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A pollen-specific DEAD-box protein related to translation initiation factor eIF-4A from tobacco

Karl A. Brander and Cris Kuhlemeier*
*Institute of Plant Physiology, University of Berne, Altenbergrain 21, CH-3013 Berne, Switzerland (*author for correspondence)*

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Abstract

A pollen-specific sequence, *NeIF-4A8*, has been isolated from a cDNA library from mature pollen of *Nicotiana tabacum* cv. Samsun. *NeIF-4A8* is a full-length cDNA whose deduced amino acid sequence exhibits high homology to the eucaryotic translation initiation factor eIF-4A from mouse, *Drosophila* and tobacco. eIF-4A is an RNA helicase which belongs to the supergene family of DEAD-box proteins. Northern blot analysis with a gene-specific probe showed strict anther-specific expression of *NeIF-4A8* starting at microspore mitosis. With antibodies raised against tobacco eIF-4A the presence of abundant eIF-4A-related proteins in developing anthers and pollen grains was demonstrated. The genomic analysis shows that the coding region is split by three introns whereas a large, fourth intron is situated in the 5′-untranslated region. A promoter construct with 2137 bp of upstream sequence fused to the GUS reporter gene was used to confirm that the expression is confined to the haploid cells within the anther. NeIF-4A8 is a prime candidate for mediating translational control in the developing male gametophyte.

Introduction

The life cycle of higher plants is characterized by the alteration between a diploid generation (sporophyte), and a haploid generation (gametophyte). The haploid male gametophyte or pollen grain is formed within diploid sporangia, the anthers. The meiotic division of pollen mother cells leads to the differentiation of haploid microspores in a tetrad. After release from the tetrad these free unicellular microspores develop further and undergo a haploid mitosis [25, 7]. In tobacco, this leads to mature pollen grains which enclose in their strong and durable wall two cells, the generative and the vegetative cell. The generative cell undergoes a second mitotic division during pollen tube growth.

During the production of pollen, two distinct sets of transcripts appear in the developing male gametophyte [reviewed 21]. The first set (early genes) is present during microspore development, reaching a maximal level at the haploid mitosis

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X79004 (NeIF-4A8 cDNA) and X79005 (NeIF-4A8 genomic).

and then decreasing until pollen maturity. The second set (late genes) appears with this mitosis and increases in its mRNA amount until maturity. This second set includes mainly pollen-specific genes whose transcripts are thought to be translated during pollen germination and pollen tube growth. It has been estimated that about 10% of the transcripts expressed in pollen are pollen-specific. Several attempts have been made to isolate such genes [reviewed in 21 and 39].

It has been shown by Mascarenhas *et al.* [24] that proteins synthesized early during pollen germination are translated from mRNAs that were previously stored in the mature pollen grain. Inhibitor studies demonstrated that germination of pollen grains and early pollen tube growth are dependent on translation but not on transcription [reviewed in 18, 6]. During this period at least 230 distinguishable new proteins are synthesized which did not exist in the mature pollen grain [19].

We are interested in the protein factors involved in the initiation of translation. In particular, we have focused on the translation initiation factor eIF-4A, a 46 kDa protein that possesses RNA unwinding activity. It is part of the cap-binding complex and functions in the removal of secondary structure in the mRNA leader. Such RNA unwinding is thought to be necessary in order to enable the ribosome to scan the mRNA leader until it reaches the initiator AUG [reviewed in 26].

Recently Owttrim *et al.* [28] reported the first characterization of plant *eIF-4A* genes in *Nicotiana plumbaginifolia*. Two of the encoded proteins, NeIF-4A1 and NeIF-4A2 were virtually identical, whereas the third one, NeIF-4A3 is only 64% identical to NeIF-4A2. These NeIF-4As show no more identity with each other at the amino acid level than they do with mouse eIF-4AI (73%) and yeast TIF1/2 (63%). Recent experiments in our laboratory indicate that at least 10 different *eIF-4A* genes are expressed in tobacco leaves [29]. All cDNAs identified so far are more or less coordinately expressed and mRNAs could be detected in all organs. Polyclonal antibodies raised against rabbit reticulocyte eIF-4A cross-reacted with *NeIF-4A2* overexpressed in *Escherichia coli* and *vice versa*. Anti tobacco eIF-4A antibodies react with eIF-4A from rabbit reticulocytes. This again indicates that NeIF-4A1, 2 and 3 are tobacco homologues of mammalian and yeast eIF-4A.

Translation initiation factor eIF-4A belongs to a large superfamily of proteins which have a number of amino acid sequence motifs in common. These so-called DEAD-box proteins which have been isolated from many different species [reviewed in 32] are not necessarily involved in translation initiation. Based on the sequence homology to eIF-4A they are thought to be RNA helicases, although they have not been characterized biochemically. Of particular interest are the *Drosophila vasa* gene [15] and the mouse PL10 gene [16], which are specifically expressed in the germ line. Mutation of *vasa* leads to a maternal effect phenotype, indicating that it has an important function early in development. So far in plants no homologies of this subfamily of putative RNA helicases have been isolated.

In order to find DEAD-box type genes that are specifically expressed in the male germ line of tobacco, we constructed a cDNA library from mature tobacco pollen and screened it at low stringency with a tobacco *eIF-4A* cDNA probe. Here we describe the isolation of the *NeIF-4A8* cDNA and the corresponding genomic clone. We show that *NeIF-4A8* is closely related to the previously identified *NeIF-4A2*, yet its expression could be detected exclusively late in pollen development. We suggest that this sex- and cell-specific translation initiation factor could have a regulatory function during postmitotic pollen development and perhaps during pollen germination. The results are discussed in relation to the unique development of pollen and to germ line-specific DEAD-box proteins.

Materials and methods

Plant material

Plants of *Nicotiana tabacum* cv. Samsun were either grown under sterile conditions on MS me-

dium supplemented with antibiotics or in soil under greenhouse conditions. Plant tissue was collected and, if necessary, stored at −80 °C. Flower buds were staged and collected by measuring flower bud length as described by Koltunow *et al.* [14] and dissected flower organs were frozen in liquid N_2 and stored at −80 °C. Developmental stages 1 to 11 were collected as intact anthers whereas mature pollen grains were separated from the dehiscent anthers by vortexing or for RNA extractions by washing off quickly with cold 0.3 M mannitol prior to storage at −80 °C.

