T

Translation initiation factors eIF4E and eIFiso4E are required for polysome formation and regulate plant growth in tobacco

Jonathan P. Combe[a], Marie E. Petracek[1], Gerben van Eldik[2], Frank Meulewaeter[2] and David Twell*

Department of Biology, University of Leicester, Leicester LE1 7RH, United Kingdom

Department of Biochemistry and Molecular Biology[1], Oklahoma State University, 246 NRC, Stillwater, OK 74078, USA

Bayer BioScience N.V.[2], Technologiepark 38, B-9052 Gent, Belgium

*Corresponding author: Department of Biology, University of Leicester, Leicester LE1 7RH, United Kingdom. Fax: 44-116-2522791; E-mail: twe@le.ac.uk

[a] Present address: Department of Biochemistry, University of Leicester, Leicester LE1 7RH, United Kingdom.

Key words: *Nicotiana tabacum*; translation initiation; eukaryotic initiation factors eIF4E and eIFiso4E

Abstract

Eukaryotic initiation factor eIF4E plays a pivotal role in translation initiation. As a component of the ternary eIF4F complex, eIF4E interacts with the mRNA cap structure to facilitate recruitment of the 40S ribosomal subunit onto mRNA. Plants contain two distinct cap-binding proteins, eIF4E and eIFiso4E, that assemble into different eIF4F complexes. To study the functional roles of eIF4E and eIFiso4E in tobacco, we isolated two corresponding cDNAs, NteIF4E1 and NteIFiso4E1, and used these to deplete cap-binding protein levels *in planta* by antisense downregulation. Antibodies raised against recombinant NteIF4E1 detected three distinct cap-binding proteins in tobacco leaf extracts; NteIF4E and two isoforms of NteIFiso4E. The three cap-binding proteins were immuno-detected in all tissues analysed and were coordinately regulated, with peak expression in anthers and pollen. Transgenic tobacco plants showing significant depletion of either NteIF4E or the two NteIFiso4E isoforms displayed normal vegetative development and were fully fertile. Interestingly, NteIFiso4E depletion resulted in a compensatory increase in NteIF4E levels, whereas the down-regulation of NteIF4E did not trigger a reciprocal increase in NteIFiso4E levels. The antisense depletion of both NteIF4E and NteIFiso4E resulted in plants with a semi-dwarf phenotype and an overall reduction in polyribosome loading, demonstrating that both eIF4E and eIFiso4E support translation initiation *in planta*, which suggests their potential role in the regulation of plant growth.

Introduction

Translation initiation, the recruitment of ribosomes onto the 5' end of mRNAs, is a multi-step process involving at least 10 different eukaryotic translation initiation factors (eIFs). A key regulatory point for translation initiation is the binding of the 43S preinitiation complex (40S ribosomal subunit charged with the initiator methionine t-RNA) to the 5' end of the mRNA, catalyzed by an initiation factor complex, eIF4F (reviewed in Browning, 1996; Pestova and Hellen, 2001; Gallie, 2002; Gingras *et al*., 1999). eIF4F is a ternary complex consisting of eIF4E, the cap-binding subunit, eIF4A, a DEAD-box RNA helicase, and eIF4G, a bridging polypeptide that interacts with eIF4A, eIF4E and the 40S ribosomal subunit via contacts with the eIF3 multi-subunit complex. eIF4F binds the mRNA 5' cap and unwinds secondary structure within the 5' UTR to facilitate the recruitment and scanning of the 43S preinitiation complex. Following a "pioneer round" of translation, eIF4F is also thought to promote the "re-initiation" of ribosomes by circularizing mRNAs through interactions between eIF4G and the poly A binding protein, PABP (Gallie, 1998).

In animal cells, eIF4F activity is regulated by the cap-binding subunit, eIF4E, through two separate mechanisms (reviewed in Raught and Gingras, 1999; Sonenberg and Dever, 2003). Firstly, the cellular pool of active eIF4E is modulated in response to physiological stimuli by a class of negative regulatory eIF4E-binding proteins (4E-BPs), which inhibit the incorporation of eIF4E into eIF4F. The inhibitory activity of the 4E-BPs is regulated by phosphorylation (reviewed in Gingras *et al*., 2004). Secondly, in mammalian cells, eIF4E is itself phosphorylated at serine 209, concomitant with changes in the overall rate of protein synthesis (reviewed in Sonenberg, and Gingras, 1998). For example, the phosphorylation of eIF4E has been

correlated with the stimulation of protein synthesis in quiescent cells treated with hormones, growth factors, and mitogens, whereas the dephosphorylation of mammalian eIF4E coincides with the inhibition of translation during mitosis, viral infection and following heat shock (Gingras e*t al*., 1999). eIF4E also plays a critical role in cell growth control, as the overexpression of eIF4E results in accelerated cell growth, transformation in culture and tumorigenesis in nude mice (De Benedetti, and Rhoads, 1990; Lazaris-Karatzas *et al*., 1990). Conversely, reducing the cellular pool of active eIF4E, either by expressing antisense eIF4E RNA or overexpressing 4E-BP, inhibits cell transformation and growth (De Benedetti *et al*., 1991; Rousseau *et al*., 1996).

