1* NVADL1* NVADL1* NVADL1* DIATLLN* KLLQ VLENMAF	•• 6 ••••••L
NeIF-4A2 NeIF-4A3 eIF-4A1 eIF-4A11 TIF1/TIF2 p68 PL10 v410	VE-K ITTDLLARGI ITTDUARGI ITTDUARGI ITTDLLARGI ISTDLLARGI IATDVASRGL VATAVAARGI IATSVASRGL	LYQLIHERSE LL DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN DVQQYSLYIN DYQQYSLYIN DYQQYSLYIN DISNYXXYIN DISNYXXYIN DISNYXXYIN	YDLPTOPENY YDLPTOPENY YDLPTORENY YDLPTORENY YDLPTORENY YDLPANKENY YDYPASSEDY FOLPSDIEEY YDMPSKIDDY	GQSSDVEEVD LHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSGRF IHRIGRSTGRV VHRIGRTGRV VHRIGRTGRV	A.KAIIFVSS VIFT GRKEVAIN.S GRKEVAIN.F GRKEVAIN.F GRKEVAIN.F GRKEVAIN.F GRKEVAIN.F GRKEVAIN.F GNLGLA.TSF GNNGRA.TSF	QNICDFISK +RO-LK VT.KDDERHL VK.SDDIKIL V7.EEDKRIL V7.EEDKRIL V7.KEDVGAM FTPHNIKQ FNERNINI FHPEK.DRAI	LLRAGIV.TC F.DIQKFY R.DIEQYY R.DIETFY RH.IETFY VSDLISVLRE TKDLLDLLVE AADLVKIL.E	.AIHAGKPYQ SH-GDQ .NYYIEELPA .STQIDENPM .NTSIEENPH .NTTVEENPM .STQIEELPS AN.Q.AIMP. AK.Q.EYPS GSGQ.TYPD	ERLIMILEKF NVADLL* NVADLI* NVADLI* NVADLI* NGADLI* DIATLLN* KLLQ VLENNAF FLRTCGA	** 6 *****
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Figure 2. Peptide Sequence Alignment of the *Nicotiana* eIF-4A genes with selected DEAD box family members. The deduced amino acid sequences of NeIF-4A2 and NeIF-4A3 are aligned with the mouse eIF-4A1 and eIF-4AII (4); and the yeast TIF1/TIF2 (13) proteins which function in translation initiation; p68, a human nuclear protein involved in cell growth and division (19); PL10, specifically expressed during mouse spermatogenesis (20); vasa, a Drosophila protein involved in oogenesis and anterior-posterior orientation of the embryo (21,22); and PRP5, a yeast protein involved in mRNA splicing (23). Alignments were performed manually using LINEUP after performing pairwise comparisons with GAP. Residues which are identical in seven of the nine sequences are shown on the Consensus line.

were rescreened, in duplicate, with both the yeast TIF1 and mouse eIF-4AI genes. Three clones, designated NeIF-4A1, NeIF-4A2, and NeIF-4A3 (N for *Nicotiana*), hybridizing to both eIF-4A probes were selected for further study.

DNA and Deduced Amino Acid Sequence Analysis

The DNA and deduced amino acid sequences of the two full length *N. plumbaginifolia* cDNA clones are not shown but are available under EMBL accession numbers X61205 and X61206. NeIF-4A1 is a partial cDNA containing a 266 amino acid open reading frame and a 254 bp 3' untranslated region. The NeIF-4A1 open reading frame differs from NeIF-4A2 at 27 bp which result in three amino acid substitutions. NeIF-4A2 is a 1,368 bp cDNA clone consisting of a complete open reading frame of 413 amino acids, 116 bp of 5' untranslated region and 13 bp of 3' untranslated region. The 1573 bp NeIF-4A3 cDNA encodes a complete open reading frame of 391 amino acids and 5' and 3' untranslated regions of 14 and 386 bp, respectively.