Extraction of mRNA and Northern blot analysis

RNA was extracted from various tissues and from mature pollen grains essentially as described by Schrauwen *et al.* [33]. The isolation of poly(A) RNA from total RNA of mature pollen of *Nicotiana tabacum* cv. Samsun was essentially as described in Sambrook *et al.* [31]. RNA either was stored under ethanol, as a dry pellet, or as an aqueous solution at −80 °C. Samples of glyoxal denatured total RNA (10 μg) were electrophoresed through 1.2% agarose gels and further treatment was essentially as described by Bucher and Kuhlemeier [5]. Northern blots hybridized with oligonucleotides were washed with SSC buffers ranging from 2 × to 0.1 × in concentration, containing 0.1% SDS. Each washing step was for 5 min. The last washing step after a hybridization at 45 °C was 5 min at 39 °C with 0.1 × SSC / 0.1% SDS. Blots hybridized with riboprobes were hybridized overnight at 65 °C. Blots were washed as described above, except the washing steps were prolonged and the last washing step was at 65 °C with 0.1 × SSC / 0.1% SDS. The relative loads of RNA on Northern blots were routinely checked by staining with methylene blue.

Generation of a pollen cDNA library and library screening

For the generation of a cDNA library from mature pollen of *Nicotiana tabacum* cv. Samsun, a Pharmacia cDNA Synthesis Kit (Pharmacia LKB) was used according to the manufacturer's instructions. Four μg of poly(A) RNA were used to synthesize the cDNA. *Eco* RI-ended cDNAs were ligated into predigested λ ZAPII/*Eco* RI arms using a λ ZAPII/*Eco* RI Cloning Kit (Stratagene, La Jolla, CA). Gigapack II Gold Packaging Extract (Stratagene) was used for packaging the recombinant phage DNA. The library subsequently was titered and amplified once as described in the manufacturer's instructions (Stratagene). 3.5×10^5 pfu of the amplified library were used for screening on duplicate plaque filters at 48 °C with a ^{32}P-labelled fragment of *NeIF-4A2* cDNA [28] encompassing the region from the GKT motif to the DEAD-box. Positive clones were isolated and the inserts were visualized in agarose gels after digestion with *Eco* RI. Clones of interest were subsequently sequenced and the sequences were compared to all tobacco eIF-4As isolated so far and to other members of the DEAD-box family [32].

About 1×10^6 pfu of a λEMBL3 genomic library of *Nicotiana tabacum* cv. Samsun (kindly provided by R. Fluhr, Rehovot, Israel) were screened in the same manner as the cDNA library, except that the full-length *NeIF-4A2* cDNA was used as ^{32}P-labelled probe. Isolation of clones of interest was supported by Southern blot analysis. One clone was further analysed by DNA sequencing and subsequently compared to *NeIF-4A8* as well as to other known *eIF-4As*.

DNA sequencing was performed by the dideoxy chain termination method. For this purpose either subcloned fragments of the *NeIF-4A8* cDNA, or the genomic clone, or nested deletions of the genomic clone were used. Analyses of the DNA sequence and predicted amino acid sequence were performed using the University of Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package, version 7.

Preparation of constructs

2137 bp sequence of *NeIF-4A8* upstream of the ATG was ligated to the GUS-coding region via

an oligonucleotide mediated introduction of a *Nco* I site over the ATG. This changed the sequence in Fig. 1 as indicated by double underlining: 5′-TAAAGTACGTACATTACAGCC-ATGGAGCT-3′. A 970 bp fragment of the 35S cauliflower mosaic virus (CaMV) promoter was fused to the GUS-coding region, and the NOS 3′ sequence used as the 3′- terminator. The constructs were transferred into the binary vector pMON505 [11].

Transformation of tobacco

Prior to the transformation of tobacco, *Agrobacterium tumefaciens* (LBA4404, kindly provided by R. Vögeli-Lange, Basel) was transformed via triparental mating [11]. After selection for transformed *Agrobacterium* mutant strains, positive clones were grown on solid medium, scratched off with a needle and leaf disks were inoculated with *Agrobacterium* by wounding. Sterile plants were regenerated from leaf discs on selective MS medium as described by Draper *et al.* [11].

Protein analysis

Total soluble protein was extracted from plant material in a buffer consisting of 100 mM Tris pH 7.5, 0.1% 2-mercaptoethanol, 0.2% PVP, 5% PVPP, directly in a 1.5 ml microfuge tube with a pestle fitting the tube (Kontes) and an electric drill. Aliquots of 10 μg were separated through a 12% SDS-PAGE gel and electroblotted onto nitrocellulose (Schleicher and Schuell). Western analysis was performed as described by Bucher and Kuhlemeier [5] except that the first antibody was polyclonal rabbit anti-tobacco eIF-4A. Immuno-reacting polypeptides were revealed using goat anti-rabbit linked peroxidase.

Analysis of GUS activity

Transgenic plants containing promoter-GUS constructs were analysed qualitatively for the presence of GUS activity in various tissues and cell types by histochemical analysis [17]. Pollen and hand-cut sections (ca. 1 mm) of fresh tissues were immediately immersed in 50–500 μl of fresh of X-Gluc substrate solution (1 mM 5-bromo-5-chloro-3-indolyl-β-*D*-glucuronic-acid [Biosynth], 50 mM sodium phosphate buffer pH 7.5) in a microwell plate (Nunc). The substrate solution was supplemented with 0.5% Triton X-100, 2.5 μM $Fe^{2+}CN$ and 2.5 μM $Fe^{3+}CN$. In our hands the addition of 20% methanol [17] did not affect the suppression of artifactual blue colour. Incubations were at 37 °C for 0.5 to 8 h. After incubation, the sections were cleared, if necessary, from chlorophyll by consecutive incubation in the following solutions: 10 min FAA (5% formaldehyde, 5% acetic acid, 38% EtOH), 3 min 50% EtOH, 100% EtOH until tissue was white, then 3 min in H_2O. Sections and pollen grains were analysed either under a microscope or under a binocular microscope equipped with a polarization filter and documented by colour slides.