In addition to eIF4F, flowering plants contain a second cap-binding complex called eIFiso4F, which is composed of different eIF4E and eIF4G subunits (reviewed in Browning 1996). In wheat germ, eIF4F consists of a 220 KDa eIF4G polypeptide and a 26 KDa cap-binding protein called eIF4E. eIFiso4F consists of eIFiso4G, an 86 KDa polypeptide, and a 28 KDa cap-binding subunit called eIFiso4E (Browning *et al*., 1992). The published eIF4E and eIFiso4E sequences, from monocot and dicot plants species, share 45-50% identity at the amino acid level, whereas *Arabidopsis* and wheat eIFiso4G and eIF4G are only 30-40% identical. Wheat eIF4F and eIFiso4F have similar biochemical properties and are both able to support protein synthesis within a fractionated wheat germ translation system (Browning, 1996). When present at rate-limiting amounts, eIF4F and eIFiso4F differentially support the *in vitro* translation of some mRNAs, suggesting that the two cap-binding complexes have evolved to regulate plant translation by mRNA selection (Gallie, 2001; Gallie and Browning, 2001). However nothing is currently known about the *in vivo* functionality

of eIFiso4F and eIF4F and the regulatory significance of the eIF4E and eIFiso4E subunits.

Recently, a transposon insertion within the single-copy *Arabidopsis* eIFiso4E gene has been isolated (Duprat *et al*., 2002). Plants homozygous for this insertion were null for the eIFiso4E gene, but surprisingly, exhibited wild-type growth and fertility. This suggests that eIFiso4E is not required for translation *in planta* under normal growth conditions, and may have a specialized role, such as promoting mRNA translation during a stress response. A similar role has recently been proposed for eIF4E2 in *Schizosaccharomyces pombe* (Ptushkina *et al*., 2004).

Here we investigated the functional and regulatory significance of both eIF4E and eIFiso4E using tobacco as a model. cDNAs encoding tobacco eIF4E and eIFiso4E were isolated and used to deplete cap-binding proteins *in planta* by antisense downregulation. Transgenic tobacco plants depleted for either eIF4E of eIFiso4E displayed normal growth and development. However, plants in which eIF4E and eIFiso4E were both depleted showed a semi-dwarf phenotype and a concomitant reduction in polysome loading. Our results demonstrate for the first time that both eIF4E and eIFiso4E support general translation in plants and suggest possible roles as cell growth regulators.

Experimental Procedures

Materials

Nicotiana tabacum cv. Samsun plants were grown in soil under standard greenhouse conditions with 16 hours day length.

cDNA cloning of tobacco eIF4E and eIFisoE cDNAs and sequence analysis

A 230 bp fragment encompassing the central coding region of a tobacco eIF4E cDNA was amplified by RT-PCR from pollen RNA, using degenerate primers, 4E5' (5'-CGTCTAGACT($^{T}/_{G}$)TACAACT($^{C}/_{A}$)ATATCC-3') and 4E3' (5'-CGGATCCGCTCC AC($^{A}/_{T}$)A($^{A}/_{T}$)A($^{A}/_{T}$)C($^{A}/_{T}$)TCATC-3'). The RT PCR product was used to isolate a full length eIF4E cDNA from a library constructed from mature poly A+ RNA (Sweetmen *et al*., 2000). Plaque lifts were washed at high stringency according to Church and Gilbert, 1984; 65 °C in 20 mM sodium phosphate buffer (pH 7.4) containing 5% sodium dodecyl sulphate with a final wash containing 1% SDS. Automated DNA sequencing was carried out by the protein and nucleic acid sequencing laboratory, University of Leicester. An *Arabidopsis* eIFiso4E cDNA clone (gift from J.F. Laliberté, University du Quebec; Wittmann et al., 1997) was used to isolate a tobacco eIFiso4E cDNA clone from a tobacco leaf cDNA library (Stratagene). Plaque lifts were washed at 55 °C in 0.1 x SSC containing 0.1% sodium dodecyl sulphate. As the isolated tobacco eIFiso4E cDNA clone was not full length, 5'RACE PCR (Marathon cDNA amplification kit, Clontech) was used to isolate the missing 5' end.

Plant protein extraction, SDS PAGE and Western analysis

Approximately 500 mg of plant material was flash frozen in liquid nitrogen, ground to a fine powder with a mortar and pestle and resuspended in 500 µl protein extraction buffer (50 mM MOPS-KOH pH 7.2, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10 % (v/v) glycerol, 14 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin). The cell extracts were centrifuged twice at 10,000 *g* to remove insoluble debris. Total protein concentration was determined according to Bradford, 1976. Approximately 10 µg of each protein sample was fractionated on 12 % SDS PAGE mini-gels (Protean II, Bio-Rad) and

transferred onto polyvinylidene difluoride membrane (Immobilon P, Millopore) by the semi dry method according to the manufacturers instructions.

Production of polyclonal antibodies against eIF4E

Rabbit polyclonal antibodies were raised against bacterially expressed eIF4E. The complete coding region of tobacco eIF4E was amplified by PCR with forward (5'-ACCACCATGGTTGATGAAGTAG-3') and reverse (5'-GTTCTAGACAATTCCTATTGTAACG-3') primers that incorporated *Nco*I and *Xba*I restriction sites at the 5' and 3' ends, respectively. The *Nco*I site was inserted at the predicted initiator ATG of the tobacco eIF4E sequence without altering the neighbouring codon to allow the generation of transcriptional gene fusions at the ATG, and the 3' *Xba*I site was inserted 24 bp 3' of the predicted stop codon. Following amplification, the PCR product was cloned into pET23d as an *Nco*I-*Xba*I fragment and checked by sequencing. Recombinant eIF4E was synthesized in *E. coli* strain BL21 (DE3), by induction of mid-exponential phase liquid cultures with 0.5 mM Isopropyl-1-thio-ß-D-galactoside (IPGT) for 5 hours at 37 °C, and purified by preparative SDS PAGE according to Harlow and Lane, 1988. Following gel isolation, eIF4E protein was eluted out of polacrylamide gel slabs into Tris-buffered saline (TBS), further dialysed against TBS and then used to raise rabbit polyclonal antibodies. Immunisations and test bleeds were carried out by Biomedical services, Leicester University.