In contrast to all other DEAD box family members, which have either a DEAD or a DEAH box, NeIF-4A3 contains a DESD box. The Ala to Ser alteration is a conservative amino acid change and results from a G to T transversion.

The NeIF-4A2 and NeIF-4A3 open reading frames code for polypeptides having calculated molecular weights of 46,828 and 44,194, respectively. The difference in amino acid length and calculated molecular weight between NeIF-4A2 and NeIF-4A3 results primarily from a 23 amino acid addition to the N-terminus of NeIF-4A2.

The 3' untranslated regions of both NeIF-4A1 and NeIF-4A3 have the potential to form extensive secondary structures (Figure 1). A potential 25 nucleotide stem/4 nucleotide loop with a single nucleotide bulge having a ΔG° of -41 kcal/mol is encoded by bp 892 to 946 of the NeIF-4A1 DNA sequence. An 11 nucleotide stem/4 nucleotide loop structure with a ΔG° of -24 kcal/mol has the potential to form from bp 1502 to 1527 in NeIF-4A3.

The deduced *Nicotiana* eIF-4A amino acid sequences have been aligned with the classical eIF-4A sequences from mouse and yeast and a selected subset of DEAD box proteins (Figure 2). The conserved amino acid domains traditionally associated with the DEAD box supergene family are obvious in this alignment, however other domains which are highly conserved between these sequences are also evident. These domains are conserved not only in amino acid sequence but also spatially with respect to each other in the *Nicotiana*, yeast and mouse eIF-4A genes.

A matrix of identity and similarity between NeIF-4A2 and NeIF-4A3 and the other DEAD box polypeptides indicates that NeIF-4A2 and NeIF-4A3 are as divergent from each other (64% identity) as they are from the most closely related DEAD box members, the mouse eIF-4A and yeast TIF genes (Table 1). The other DEAD box members all share approximately 30% identity with the *Nicotiana* members.

TABLE I. Percent Identity and Similarity (in brackets) between NeIF-4A2, NeIF-4A3 and selected members of the DEAD box gene family.

	NeIF-4A2	NeIF-4A3	eIF-4AI	eIF-4AII	TIF1/2	vasa	PLIO	p68	PRP5
NeIF-4A2		64 (77)	73 (83)	69 (81)	62 (78)	32 (53)	33 (54)	32 (53)	28 (50)
NeIF-4A3	64 (77)		64 (80)	65 (79)	59 (79)	32 (55)	33 (54)	34 (53)	30 (53)



Figure 3. Southern Blot Analysis of *N. plumbaginifolia* and *N. tabacum* Genomic DNA. Genomic DNA (5 μ g) from each plant was digested with either *Eco*RI, (E); *Hin*dIII, (H); *Xba*I, (X); or *Eco*RV, (EV), and size fractionated by electrophoresis through identical 1% agarose gels and transferred to nitrocellulose. The blots were cut in half and each half was hybridized separately with either the NeIF-4A2 or the NeIF-4A3 cDNA insert randomly labelled with ³²P-dC-TP. The positions of faint hybridizing bands are indicated to the right of the lane by a < . Migration positions of a 1 kb DNA ladder (Gibco BRL) are indicated.

Southern Blot Analysis of Genomic DNA

Southern blot analysis was performed at high stringency to determine the genomic copy number of the NeIF-4A2 and NeIF-4A3 genes in *N. plumbaginifolia* and *N. tabacum* (Figure 3). In *N. plumbaginifolia*, NeIF-4A3 hybridized to fewer genomic DNA fragments per digest than NeIF-4A2. Although a few of the bands align, the NeIF-4A2 hybridization pattern is distinct from the NeIF-4A3 generated pattern. Similar hybridizations using *N. tabacum* genomic DNA also indicated that NeIF-4A3 hybridized to fewer genomic fragments per digest than did NeIF-4A2. Again the NeIF-4A2 banding pattern differs from that observed with NeIF-4A3. NeIF-4A2 and NeIF-4A3 both hybridize to a larger number of genomic DNA restriction fragments in *N. tabacum* than in *N. plumbaginifolia*.