Fluorometric analysis was used to quantitate GUS activity in different tissues [13]. Protein was extracted from fresh tissue by homogenizing about 100 mg tissue or pollen grains from 3 to 5 flowers directly in a microfuge tube containing 100 μl pre-cooled extraction buffer consisting of 50 mM sodium phosphate buffer pH 7, 1 mM EDTA, 10 mM 2-mercaptoethanol and 0.1% Triton X-100. The homogenizer was washed with 700 μl extraction buffer which was combined with the homogenate on ice. The cell debris was pelleted for 5 min at room temperature and the protein concentration in the clarified supernatant was determined with the BioRad protein assay. For the enzymatic reaction, 25 μl supernatant was mixed with 250 μl MUG substrate solution (1 mM 4-methylumbelliferyl glucuronide [Serva] in extraction buffer). Samples were incubated in a 37 °C water bath for 45 min. The reaction was terminated by adding 1.725 ml 0.2 M Na_2CO_3. Fluorescence at 460 nm was measured in a TKO 100 mini-fluorometer (Hoefer) or SFM 25 (Kontron) at an excitation of 365 nm. The values were corrected for intrinsic degradation of the substrate (MUG) itself over the incubation time of 45 min

at 37 °C which was 0.03 nmol MU per minute per mg protein, and which was exactly the same activity as in wild-type tissues and for tissues other than pollen of the transformants.

Results

Generation of a pollen cDNA library and screening for eIF-4A

A cDNA library derived from poly(A) RNA from mature pollen grains of *Nicotiana tabacum* cv. Samsun was generated, resulting in 2.2×10^5 independent clones. The amplified library was screened with an internal fragment of the isolated cDNA *NeIF-4A2*, coding for *N. plumbaginifolia* eIF-4A [28]. This fragment encompassed a region (GKT motif to DEAD-box) which has high homology in all *eIF-4As* isolated so far from tobacco. The longest clone with a 1.5 kb insert was further analysed. Subsequent sequencing and sequence comparisons confirmed that this clone contained the entire protein coding region. This clone, termed *NeIF-4A8*, was 1497 bp in length, consisting of 48 bp 5′-untranslated region and 210 bp 3′-untranslated region (Fig. 1). The deduced amino acid sequence (Fig. 1) shows strong homology throughout the amino acid sequence to NeIF-4A2 [28] as well as to other members of the DEAD-box supergene family [32, 4]. All five conserved boxes that are characteristic for the DEAD-box family (GKT, PTRELA, DEAD, SAT and HRIGR) are present. In addition, a second SAT-box is located between the conserved SAT-box and the HRIGR-box. This second SAT-box at this position is observed only in plants so far [4].

The deduced amino acid sequence of the cDNA of NeIF-4A8 (Fig. 1) is a 413 amino acid open reading frame that shares 93% identity with NeIF-4A2 [28] and 68% with the mouse eIF-4AI [27]. The protein of NeIF-4A8 has a calculated molecular mass of 46.9 kDa and an estimated pI of 6.1. Through sequence comparison with other members of the *NeIF-4A* gene family in tobacco [28, 29], the 5′-untranslated region of *NeIF-4A8*