m^7GTP-Sepharose chromatography

m^7GTP-sepharose chromatography (adapted from Morley and Traugh, 1990) was used to partially purify tobacco cap-binding proteins from plant cell extracts. Approximately 500 mg of tobacco leaf material was ground in liquid nitrogen and resuspended in 300 µl buffer A (50 mM MOPS-KOH pH 7.2, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM GTP, 10 % (v/v) glycerol, 14 mM 2-mercaptoethanol,

0.5 mM phenylmethylsulphonyl fluoride, 1 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml pepstatin). The cell extract was centrifuged at 10,000 *g* and the resulting supernatant was applied to a 0.2 ml m^7GTP-sepharose column (Pharmacia,) pre-equilibriated in buffer A. The column was washed twice with 300 µl buffer A and bound cap-binding proteins were eluted with 300 µl buffer A containing 2 mM m^7GTP. The resulting eluate was concentrated using microcon 10 micro-concentrators (Amicon) and analysed by SDS PAGE and immunoblotting.

Construction of antisense vectors, tobacco transformation and analysis

The full length *NteIF4E1* and *NteIFiso4E1* cDNAs were expressed in the antisense orientation under the control of 35S promoter and terminator sequences derived from pRTL2GUS (Carrington and Freed, 1990). The NteIF4E1 cDNA was cloned into a derivative of pRTL2GUS as an *Xho*I-*Xba*I fragment, displacing the TEV-GUS fragment. The resulting 35S5'-NteIF4E1a/s-35S3' cassette was introduced into *Hind*III-*Sac*I cut pBIN19, producing pBIN35S-NteF4E1a/s. pBIN35S-NteIFiso4E1a/s was generated by removing the NteIF4E1 cDNA in pBIN35S-NteIF4E1a/s and inserting the NteIFiso4E1 cDNA in the antisense orientation as an *Xho*I-*Xba*I fragment. pBIN35S-NteIF4E1a/s and pBIN35S-NteIFiso4Ea/s were introduced into *Agrobacterium* strain LBA4404 (Hofgen, and Willmitzer, 1988). For each construct, at least 150 independent tobacco transformants were generated by the leaf disc transformation method (Draper *et al*., 1988). Tobacco eIF4E and eIFiso4E levels were analysed in transgenic lines by immunoblotting using the tobacco eIF4E and eIF4A (Owttrim *et al*., 1994) antibodies.

Polyribosome analysis

One half of a leaf (10 cm length) from wild-type and eIF4E/eIFiso4E single and double antisense lines, was flash-frozen in liquid nitrogen and polyribosomes were

isolated as previously described (Petracek *et al*., 1997). Equal amounts of total polyribosome RNA were loaded and resolved on a 15% to 60% sucrose gradient. The sucrose gradients were sampled using model 640 Density gradient fractionator (ISCO, Nebraska) pumping through a type 6 optical unit (ISCO). Polyribosomes traces were recorded at OD_{254} using a UA05 Absorbance/fluorescence detector (ISCO).

Results

The isolation of tobacco eIF4E and eIFiso4E cDNAs

Full-length cDNAs encoding tobacco eIF4E and eIFiso4E orthologues were isolated by a combination of RT-PCR and cDNA library screening. The full-length tobacco eIF4E cDNA clone, *NeIF4E1*, encoded a predicted 222 amino acid polypeptide with a molecular mass of 25 KDa and an isolelectric point of 5.04. Whereas the isolated tobacco eIFiso4E cDNA clone, NteIFiso4E1, encoded a predicted 200 amino acid polypeptide with a molecular mass of 23 KDa and an isoelectric point of 5.97. In pairwise alignments with plant, animal and fungal eIF4E polypeptide sequences, NteIF4E1 was most related to the known plant eIF4E sequences from tomato, red pepper, rice, wheat, maize and *Arabidopsis*, with identity scores of 72 %, 71 %, 68 %, 68 %, 66 %, and 64 %, respectively. This compared with 47 % identity between NteIF4E1 and NteIFiso4E1. NteIFiso4E1 was most related to the eIFiso4E sequences identified in *Arabidopsis*, wheat, rice and maize with identity scores of 59 %, 68 %, 68 % and 66 %, respectively. The assignment of the tobacco cDNA clones to the plant eIF4E and eIFiso4E sub-families was confirmed by constructing a phylogenetic tree from the conserved ca. 175 carboxy-terminal residues of plant, human and yeast eIF4E sequences (Figure 1A). NteIF4E1 formed a distinct clade with all of the published monocot and dicot eIF4E sequences, whereas NteIFiso4E1 was grouped with *Arabidopsis*, maize, wheat and rice eIFiso4E.

A multiple alignment between the tobacco and mouse eIF4E sequences revealed that both NteIF4E1 and NteIFiso4E1 contain a number of critical amino acid residues involved in interactions between eIF4E and either m^7GTP or eIF4G (Marcotrigiano *et al*., 1997; Ptushkina *et al*., 1999). These include residues determined from the mouse eIF4E crystal structure to either directly contact 7-methyl guanine (Trp-56, Trp-102, Glu-103, and Trp-166 of mouse eIF4E), or interact with the phosphate groups of m^7GTP (Arg-157 and Lys-162 of mouse eIF4E). Amino acid residues required for the eIF4E-eIF4G and eIF4E-4EBP interactions, located on the dorsal surface of the mouse crystal structure, (His-37, , Leu-39, Val-69, Trp-73, Gly-139), were also absolutely conserved in NteIF4E1 and NteIFiso4E1. In common with all the previously published plant eIF4E/eIFiso4E sequences, both NteIF4E1 and NteIFiso4E1 do not possess the conserved Serine 209 residue that is phosphorylated in animal cells.