Immunological Characterization of the NeIF-4A2 cDNA Encoded Polypeptide

The NeIF-4A2 cDNA was cloned directly behind the bacteriophage T7 expression signals and the NeIF-4A2 polypeptide overexpressed in *E. coli*. A 47 kD polypeptide, corresponding to the molecular weight of the NeIF-4A2 polypeptide, was only observed in cells transformed with the NeIF-4A2 coding region in the correct orientation and in a time dependent manner, after induction with IPTG. Western analysis, shown in Figure 4, indicates that a polyclonal antibody directed against rabbit reticulocyte eIF-4A cross reacts with the 47 kD NeIF-4A2 polypeptide (Lane 1). As expected, a 46 kD polypeptide present in a rabbit reticulocyte lysate was also detected (Lane 2). Cross reactivity was not observed between the NeIF-4A2 polypeptide and an antibody directed against the yeast TIF polypeptide (data not shown).

NeIF-4A mRNA Transcript Analysis

Northern analysis was performed to determine the size and abundance of the NeIF-4A2 mRNA transcript in N.



Figure 4. Western Analysis of the NeIF-4A2 Polypeptide. A crude lysate from *E. coli* overexpressing the NeIF-4A2 polypeptide (Lane 1) and mammalian eIF-4A partially purified from rabbit reticulocytes (Lane 2) were separated by SDS-polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose and immunodecorated with polyclonal antisera raised against rabbit reticulocyte eIF-4A. Migration of molecular size markers (Bio-Rad) are shown on the left side: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).



Figure 5. Northern analysis. Total *Nicotiana* RNA (5 μ g), isolated from the indicated organs, was separated by agarose gel electrophoresis, after denaturation in the presence of glyoxal, and transferred to a nylon membrane. The blot was probed with the complete cDNA inserts from either NeIF-4A2 or NeIF-4A3 labelled with ³²P-dCTP. The approximate size of the hybridizing bands are shown in kb to the left and right and were calculated from the migration of an RNA ladder (Gibco BRL). PIST + ST represents pistil plus stamen.

plumbaginifolia and N. tabacum. Total RNA from leaves and roots of both species was probed with the NeIF-4A2 cDNA. A single hybridizing band was observed in both organ types and in both species, however the 1.6 kb transcript observed in N. tabacum is slightly larger than that observed in N. plumbaginifolia. NeIF-4A2 transcript levels are also higher in roots than in leaves in both species (data not shown). To investigate possible organ specific expression, the Nicotiana eIF-4A cDNA clones were hybridized separately to equal amounts of total RNA isolated from six N. tabacum organs (Figure 5). Single hybridizing transcripts were observed with both eIF-4A clones although NeIF-4A3 hybridized to a slightly larger transcript than did NeIF-4A2, 1.7 versus 1.6 kb, respectively. The overall hybridization pattern indicates that NeIF-4A2 and NeIF-4A3 are coordinately expressed in each of the organs examined, however, the level of expression varied between organs with the lowest expression in leaves and the highest in root, stem and shoot apex.



Figure 6. S1 nuclease protection analysis. Total RNA (20 μ g) isolated from the indicated organs was hybridized with 1×10^5 cpm of ssDNA probes specific for NeIF-4A2 or NeIF-4A3. A ssDNA probe specific for *atp*1, the nuclear-encoded gene for the beta-subunit of the *N. plumbaginifolia* mitochondrial ATPase, was included as an internal control in each reaction. Reactions were digested with 50 U S1 nuclease, denatured and separated on a 8% polyacrylamide sequencing gel. The expected 153 and 61 nucleotide protected transcript fragments specific for NeIF-4A2 and NeIF-4A3, respectively, are shown, as are the multiple bands expected for the protected *atp*1 gene transcript (16). YNG LF and PIST + ST represent young leaf and pistil plus stamen, respectively.