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1    GTTTAATTCTCAGTTTTGCTAAAGTTATTATCATCGTCATTCCGAGTCATGGCACGTTTG   60
                                                  M  A  R  L
61   GCACCAGATGGAGCTCAATTTGATGCCCGTCAATATGATTCTAAGATGAACGATTTACTT  120
     A  P  D  G  A  Q  F  D  A  R  Q  Y  D  S  K  M  N  D  L  L
121  GCCGCTGATGGAAAAGATTTCTTTACATCATATGACGAAGTTTATGACAGTTTTGATGCT  180
     A  A  D  G  K  D  F  F  T  S  Y  D  E  V  Y  D  S  F  D  A
181  ATGGGTCTGCAAGAGAACCTTCTCAGGGGCATTTATGCCTATGGTTTTGAGAAACCTTCT  240
     M  G  L  Q  E  N  L  L  R  G  I  Y  A  Y  G  F  E  K  P  S
241  GCAATTCAACAAAGAGGTATAGTTCCATTTTGCAAGGGACTTGATGTAATTCAGCAGGCT  300
     A  I  Q  Q  R  G  I  V  P  F  C  K  G  L  D  V  I  Q  Q  A
301  CAGTCTGGCACAGGAAAAACAGCTACTTTTTGTTCTGGAATTTTGCAGCAGCTTGATTAT  360
     Q  S  G  T  G  K  T  A  T  F  C  S  G  I  L  Q  Q  L  D  Y
361  GGTTTAGTTCAATGTCAAGCGTTGGTGTTAGCACCTACTCGTGAACTTGCTCAACAGATT  420
     G  L  V  Q  C  Q  A  L  V  L  A  P  T  R  E  L  A  Q  Q  I
421  GAGAAGGTGATGCGAGCACTTGGTGACTACCTTGGGGTTAAGGTCCATGCTTGTGTAGGT  480
     E  K  V  M  R  A  L  G  D  Y  L  G  V  K  V  H  A  C  V  G
481  GGGACTAGTGTCAGGGAGGATCAACGTATTCTCGCAGCTGGTGTTCATGTTATTGTTGGC  540
     G  T  S  V  R  E  D  Q  R  I  L  A  A  G  V  H  V  I  V  G
541  ACCCCTGGACGTGTGTTTGACATGCTGCGAAGACAGTCTCTCCGTCCTGATTACCTCAGA  600
     T  P  G  R  V  F  D  M  L  R  R  Q  S  L  R  P  D  Y  L  R
601  ATGTTTGTGCTAGACGAGGCTGATGAAATGCTGTCACGTGGTTTTAAGGATCAGATATAT  660
     M  F  V  L  D  E  A  D  E  M  L  S  R  G  F  K  D  Q  I  Y
661  GATATTTTTCAGATGCTGCCTACAAAAGTCCAAGTCGGAGTGTTTTCTGCGACCATGCCA  720
     D  I  F  Q  M  L  P  T  K  V  Q  V  G  V  F  S  A  T  M  P
721  CCAGAAGCCCTTGACATCACAAGAAAGTTCATGAATAAGCCCGTGAGAATCTTGGTTAAA  780
     P  E  A  L  D  I  T  R  K  F  M  N  K  P  V  R  I  L  V  K
781  CGCGATGAATTGACACTTGAGGGTATCAAACAGTTTTATGTCAATGTTGATAAGGAGGAA  840
     R  D  E  L  T  L  E  G  I  K  Q  F  Y  V  N  V  D  K  E  E
841  TGGAAGCTCGAGACACTCTGCGATCTATACGAGACGCTAGCAATTACACAGAGTGTCATA  900
     W  K  L  E  T  L  C  D  L  Y  E  T  L  A  I  T  Q  S  V  I
901  TTTGTGAACACCAGGCGCAAGGTTGATTGGTTAACAGACAAAATGCGAACGCGTGATCAC  960
     F  V  N  T  R  R  K  V  D  W  L  T  D  K  M  R  T  R  D  H
961  ACAGTCTCAGCTACACATGGAGATATGGACCAGAACACTAGGGACATAATCATGCGCGAG 1020
     T  V  S  A  T  H  G  D  M  D  Q  N  T  R  D  I  I  M  R  E
1021 TTTCGCTCTGGTTCTTCTCGTGTCCTTATCACAACCGATCTGTTGGCTCGTGGTATAGAT 1080
     F  R  S  G  S  S  R  V  L  I  T  T  D  L  L  A  R  G  I  D
1081 GTACAACAAGTATCACTTGTGATCAACTATGATCTCCCGACTCAGCCAGAGAATTATCTC 1140
     V  Q  Q  V  S  L  V  I  N  Y  D  L  P  T  Q  P  E  N  Y  L
1141 CATCGTATTGGAAGAAGTGGAAGGTTTGGAAGGAAAGGAGTTGCTATCAACTTTGTGACA 1200
     H  R  I  G  R  S  G  R  F  G  R  K  G  V  A  I  N  F  V  T
1201 ACAGACGACGAAAGAATGTTGTTCGATATTCAGAAGTTTTACAACGTGATAATCGAAGAA 1260
     T  D  D  E  R  M  L  F  D  I  Q  K  F  Y  N  V  I  I  E  E
1261 CTCCCCTCAAATGTTGCTGATCTCCTCTGAAAAACTTATGTTTGTGGCTGGAGCTTTAAG 1320
     L  P  S  N  V  A  D  L  L  #
1321 CAGAAAGAGTAACCATGTTATAGTGTCCACACCATTCCATAGAAATTAGTCTTTTAACTA 1380
1381 CCATTATCATCTAGTGTCTAAGACAGACCTGAGGCAGTAATGCTGCTCCAAATTTTGCAG 1440
1441 TAGCACATTTTGTTTTTCATTTCCTAATTCTTAAACTTCTTTGGCATTTTGTAGTTT     1497
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of the tobacco NeIF-4A8 cDNA. The five characteristic conserved motifs of the DEAD-box family are underlined. * denotes positions of introns; # end of coding region (TGA). The 5′ UTR is double-underlined. The deduced amino acid sequence in one-letter code is below the nucleotide sequence.

(double-underlined), encompassing the position 1 to 48 (Fig. 1) was determined to be unique for *NeIF-4A8*.

Northern blot analysis

The DNA sequence of *NeIF-4A8* is highly homologous to the other members of the *NeIF-4A*

family, except for *NeIF-4A3*. Also in the 3′ noncoding region there are considerable stretches of sequence identity. In the 5′UTR, however, no sequence homology with other *eIF-4A* genes could be found. In order to distinguish the *NeIF-4A8* mRNA from other *eIF-4A* mRNAs, an oligonucleotide was synthesized encompassing 36 bp of the 5′UTR (position 9 to 44, Fig. 1) to be used as a gene-specific probe for *NeIF-4A8*. It now was possible to specifically measure *NeIF-4A8* mRNA levels. An antisense riboprobe made of the coding region of a constitutively expressed *eIF-4A* gene, *NeIF-4A2* [4] was used as a (internal) control for northern blot analysis. This latter probe has high sequence homology to all known members of the *NeIF-4A* family including *NeIF-4A8*. Northern blots were prepared as described in Materials and methods with equal amounts of total RNA isolated from different tobacco organs. In order to obtain direct comparisons, northern blots were first hybridized with the *NeIF-4A8* gene-specific oligonucleotide and, after exposure of the blot to the X-ray film, the blot was stripped. Subsequently, the *NeIF-4A2* probe was used for detection of all *eIF-4A*-related transcripts.

The gene-specific oligonucleotide for *NeIF-4A8* gave a strong hybridization signal exclusively in mRNA preparations containing pollen RNA (Fig. 2). Also after overexposure (not shown) signals only could be observed in mRNA preparations from anthers at stage 11 [14] and from mature pollen (Fig. 2). In contrast to this pollen-specific pattern, the *NeIF-4A2* probe which detects all *NeIF-4A* species, hybridizes to transcripts in mRNA from all organs of tobacco.