Expression analysis of NteIF4E and NteIFiso4E

One possible explanation for the existence of two distinct cap-binding complexes in plants is that eIF4F and eIFiso4F are adapted to support translation in different cell types. To test this hypothesis, the expression patterns of NteIF4E and NteIFiso4E were investigated by western blot analyses of different tobacco tissues, using an antibody raised against recombinant NteIF4E1 that cross-reacted with tobacco eIFiso4E. The full length *NteIF4E1* open reading frame was expressed in *E. coli*, producing a polypeptide migrating at 30 kDa (data not shown). A rabbit polyclonal antibody raised against recombinant NteIF4E1 detected three polypeptides from a tobacco leaf extract that were all enriched by m^7GTP sepharose chromatography (Figure 2a). The three polypeptides, migrating at 30 kDa, 25 kDa and 22 kDa, likely represent NteIF4E and two isoforms of NteIF4iso4E (NteIFiso4Ea

and NteIFiso4Eb), respectively. The identities of the three polypeptides were confirmed in the antisense experiments detailed below. In particular, the 30 kDa polypeptide band was specifically depleted by the *NteIF4E1* antisense construct and the 22 and 25 kDa polypeptide species were both specifically depleted by the *NteIFiso4E1* antisense construct (Figure 3).

NteIF4E, and the two NteIFiso4E isoforms, were detected in all tissues analysed on immunoblots of total soluble protein isolated from different tobacco tissues, including 2 week-old seedlings, roots, leaves, sepals, petals, anthers and dehisced pollen (Figure 2b). Changes in the relative abundance of NteIF4E, NteIFiso4Ea and NteIFiso4Eb between tissues followed a broadly similar pattern to tobacco eIF4A, with the highest levels of all four initiation factors being detected in isolated pollen, maturing anthers and roots. However, alterations in relative molar ratios of NteIF4E, NteIFiso4E1 and NteIFiso4E2 between tissues were also observed. Whereas the ratio of NteIFiso4E1 to NteIFiso4E2 remained constant, relatively higher molar ratios of NteIF4E to the two NteIFiso4E isoforms were detected in anthers and pollen compared with seedlings, roots, leaves, sepals and petals (Figure 2b).

Antisense down-regulation of NteIF4E and NteIFiso4E

To investigate the *in vivo* functionality of tobacco eIFiso4E and eIF4E, we used an antisense approach. Two plant transformation vectors were created containing the full length *NteIF4E1* and *NteIFiso4E1* cDNAs in antisense orientation with expression driven by the Cauliflower mosaic virus *35S* promoter. Approximately 150 independent lines were generated with each vector, and plants were screened by immunoblotting for reduction in either NteIF4E or NteIFiso4E. A polyclonal antibody raised against tobacco eIF4A was used as a loading control (Owttrim, et al, 1994). Several *35S::NteIF4E1* antisense plants showing significant NeIF4E down-regulation

were isolated in the T1 generation. Two of the most effective *NteIF4E* antisense lines, 9 and 23, were selected for further study. The T2 progeny displayed reproducible reductions of NeIF4E to 30 % or 40 % of wild-type protein levels, respectively. These plants showed no significant effects on either NteIFiso4E or NteIF4A levels (Figure 3a). Moreover, these *NteIF4E* antisense lines did not show obvious developmental or reproductive defects in T1 or T2 generations (data not shown). Screening of T1 generation *NteIFiso4E1* antisense lines by immunoblotting identified a number of plants that were partially depleted for both 22 KDa and 25 KDa NteIFiso4E isoforms. NteIFiso4Ea and NteIFiso4Eb down-regulation was confirmed in the T2 generation in the progeny of lines 21 and 106. These lines showed levels of both NteIFiso4E isoforms that were approximately 60 % of wild-type protein levels (Figure 3a). Interestingly, the reduction in NteIFiso4E was compensated by an increase in NteIF4E levels to approximately 200 % of wild type levels, whereas NteIF4A was unaffected. Similar to *NteIF4E1* antisense plants, these plants showed no obvious vegetative or fertility defects (data not shown).

The effect of simultaneously depleting both NteIF4E and NteIFiso4E was investigated by combining different antisense lines. *NteIF4E1* antisense line 23 was crossed with *NteIFiso4E1* antisense line 21. The resulting F1 progeny were grown on soil under greenhouse conditions with wild-type and parental antisense plants as controls. Of 10 double-antisense plants, 6 individuals showed significant down-regulation of both NteIF4E and the two NteIFiso4E isoforms to approximately 26 % and 31 % of wild-type protein levels, respectively (Figure 3b). The growth rates of the effective double antisense plants were significantly reduced compared wild-type plants, and the parental antisense lines (Figure 4). Flowering time of the effective double antisense plants was also delayed by up to 3 days, and terminal plant height

was reduced to 80 % of wild-type plants, producing a semi-dwarf phenotype (Figure 4). Although there was no significant difference in terminal plant height between the single antisense lines and isogenic wild-type plants, a lag in growth was observed for *NeIF4E1* antisense line 23 (Figure 4B). A similar growth lag was observed for independent NeIF4E antisense line 9 (data not shown). In contrast, the growth rate of *NteIFiso4E1* antisense line 21 and line 106 were indistinguishable from wild-type (Figure 4B and data not shown). The semi-dwarf phenotype of the double-antisense plants was recapitulated in an independent cross between *NteIFiso4E1* antisense line 21 and *NteIF4E1* antisense line 9 (data not shown).