In order to verify that NeIF-4A2 and NeIF-4A3 are coordinately expressed, quantitative S1 nuclease protection analysis was performed using NeIF-4A2 and NeIF-4A3 specific ssDNA probes (Figure 6). The resulting data confirm the coordinate expression of both NeIF-4A2 and NeIF-4A3 in all N. tabacum organs examined. Absolute transcript levels of the two genes also vary between organs by the criteria of equivalent RNA loads. This probably does not reflect organ-specific variation of expression levels, but is a peculiarity of plant systems as a result of the high proportion of chloroplastic RNA present in photosynthetic tissues. Transcript levels for the atp1 gene, a popular control gene for equivalent RNA loading, also vary between the various organs, being higher when NeIF-4A levels are high and vice versa. This correspondence holds for all organs except the shoot apex where atp1 levels are low and both NeIF-4A2 and NeIF-4A3 transcript levels are high.

DISCUSSION

We have isolated and characterized three cDNA clones from the dicotyledonous plant *Nicotiana plumbaginifolia* by heterologous hybridization to both mammalian eIF-4A and yeast TIF genes. The three *Nicotiana* clones comprise two distinct gene classes as NeIF-4A1 and NeIF-4A2 share high amino acid similarity, while NeIF-4A3 is only 64% identical to NeIF-4A2. This is in contrast to other eukaryotic systems where two eIF-4A genes are also observed but their deduced amino acid sequences are highly similar, exhibiting 100% identity in yeast (13) and 91% identity in mouse (4). Thus NeIF-4A1 and NeIF-4A2 may be the plant homologues of the mouse eIF-4AI and eIF-4AII genes while NeIF-4A3 is clearly different. It is not known whether a gene corresponding to NeIF-4A3 is present in mammalian cells. Although the two *Nicotiana* eIF-4A gene classes are divergent, they both exhibit significant similarity to the rapidly growing

DEAD box supergene family. This family is characterized by a series of highly conserved amino acid domains (8), one of which, the DEAD box motif, has only been observed in this family. The *Nicotiana* cDNA clones contain all of the conserved amino acid domains although, interestingly, a conservative amino acid substitution converts the DEAD box into a DESD box in NeIF-4A3, further emphasizing the divergence between NeIF-4A2 and NeIF-4A3.

NeIF-4A2 and NeIF-4A3 exhibit the highest sequence identity with the mouse eIF-4A and yeast TIF genes which are known to be involved in the initiation of protein synthesis. The Nicotiana, mouse and yeast sequences are also spatially conserved over their entire amino acid sequences and thus encode polypeptides of approximately the same size. Both spatial and sequence identity decrease when the Nicotiana eIF-4A genes are compared with other members of the DEAD box family. The amino acid conservation existing between all DEAD box family members is still quite remarkable, however, especially when the diverse functions performed by these polypeptides is taken into consideration. The conservation not only spans the six previously identified DEAD box family domains (8) but exists throughout the eIF-4A-like region. The high level of conservation observed within these domains suggests that they perform an essential structural or functional role.

The similarity observed between NeIF-4A2 and the mammalian eIF-4A genes exists not only on the deduced amino acid level but also with respect to immunological cross reactivity. The 47 kD NeIF-4A2 polypeptide cross reacts with antisera raised against the rabbit reticulocyte eIF-4A protein but not with anti-TIF antisera. Immunological cross reactivity is not always detected between eIF-4A proteins from diverse organisms (24), an observation which emphasizes the similarity between mammalian eIF-4A and the NeIF-4A2 clone.

Therefore, on the basis of both sequence similarity and immunological cross reactivity we believe that the two classes of *Nicotiana* genes characterized in this paper represent genuine eIF-4As involved in translation initiation. Whether NeIF-4A3 performs a different role in plant translation initiation than do NeIF-4A1 and NeIF-4A2 remains to be determined.