Because *NeIF-4A8* expression is exclusively detectable in pollen we were interested in the determination of the time course of *NeIF-4A8* expression during pollen development (Fig. 3). Therefore both probes, *NeIF-4A8*-specific and general *NeIF-4A* probe, were hybridized to a blot containing mRNA from 12 different stages of pollen development which range from meiotic microspores (stage 1) up to mature pollen (stage 12). The staging of pollen development was according to Koltunow *et al.* [14] and the mRNAs were prepared from whole anthers except for mature pollen which were collected after anthesis and thus contained only male gametophytic material. The *NeIF4A2* probe which hybridizes to all *NeIF-4A* mRNAs, detects a single mRNA species at all stages of anther development. There is an increase at stage 6 (haploid microspore mitosis) in agreement with other observations that document a general activation of transcription at this stage [33]. The *NeIF-4A8* gene-specific probe hybridized only to transcripts from anthers that have passed microspore mitosis (Fig. 3). Interestingly, a decrease of the hybridization signal can be observed after stage 10, which is not usual for *late*

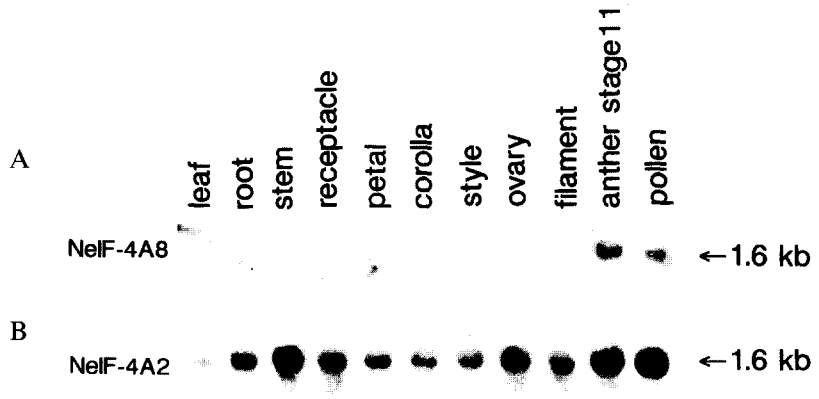

Fig. 2. Expression of NeIF-4A8 is restricted to anthers and mature pollen grains. A. Northern blot with total RNA from different organs of tobacco as indicated at the top of each line. 10 μg of total RNA was loaded per lane and hybridized with the gene-specific oligonucleotide for NeIF-4A8. B. The same blot as in A after it has been stripped. The blot was hybridized with the NeIF-4A2 riboprobe as an internal control in order to detect all tobacco eIF-4A like transcripts.

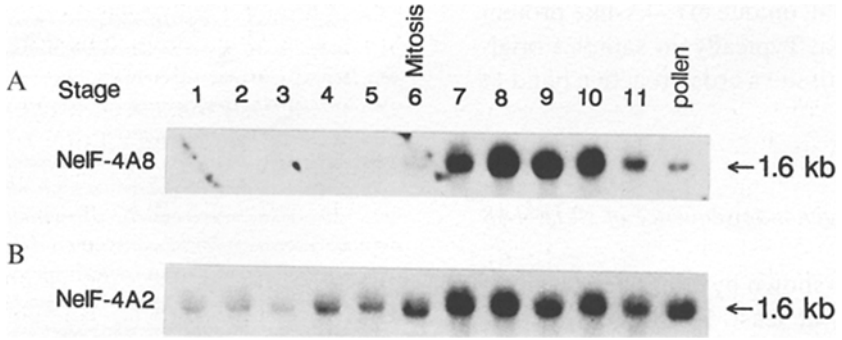

Fig. 3. Expression of NeIF-4A8 is first detectable at microspore mitosis. Total RNA was isolated from tobacco anthers, except that the lane pollen contains total RNA from mature pollen grains only (stage 12). Stage 6 corresponds to microspore mitosis as indicated. A. Northern blot containing 10 μg total RNA from intact anthers of the stages 1 to11 and from mature pollen grains. The probe was the gene-specific oligonucleotide for NeIF-4A8. B. The same blot as A after stripping and after hybridization with the NeIF-4A2 riboprobe as internal control in order to detect all tobacco eIF-4A like transcripts.

genes. Thus, *NeIF-4A8* belongs to the group of *late genes* [36] that are only expressed after microspore mitosis and that play a role during the development of binucleate pollen and possibly during pollen germination. Because in all RNA isolations, except for the lane 'pollen' (mature pollen only), the RNA was isolated from complete anthers, the results of Fig. 3 do not show whether the detected *NeIF-4A8* expression is specific for a particular cell type within the anther.

Immunological characterization of eIF-4A proteins

Messenger RNAs of the late group may not be translated immediately. They can be stored as transcripts and be used as templates for translation during a later stage in the pollen life cycle, such as pollen germination and pollen tube growth [reviewed in 23]. Therefore we verified the possible presence of eIF-4A protein in pollen grains via Western blotting. Total protein from leaf, anthers before and after microspore mitosis, as well as from mature pollen of tobacco were extracted and equal amounts of soluble total protein were separated on SDS-PAGE gels and transferred to nitrocellulose. A polyclonal antibody directed against tobacco eIF-4A reacted with several proteins in the range from 45 to 48 kDa, indicating that eIF-4A protein is present in all tissues analysed (Fig. 4). In pollen two major and one minor band, which only differ marginally in size, show eIF-4A to be an abundant protein in mature pollen grains. NeIF-4A immuno-reactive proteins from anthers before microspore mitosis are slightly smaller than after mitosis. In premitotic

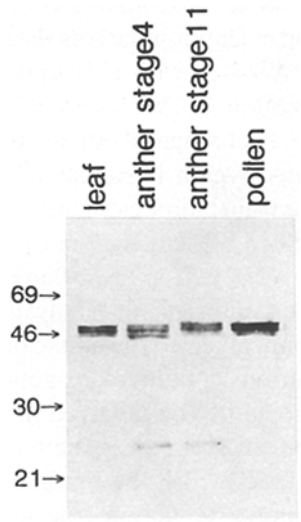

Fig. 4. Western blot analysis for eIF-4A. Total protein from different organs and anthers containing microspores at different developmental stages as indicated. 10 μg protein of each preparation was loaded and separated on 12% SDS-polyacrylamide gel. Immuno-detection was performed with rabbit antibodies against *Escherichia coli* recombinant tobacco NeIF-4A2. Numbers on the left indicate the molecular mass in kDa.

anthers an additional, unique eIF-4A-like protein is present at 45 kDa. Typically, in samples originating from anther tissue a cross-reactive band at 25 kDa is present.