Reduced growth rate of the NteIF4E/NteIFiso4E double antisense plants is correlated with reduced polyribosome loading

To determine whether translation initiation is affected by the down-regulation of NteIF4E and NteIFiso4E, polyribosomes were isolated from the crude leaf extracts of single antisense, double antisense and wild-type plants, and fractionated by sucrose density gradient centrifugation (Petracek, *et al.*, 1997). Four separate polyribosome fractionation experiments revealed no reproducible difference between the polyribosome profiles of NteIF4E antisense line 23, NteIFiso4E antisense line 21, and wild-type plants (data not shown). However, a reproducible shift in the polyribosome distribution was detected between four replicate semi-dwarf double-antisense plants and four wild-type plants. Figure 5A shows a typical polyribosome trace isolated from 10 mm long leaves ofa 55 day-old wild-type and a double antisense plant. Compared to wild-type, the four double antisense plants consistently displayed a higher monosome/polyribosome ratio (Figure 5B) suggesting the rate of translation initiation is significantly reduced by the down-regulation of both NteIF4E and NteIFiso4E.

Discussion

In this study, cDNAs encoding tobacco eIF4E and eIFiso4E ortholoques were isolated and characterised. Rabbit polyclonal antibodies were raised against recombinant NteIF4E protein, which detected both NteIF4E and NteIFiso4E from tobacco protein extracts. Whereas NteIF4E was detected as a 30 kDa polypeptide band, two distinct NteIFiso4E isoforms, (NteIF4Ea and NteIFiso4Eb) were resolved, migrating at 22 kDa and 25 kDa. The molecular weight difference between NteIFiso4Ea and NteIFiso4Eb is too great to be accounted for by phosphorylation alone, and probably represents two distinct eIFiso4E polypeptides. In wheat, maize and *Arabidopsis*, only single eIFiso4E and eIF4E polypeptides have been detected (Duprat *et al*., 2002; Gallie *et al.*, 1998), although two distinct eIFiso4E isoforms have been purified from cauliflower (Browning *et al*., 1992). It remains to be determined whether the two eIFiso4E polypeptide isoforms in tobacco and cauliflower perform specialized roles.

In a western blot of total soluble protein isolated from different tissuesNteIF4E, the two NteIFiso4E polypeptide isoforms and tobacco eIF4A were most abundant in mature pollen, maturing anthers and roots. The presence of enhanced levels of these factors in anthers and pollen is consistent with previous expression studies showing higher levels of initiation factors in the meristematic regions that contain higher numbers of dividing and expanding cells (Gallie, et al., 1998; Rodriguez *et al*., 1998). Whereas anthers contain actively expanding immature pollen grains, there is good evidence to indicate that early pollen tube growth following germination is strictly dependent upon the efficient translation of mRNAs stored as mRNPs in mature dehydrated pollen (Mascarenhas, 1990; Honys *et al*., 2000). The translation initiation factors, including NteIF4E, NteIFiso4E, and tobacco

eIF4A are likely to be major constituents of these mRNP complexes. Compared with the other tissues analysed, maturing anthers and pollen also displayed a higher molar ratio of NteIF4E to the two NteIFiso4E.isoforms. Stoichiometric changes between eIF4E and eIFiso4E have also been observed during seedling development in monocots (Gallie, et al., 1998) that may reflect developmental changes in the translation of mRNAs with differences in their preference for the two cap-binding complexes, eIF4F and eIFiso4F.

The main objective of this study was to determine the *in vivo* functional significance of tobacco eIF4E and eIFiso4E using an antisense approach. Transgenic tobacco plants were generated containing the *NteIF4E1* or *NteIFiso4E1* cDNAs transcribed in the antisense orientation from the constitutive *CaMV 35S* promoter. Two independent transformants were isolated for each antisense construct, which displayed stable depletion of either NteIF4E or NteIFiso4E. *NteIF4E1* antisense plants had less than 50 % of wild-type NeIF4E levels, but normal NeIFiso4E and NteIF4A levels. Apart from a consistentlag in growth, both *NteIF4E1* antisense lines displayed no obvious vegetative or reproductive defects. The *NteIFiso4E1* antisense plants were depleted for the two NteIFiso4E polypeptide isoforms to approximately 60 % of wild-type levels, but, unlike the *NteIF4E1* antisense plants, no growth lag was observed compared to wild-type plants. Interestingly, NteIFiso4E depletion was compensated by the overexpression of NteIF4E which provides an explanation for the absence of any growth lag in the *NteIFiso4E1* antisense plants. Recently, a transposon insertion in the single-copy *Arabidopsis AteIFiso4E-1* gene has been isolated (Duprat *et al*., 2002). The insertion is a null allele and, like the tobacco *eIFiso4E* antisense plants, has no effect on plant growth and development. Furthermore, *Arabidopsis* eIF4E polypeptide levels are up-regulated in the *AteIFiso4E-1* knock-out plants,

strongly suggesting that a common homeostatic control mechanism exists in both tobacco and *Arabidopsis* which serves to maintain constant cap-binding protein levels.

In *Arabidopsis*, the regulation of *AteIF4E* expression by either *AteIFiso4E* polypeptide or mRNA levels appears to be operating at the translational level, as higher levels of *AteIF4E* mRNA were co-fractionated with polyribosomes in *AteIFiso4E-1* mutant plants than wild type, despite there being no difference in the steady state *AteIF4E* transcript abundance (Duprat *et al*., 2002). The precise mechanism of eIF4E translational control by eIFiso4E is currently unknown, but probably involves specific sequence elements in the 5' or 3' UTRs of the tobacco and *Arabidopsis eIF4E* mRNAs. Interestingly, the control mechanism appears to be operating in one direction only in tobacco, as the down-regulation of NteIF4E by antisense does not result in the reciprocal up-regulation of NteIFiso4E.