The distinct genomic Southern hybridization patterns produced by NeIF-4A2 and NeIF-4A3 within each Nicotiana species indicates that the two genes belong to separate gene families in Nicotiana. The presence of multiple hybridizing bands suggests that both families are composed of more than one member. NeIF-4A2 is a representative of a small multigene family and NeIF-4A3 is a member of a gene family containing one or at most two members in N. plumbaginifolia, while both Nicotiana eIF-4A genes are members of small multigene families in N. tabacum. The NeIF-4A2 family appears to contain more members than that of NeIF-4A3, although the exact number of family members is unknown as the intron/exon arrangement within each family has not been exactly determined. Preliminary sequence data suggests that only three small introns occur in a Nicotiana genomic eIF-4A clone which is a member of the NeIF-4A2 family (Brander, Owttrim and Kuhlemeier, unpublished observation). The observation that more family members are present in N. tabacum, than in N. plumbaginifolia can be explained by the evolutionary origin of the two species. N. plumbaginifolia is a diploid species while N. tabacum is an amphiploid species whose genome complement was formed by the fusion of two diploid Nicotiana species, N. sylvestris and N. tomentosiformis (25). N. tabacum would therefore be expected to contain more gene family members, as is evident from the Southern blot analysis presented here.

It has recently been suggested that mammalian translation factor genes are present as duplicate, highly conserved copies (5). The results presented here indicate that this generalization does not extend to *Nicotiana* eIF-4A genes, as NeIF-4A2 and NeIF-4A3 are highly divergent and both are representatives of small multigene families. This raises the possibility that mammalian systems may also contain other eIF-4A-like genes which are so divergent that they may not be detectable by routine screening procedures.

The two *Nicotiana* eIF-4A gene classes are coordinately expressed in each organ examined with the ratio of NeIF-4A2 to NeIF-4A3 expression remaining approximately constant. The relative level of expression of each gene class changes considerably, however, between organs; the lowest levels are observed in the sepals and leaves while the highest levels occur in the root, stem and shoot apex, on the basis of total RNA. When compared to the expression levels of the atp1 housekeeping gene, however, NeIF-4A transcript levels are significantly elevated only in the shoot apex. The apical region dictates the formation and development of the aerial portion of a plant and therefore represents a region of high metabolic activity and cell division. Elevated expression of protein translation initiation factors may thus be required to complement the high metabolic requirements in the apical region, with different eIF-4A gene products possibly contributing to the switch from one developmental pathway to another. We are currently investigating the possibility that additional eIF-4A gene products are expressed in the apex in contrast to other Nicotiana organs.

The stability and processing of RNA coding for the two classes of *Nicotiana* eIF-4A genes may be controlled by RNA secondary structure present in the 3' noncoding regions. Similar RNA stemloop structures are required for 3' end formation and mRNA stability of both mammalian histone genes (26) and numerous chloroplast encoded genes (27). These structures may therefore play a role in dictating when during the cell cycle or developmental program various eIF-4A members are themselves translated.

The presence of two divergent Nicotiana eIF-4A genes is also interesting in light of the biochemical analysis of wheat germ in which only one eIF-4A polypeptide is observed (10). The 50 kD wheat germ eIF-4A polypeptide is significantly larger than the 47 and 44 kD NeIF-4A2 and NeIF-4A3 polypeptides. The eIF-4A polypeptides required for the initiation of protein synthesis may differ between dormant wheat germ and actively growing mature plants as a result of the different developmental states of the two systems. It is tempting to speculate that, in vivo, the single wheat germ eIF-4A polypeptide initiates translation of mRNA transcripts required to maintain the quiescent state, while the expression of additional eIF-4A genes may be specifically correlated with later stages of development. Nicotiana is an excellent system in which to observe differential eIF-4A function because of the divergence between the two eIF-4A genes which we have characterized in this paper.

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