Identification of the genomic sequence of NeIF-4A8

We have previously shown by characterization of cDNA clones that at least 10 different *eIF-4A* genes are expressed in tobacco leaves [29]. We also screened a λEMBL3 genomic library of *Nicotiana tabacum* cv. Samsun and characterised four *NeIF-4A* genes [4]. One of these genes corresponds in sequence to the *NeIF-4A8* cDNA isolated from the pollen library. The major part of this clone consists of the complete coding sequence, ca. 4.4 kb of 5′-flanking region and 386 bp of 3′-flanking region. The coding part of the nucleotide sequence shows 100% identity to *NeIF-4A8* cDNA. The region coding for *NeIF-4A8* is split by 3 introns of various length into 4 exons as indicated by asterisks in Fig. 1 [4]. A fourth intron is situated within the 5′-untranslated region at position −826 to −2 (Fig. 5). This intron of 825 bp in length separates the gene-specific 5′-region of *NeIF-4A8* (double-underlined) from the coding region of *NeIF-4A8* at position −2 (Fig. 5). The occurence of an intron within the 5′-untranslated region is similar to the situation found for the translation elongation factor *eEF-1* in *Arabidopsis* [8, 9] and soybean [1].

Sequencing of 2137 bp of 5′-flanking region of *NeIF-4A8* and subsequent analysis of the sequence not only revealed the first exon (= 5′UTR) but also various putative *cis*-acting elements which are thought to be involved in gene expression in pollen. In Fig. 5 only the motifs are indicated (bold face) which, by mutational analysis, have been shown to play a role in pollen gene expression [38]. In *NeIF-4A8* the pollen-box motifs (PB) were identified as at least 85% matches to the core motif at seven positions (Fig. 5). Interestingly an additional eighth PB motif which is 100% identical to the consensus sequence is situated within the first intron at position −291 (Fig. 5). Other *cis*-acting elements like the 52/56

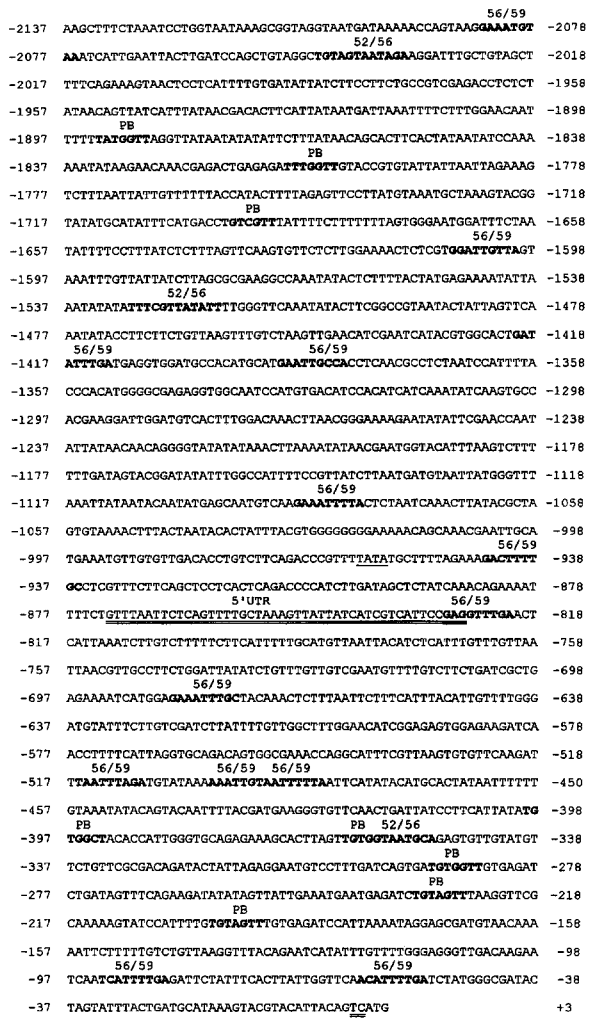

Fig. 5. Nucleotide sequence of the 5′-flanking region in the NeIF-4A8 genomic clone. Positions +1 to +3 are the translation start codon ATG, the double underlined sequence is the first exon which corresponds to the 5′UTR. The putative TATA-box is underlined and *cis*-acting elements involved in pollen gene expression (PB; 52/56; 56/59) are in bold face as indicated in the superscript.

motif is present three times as 75% matches and the 56/59 motif could be identified 13 times as 77% matches [38]. Interestingly, 13 out of these 24 putative *cis*-acting elements for pollen gene expression situated in the 5′-flanking region, reside within the first intron. This may indicate that the intron possesses regulatory functions for *NeIF-4A8* gene expression, as it has been demonstrated for *eEF-1α* [9].

Analysis of NeIF-4A8 expression with the GUS reporter gene system

In order to determine the expression pattern within the anther and to delineate the DNA sequences that control this pattern of *NeIF-4A8* gene expression, we combined the 5'-upstream region of *NeIF-4A8* with the GUS reporter gene system [13] by making an exact fusion at the ATG (Figs. 5 and 6). This construct, containing 2137 bp of upstream region was fused to GUS. The cauliflower mosaic virus 35S promoter (35S CaMV) fused to GUS was used as positive control. The resulting constructs were introduced into the same tobacco cultivar (cv. Samsun) via *Agrobacterium*-mediated gene transfer. From each construct 22 individual independent transgenic tobacco plants were grown and seeds were collected for a detailed analysis of the stably transformed F1 generation.