Since the "single" antisense downregulation of NteIF4E and NteIFiso4E resulted in plants virtually indistinguishable from wild-type, the effect of simultaneously depleting both cap-binding proteins was investigated. The most effective NteIF4E1 and NteIFiso4E1 antisense lines were combined, giving an F1 progeny with both NteIF4E and NteIFiso4E down-regulated to approximately the same levels as in the single antisense lines. F1 plants with both cap-binding proteins depleted displayed significantly reduced growth and polysome loading, suggesting that NteIF4E and NteIFiso4E contribute additively to translation and plant growth. The observed semi-dwarf phenotype in the double antisense plants could either be caused by lower levels of general protein synthesis or the selective repression of a "weakly-translated" mRNA coding for a limiting cell cycle regulator. In support of first mechanism, similar dwarf phenotypes have been observed in transgenic plants

with reduced levels of general ribosomal proteins (Popescu and Tumer, 2004). However, the second model is suggested by observations on the effect of attenuating eIF4E activity in yeast (Brenner *et al*., 1988). In *Saccharomyces cerevisiae*, temperature sensitive mutations in the single eIF4E gene arrest cell division at G1/S phase, that can be suppressed by overexpression of an inefficiently translated D-type cyclin mRNA (Danaie *et al*., 1999).

In conclusion, we have demonstrated, using an antisense approach in transgenic tobacco, that both eIF4E and eIFiso4E support general translation *in planta.* Further analyses of these antisense plants should determine whether eIF4E and eIFiso4E can differentially select mRNAs for translation *in planta.*

Acknowledgements

We are particular grateful to Cris kuhlemeier for the kind gift of the NteIF4A antibodies, Jean-François Laliberté for the gift of the *Arabidopsis* eIFiso4E cDNA, Paul Jarvis for critical comments on the manuscript, Sybille Kubis for assistance with the phylogenetic analysis and Graham Benskin for the maintenance of the tobacco plants. J.C was supported by a BBSCR studentship and a one year industrial postdoctoral fellowship provided by Bayer BioScience N.V. M.P was supported by NSF grant MCB-0090666 and the Oklahoma Agricultural Experiment Station (project no. H-2427). GvE was supported by a post-doctoral fellowship from the Institute for the Promotion of Innovation by Science and Technology in Flanders (IWT-Flanders).

Figure Legends

Figure 1. Phylogenetic analysis and sequence conservation of NteIF4E1 and NteIFiso4E1.

A. Phylogenetic analysis of NteIF4E1 and NteIFiso4E1. The C-terminal residues (equivalent to residues 47-222 of NteIF4E1) of eIF4E homologues from

plants, yeast and humans were aligned using clustal W algorithm and used to produce a maximum parsimony phylogentic tree using PAUP v4.4. Numbers of mutations are given above the clades, with bootstrap values below. Gene and accession numbers are as follows; ateIF4E-1, At4g18040; ateIF4E-2, At1g29590; ateIF4E-3, At1g29550; ateIFiso4E, AAB66906; nteIF4E1, AY702653; nteIFiso4E1, AY699609; lseIF4E, AAP86602; leeIF4E, AAF70507; caeIF4E, AAR23918; zmeIF44E, AF076954; zmeIFiso4E, AF076955; taeIF4E, CAA78262; taeIFiso4E, AAA34296; oseIF4E, P48599; oseIFiso4E, P48600; nCBP, NP 197312; 4EHP, AAC18565; hseIF4E, AAH12611; sceIF4E, NP 014502. Species of origin of the sequences are indicated as follows; *Arabidopsis thaliana*, at; *Nicotiana tabacum*, nt; *Capsicum annum*, ca; *Lycopersicon esculentum*, le; *Lactuca sativa*, ls; *Zea mays*, zm; *Triticum aestivum*, ta; *Oryza sativa*, os*; Saccharomyces cerevisiae* sc; *Homo sapiens*, hs.

B. Polypeptide sequence conservation between NteIF4E1, NteIFiso4E1 and mouse eIF4E. The full-length predicted polypeptide sequences of NteIF4E1 and NteIFiso4E1 were aligned against mouse eIF4E using the clustal W algorithm. Absolutely conserved residues are shaded black. Conservative substitutions are shaded grey. Residues in the mouse crystal structure that interact with the m^7GTP cap are denoted by g. Residues important for the eIF4E-eIF4G and eIF4E-4EBP interactions are denoted by b. The mammalian phosphorylation site, Serine 209, is marked by an asterix. Numbers represent residue positions.

Figure 2. Detection of NteIF4E and NteIFiso4E in tobacco tissues.

T

A. NteIF4E and NteFiso4E were partially purified from a soluble leaf protein extract by m^7GTP sepharose chromatography. A western blot containing equal amounts of total soluble leaf protein (TP) and m^7GTP-purified protein (m^7GTP) was probed with antibodies raised against recombinantly expressed NteIF4E1 polypeptide.

B. Tissue distribution of NteIF4E and NteIFiso4E. A western blot containing 10 g of total soluble protein isolated from two week-old seedlings, roots, leaves, sepals, petals, anthers and dried pollen was sequentially probed with antibodies to NteIF4E1 and tobacco eIF4A.

Figure 3. Antisense down-regulation of NteIF4E and NteIFiso4E in transgenic tobacco.

A. Western blot analysis of NteIF4E and NteIFiso4E antisense lines. 10 g of total soluble protein, isolated from two week-old T2 generation seedlings of NteIF4E antisense lines 9 and 23 (NteIF4Eas), and NteIFiso4E antisense lines 21 and 106 (NteIFiso4Eas), were fractionated by SDS PAGE with wild-type seedling protein, blotted onto PVDF membrane and sequentially probed with antibodies to NteIF4E and tobacco eIF4A.

B. Western blot analysis of double-antisense plants. The F1 progeny from the cross between NteIF4E antisense line 23 and NteIFiso4E antisense line 21 (double) were grown under standard green house conditions with isogenic wild-type tobacco plants. 10 g of total soluble leaf protein, isolated from four wild-type plants and five double-antisense plants (50 days after sowing), were fractionated by SDS PAGE, blotted onto PVDF membrane and sequentially probed with antibodies to NteIF4E and tobacco eIF4A.