Several authors have recently pointed out that the GUS reporter gene system may be prone to artefacts [12, 40] especially in pollen [22, 30]. For this reason we carefully tested and calibrated both the histochemical and fluorometrical assays. The pH was set to 7.5 because lower pHs gave rise to faint histochemical GUS (-like) activity even in wild-type pollen [35], particularly in combination with 50 mM ascorbate. A pH shift up to pH 8 generally suppressed GUS activities and was therefore not suitable for plants with low expression. The addition of 20% methanol [17] did not remove endogenous activity in wild-type pollen, but gave less contrast in the blue colour of transgenic pollen grains. To prevent glucuronide leakage of the pollen grains during incubation,

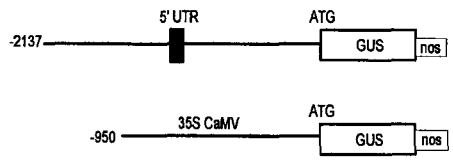

Fig. 6. GUS fusions with the upstream region of NeIF-4A8 and 35S CaMV promoter. Cartoon of the exact ATG fusions of NeIF-4A8 upstream sequence and the 35S promoter, each fused to the GUS reporter gene (not drawn to scale). The black box indicates the 5'UTR of NeIF-4A8.

addition of ferric cyanide [10] was necessary. Due to the relatively high GUS-activity in the pollen grains, the incubation times (0.5–8 h) were rather short for the −2137-GUS construct. Histochemical assays showed variations in the intensity of the blue staining of the pollen grains, probably due to the non-synchronous pollen development [33]. The direct transformants (F0) were only assayed for roughly localizing GUS expression and for optimizing histochemical and fluorometrical GUS assays. 22 direct transformants were assayed histochemically in leaves, roots, etc. GUS expression could exclusively be detected in pollen grains of these plants. For the next generation of plants (F1), five plants were chosen which had shown blue pollen in the F0 generation and 5 parallel plants were grown of each.

Plants of the F1 generation were assayed fluorometrically for GUS activity in pollen as well as in other tissues. Because of the variability within a pollen population, for the fluorometric assay pollen from 3 to 5 flowers were used for extraction and determination of GUS activity (Fig. 7) which was linear over an incubation time from 5 to over 70 min.

Through fluorometric GUS assays we could confirm the findings of the Northern blot analysis. In plants harboring the −2137-GUS construct, GUS activity could only be detected in mature pollen grains (Fig. 7A). In a closer inspection of pollen development, GUS expression was detectable starting at microspore mitosis (Fig. 7B). These findings again support the *late gene* character of pollen-specific expression of *NeIF-4A8*. 35S-GUS plants showed enzyme activity in histochemical and fluorometric assays in all organs tested, but the average GUS activity in mature pollen grains was 85 times lower than in the −2137 construct and only 3 times higher than in pollen of wild-type *Nicotiana tabacum* cv. Samsun (Fig. 7A). This low transcription from the 35S promoter in pollen is in agreement with the finding of Twell *et al.* [37].

In order to obtain not only quantitative results but also to obtain insight into the cellular specificity of GUS gene expression we performed histochemical assays. Figure 8 shows the results of

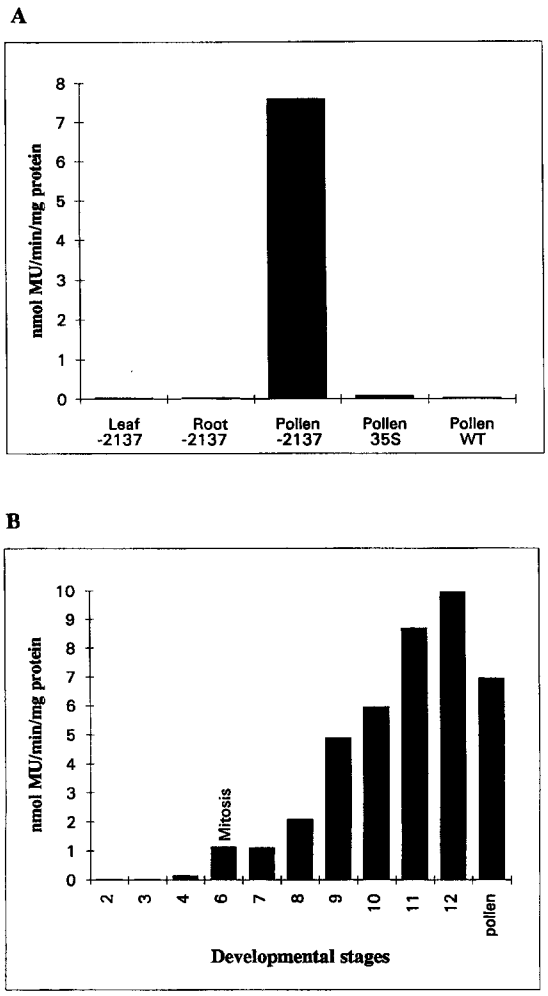

Fig. 7. GUS activity in different organs and at different stages of pollen development. A. Average GUS activity in leaves, roots and pollen of plants with the −2137-GUS, the 35S-GUS construct as well as from wild type (WT). All values represent the average enzyme activity for each construct and organ studied. The values have been corrected with the intrinsic degradation of the substrate (0.03 nmol MU per minute per mg protein). B. For representing the GUS activity in the course of pollen development one −2137-GUS plant was chosen. Whole anthers including the microspores were extracted except for lane 'Pollen' which is from mature pollen grains only. Numbers indicate the developmental stages. Microspore mitosis is at stage 6. Values were again corrected to the standard activity as described above.

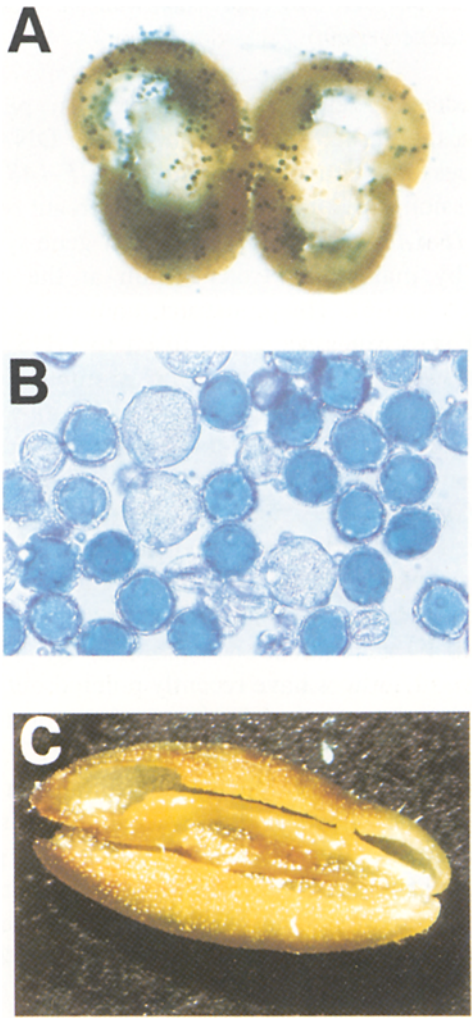

Fig. 8. Histochemical localization of GUS activity. A. Cross-section of an anther at stage 10, incubated in the substrate solution for 6 h at 37 °C. B. Pollen grains from the same plant collected just after anthesis and incubated in the substrate solution at 37 °C for 4 h. C. Anther from the same plant, cleared of pollen grains prior to an overnight incubation at 37 °C.