T

C. Quantification of NteIF4E (open bars) and NteIFiso4E (shaded bars) down-regulation in antisense plants. The relative abundance of NteIF4E and NteIFiso4E in NteIF4E antisense lines 9 and 23, and NteIFiso4E antisense lines 21 and 106, were quantified from figure 3a by scanning densitometry using NIH Image software (version 1.625 b7f). For NteIFiso4E, both the NteIFiso4Ea and NteIFiso4Eb bands were quantified and added together. The densitometry measurements were standardized as a percentage of wild-type levels using a calibration curve derived from a dilution series of wild-type protein. Each bar represents the mean from two measurements.

D. Quantification of NteIF4E (open bars) and NteIFiso4E (shaded bars) levels in double antisense plants. The relative abundance of NteIF4E and NteIFiso4E in the double antisense plants was quantified from figure 3B using NIH image software as in figure 3C. Each bar represents the mean relative abundance from four wild-type and five double antisense plants, respectively. Error bars represent standard error of the mean (n=5).

Figure 4. Plants depleted for both NteIF4E and NteIFiso4E exhibit a semi-dwarf phenotype.

Seeds of wild type tobacco, NteIF4E antisense line 23 (T2 generation), NteIFiso4E antisense line 21 (T2 generation) and the F1 progeny of the cross between antisense lines 23 and 21, were germinated in soil and grown under standard green house conditions with 16 hours day length until first flower.

A. Representative plants at 60 days after sowing for NteIF4E antisense line 23 (NteIF4Eas), NteIFiso4E antisense line 21 (NteIFiso4Eas) and the F1 progeny of the cross between NteIF4Eas line 23 and NteIFiso4Eas line 21 (double)

B. Growth rate of wild-type and transgenic plants. Each data point represents the mean stem height of 5 replicate plants. Error bars represent the standard error of the mean (n=6).

Figure 5. Polyribosome analysis of double antisense and wild-type plants

A. Polyribosomes were isolated from leaves (10 cm length) of 55 day-old wild-type and double-antisense plants (F1 progeny from NteIF4Eas line 23 X NteIFiso4Eas line 21 cross) and fractionated by sucrose density gradient centrifugation.

B. Average monosome/polyribosome ratio of polyribosomes isolated from double antisense and wild-type leaves. The monosome/polysome ratios were determined for four replicate double-antisense and wild-type polyribosome traces by measuring the enclosed areas under the peaks. The mean monosome abundance is expressed as a fraction of polyribosome abundance (n=5).

References

Bradford, M. M. 1976. A rapid and sensitive method for the quantification of migrogram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Brenner, C., Nakayama, N., Goebl, M., Tanaka, K., Toh-e, A. and Matsumoto, K. 1988. CDC33 encodes mRNA cap-binding protein eIF-4E of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 8: 3556-3559.

Browning, K. S. 1996. The plant translational apparatus. Plant Mol. Biol. 32: 107-144.

Browning, K..S., Webster, C., Roberts, J. K. M. and Ravel, J. M. 1992. Identification of an isozyme form of protein synthesis initiation factor 4F in plants. J. Biol. Chem. 267: 10096-10100.

Carberry, S. E., Darzynkiewicz, E. and Goss, D. J. 1991. A comparison of the binding of methylated cap analogues to wheat germ protein synthesis initiation factors 4F and (iso)4F Biochemistry 30; 1624-1627.

Carberry, S. E. and Goss, D. J. 1991. Wheat germ initiation factors 4F and (iso)4F interact differently with oligoribonucleotide analogues of rabbit alpha-globin mRNA. Biochemistry 30: 4542-4545.

Carrington, J. C. and Freed, D. D. 1990. Cap-independent enhancement of translation by a plant potyvirus 5' nontranslated region. J. Virol. 64: 1590-1597.

Church, G. M. and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. (USA) 81: 1991-1995.

Danaie, P., Altmann, M., Hall, M. N., Trachsel, H. and Helliwell, S. B. 1999. CLN3 expression is sufficient to restore G1-to-S-phase progression in *Saccharomyces*

cerevisiae mutants defective in translation initiation factor eIF4E. Biochem. J. 340: 135-141.

De Benedetti, A., Joshi-Barve, S., Rinker-Schaeffer, C. and Rhoads, R. E. 1991. Expression of antisense RNA against initiation factor eIF-4E mRNA in HeLa cells results in lengthened cell division times, diminished translation rates, and reduced levels of both eIF-4E and the p220 component of eIF-4F. Mol. Cell. Biol. 11: 5435-5445.

De Benedetti, A. and Rhoads, R. E. 1990. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. Proc. Natl. Acad. Sci. (U SA) 87: 8212-8216.

Draper, J., Scott, R., Armitage, P and Walden, R. 1988. Plant genetic transformation and gene expression. Blackwell scientific publications, Oxford, UK

Duprat, A., Caranta, C., Revers, F., Menand, B., Browning, K. S, and Robaglia C. 2002. The *Arabidopsis* eukaryotic initiation factor (iso)4E is dispensable for plant growth but required for susceptibility to potyviruses. Plant J. 2002 32: 927-934.

Gallie, D. R. 2002. Protein-protein interactions required during translation. Plant Mol. Biol. 50: 949-970.

Gallie, D. R. 2001. Cap-independent translation conferred by the 5' leader of tobacco etch virus is eukaryotic initiation factor 4G dependent. J. Virol. 75: 12141-12152.

Gallie, D. R. and Browning, K. S. 2001. eIF4G functionally differs from eIFiso4G in promoting internal initiation, cap-independent translation, and translation of structured mRNAs. J. Biol. Chem. 276: 36951-36960.

Gallie, D. R. 1998. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. Gene 216: 1-11.

Gallie, D. R., Le H, Tanguay, R. L and Browning, K. S. 1998. Translation initiation factors are differentially regulated in cereals during development and following heat hock in wheat. Plant J. 14: 715-722.

Gingras, A. C., Raught, B. Sonenberg, N. 2004. mTOR signaling to translation. Curr. Top. Microbiol. Immunol. 279: 169-197.

Gingras, A. C., Raught. B. and Sonenberg,N. 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Ann. Rev. Biochem. 68: 913-963.

Harlow, E. and Lane, D. 1988. Antibodies a Laboratory manual. Cold Spring Harbor Laboratory press, Cold spring Harbor, NY.

Hofgen, R. and Willmitzer, L. 1988. Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res. 16: 9877.

Honys, D., Combe, J. P., Twell, D. and Capková, V. 2000. The translationally repressed pollen-specific *ntp303* mRNA is stored in non-polysomal mRNPs during pollen maturation. Sex. Plant Reprod. 13: 135-144.

Jankowska-Anyszka, M., Lamphear, B. J., Aamodt, E. J., Harrington, T., Darzynkiewicz, E., Stolarski, R. and Rhoads, R. E. 1998. Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in *Caenorhabditis elegans* can distinguish between mono- and trimethylated mRNA cap structures. J. Biol. Chem. 273, 10538-10542.

Keiper, B. D., Lamphear, B. J., Deshpande, A. M., Jankowska-Anyszka, M., Aamodt, E. J., Blumenthal, T. and Rhoads, R. E. 2000. Functional characterization of five eIF4E isoforms in *Caenorhabditis elegans*. J. Biol. Chem. 275: 10590-10596.

Lazaris-Karatzas, A., Montine, K. S. and Sonenberg, N. 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature 345: 544-547.

Marcotrigiano, J., Gingras, A. C., Sonenberg, N. and Burley, S. K. 1997. Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP Cell 89: 951-961.

Maroney, P. A., Denker, J. A., Darzynkiewicz, E., Laneve, R. and Nilsen, T. W. 1995. Most mRNAs in the nematode *Ascaris lumbricoides* are trans-spliced: a role for spliced leader addition in translational efficiency. RNA 1: 714-723.

Mascarenhas, J. P. 1990. Gene activity during pollen development**.** Ann. Rev. Plant. Phys. Mol. Biol. 41: 317-338.

Miyoshi, H., Dwyer, D. S., Keiper, B. D., Jankowska-Anyszka ,M., Darzynkiewicz, E. and Rhoads, RE 2002. Discrimination between mono- and trimethylated cap structures by two isoforms of *Caenorhabditis elegans* eIF4E. EMBO J. 21: 4680-46890.

Morley, S. J. and Traugh ,J. A. 1990. Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters. J. Biol. Chem. 265: 10611-10616.

Owttrim, G. W., Mandel, T., Trachsel, H., Thomas A. A. and Kuhlemeier C. 1994. Characterization of the tobacco eIF-4A gene family. Plant Mol. Biol. 26:1747-1757.

Pestova, T.V. and Hellen, C. U. 2001. Functions of eukaryotic factors in initiation of translation. Cold Spring Harb. Symp. Quant. Biol. 66: 389-396.

Popescu S. C. and Tumer N. E. 2004. Silencing of ribosomal protein L3 genes in N. tabacum reveals coordinate expression and significant alterations in plant growth, development and ribosome biogenesis. Plant J. 39: 29-44.

Petracek, M. E., Dickey, L. F., Huber, S. C., Thompson W. F. 1997. Light-regulated changes in abundance and polyribosome association of ferredoxin mRNA are dependent on photosynthesis. Plant Cell 9: 2291-2300.

Ptushkina ,M., Malys, N. and McCarthy, J. E. 2004. eIF4E isoform 2 in *Schizosaccharomyces pombe* is a novel stress-response factor. EMBO Rep. 5: 311-316.

Ptushkina, M., von der Haar, T., Karim, M. M., Hughes, J.M. and McCarthy, J.E. 1999. Repressor binding to a dorsal regulatory site traps human eIF4E in a high cap-affinity state. EMBO J. 18: 4068-4075.

Raught, B. and Gingras, A. C. 1999. eIF4E activity is regulated at multiple levels. Int. J. Biochem. Cell Biol. 31: 43-57.

Rodriguez, C. M., Freire, M. A., Camilleri, C., Robaglia, C. 1998. The *Arabidopsis thaliana* cDNAs coding for eIF4E and eIF(iso)4E are not functionally equivalent for yeast complementation and are differentially expressed during plant development. Plant J. 13: 465-473.

Rousseau, D., Gingras, A. C., Pause, A. and Sonenberg, N. 1996. The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth. Oncogene 13: 2415-2420.

Sonenberg, N. and Dever, T. E. 2003. Eukaryotic translation initiation factors and regulators. Curr. Opin. Struct. Biol.13: 56-63.

Sonenberg, N., Gingras, A. C. 1998. The mRNA 5' cap-binding protein eIF4E and control of cell growth. Curr. Opin. Cell Biol. 10: 268-275.

Sweetman, J., Spur, C, Eliassson, A., Gass, N. Steinmeetz, A. and Twell, D. 2000. Isolation and characterization of two pollen-specific LIM domain protein cDNAs from *Nicotiana tabacum*. Sex. Plant Reprod. 12: 339-345.

Swofford, D.L. (2003) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. (Sunderland, MA: Sinauer Associates).

Wittmann, S., Chatel, H., Fortin, M. G. and Laliberte. J. F. 1997. Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of *Arabidopsis thaliana* using the yeast two-hybrid system. Virology 234: 84-92.