ananalysis of anthers from a plant containing the −2137-GUS construct. A positive, strong blue colour can be observed only in the pollen grains with a very faint signal detectable in the connective tissue (Fig. 8A). An analysis of pollen grains from the same transformant but separated from their anther prior to incubation in order to prevent artefactual blue colour into the surrounding anther tissue, caused by diffusion of the glucuronide [22], shows GUS expression to be restricted to the pollen grains (Figs. 8B, 8C). Other tissues of transgenic tobacco which were his-

tochemically assayed for GUS gene expression also did not show any blue staining (data not shown). These data are strong supporting evidence for the pollen specificity of *NeIF-4A8* gene expression.

Discussion

A cDNA library was prepared from poly(A) RNA isolated from mature pollen of *Nicotiana tabacum* cv. Samsun and screened with an internal fragment of the ubiquitously expressed *NeIF-4A2* clone [28]. This resulted in the isolation of a full-length cDNA with high sequence similarity to *NeIF-4A2* (93%) and mouse *eIF-4AI* (73%), as well as to other *eIF-4A*s. Sequence comparisons revealed that this was a novel cDNA and that it was not present in a tobacco leaf cDNA library, which we had screened extensively [29].

It has been estimated that in pollen ca. 20 000 genes are expressed of which only about 10% are pollen-specific [20]. Many genes described in the literature are expressed predominantly in the gametophyte, but on closer inspection sporophytic expression is often also observed [39]. We set out to prove rigorously that the expression of *NeIF-4A8* is limited to developing and mature pollen grains of tobacco. Northern blot analysis with a *NeIF-4A8*-specific probe showed expression only in anthers at developmental stages after microspore mitosis. When the *NeIF-4A8* promoter was fused to the GUS reporter gene, GUS activity was restricted to the pollen grains. Individual transgenic plants showed variability in the level of enzyme activity of the introduced GUS (data not shown). This fact is well documented [40] for many transgenes including the *LAT52*-GUS construct in pollen of tobacco [38]. In the case of *NeIF-4A8*, only quantative but no qualitative changes in the gene expression were observed. Thus *NeIF-4A8* gene expression was detected exclusively during late pollen development.

Several reports in the literature indicate that gene expression during male gametophyte development is at least partially under translational control. In the case of the *LAT52* gene, sequences in the 5′-untranslated region strongly enhance translation upon transient expression in pollen [39]. Could *NeIF-4A8*, a putative translation initiation factor, be itself under translational control? The fusion with the GUS reporter gene was made exactly at the initiator AUG and any possible translational control elements in the 5′UTR would therefore be present in the GUS mRNA. Comparison of Figs. 3 and 7B shows that the pattern of GUS activity closely matches the profile of mRNA expression. Thus at present there is no evidence to suggest that *NeIF-4A8* expression is controlled at the level of translation.

Polyclonal antibodies raised against a ubiquitously expressed tobacco eIF-4A reacted with several polypeptides of ca. 46 kDa in leaves, anthers and pollen. Because of the high sequence similarity between all NeIF-4As characterized to date, we assume that this antibody cross-reacts with NeIF-4A8. Some of the bands seen in Fig. 4 appear to be pollen-specific. We do not know yet, if these multiple bands reflect different gene products or if they result from post-translational modifications. If one of these protein species is the product of a separate gene, it could very well represent NeIF-4A8. It should be kept in mind, however, that although no evidence for posttranslational modification of eIF-4A has been obtained in yeast and mammalian systems, in maize it has been reported that eIF-4A can be phosphorylated [41]. We are presently testing whether NeIF-4A8 is phosphorylated in pollen.

NeIF-4A8 is highly related in sequence to canonical eIF-4A. And thus it seems reasonable to assume that it functions as a translation initiation factor. Yet its expression pattern is reminiscent of that observed for mouse PL10 [16] and *Drosophila vasa* which are germ line-specific; *vasa* is expressed in cells of both sexes but null mutants of *vasa* have no effect in the male [15]. PL10 is present with high levels of transcripts during the meiotic and haploid stages of mouse spermatogenesis. Both *vasa* and PL10 are members of the DEAD-box family which are putative RNA helicases, but to none of them a defined function has been ascribed yet.

We can envisage two different ways in which

NeIF-4A8 could function. First, its enzymatic properties could be similar to those of ubiquitously expressed eIF-4A proteins. The extra eIF-4A, represented by NeIF-4A8 would be required to meet an increased demand for eIF-4A activity. In *Xenopus* oocytes it was demonstrated by Audet *et al.* [2] that an injection of eIF-4A protein led to an increased translational activity, whereas no other translation factor could enhance protein synthesis. In the *Xenopus* experiment the eIF-4A concentration and the degree of secondary structure of the mRNA leaders could be the crucial factors determining whether the transcripts are translated or not. There is reason to believe that eIF-4A could be limiting in pollen as well. Between microspore mitosis and pollen maturity the protein content of the pollen grain rises more than threefold [42]. Extra eIF-4A could be required to sustain the high translational activity. In addition during pollen maturation the potassium ion concentration can reach values up to 280 mM at pollen maturity. Such a high concentration is suboptimal for translation [3], most likely because of an increase in RNA secondary structure [34]. This may lead to a reduced efficiency of mRNA translation that has to be counteracted by increased NeIF-4A8.

Second, NeIF-4A8 could be functionally distinct from other eIF-4A species. It could be important for the selective translation of pollen-specific mRNAs during postmitotic pollen development as well as later during pollen germination. Tobacco pollen germination is dependent on protein synthesis and only in a later stage on both transcription and translation [19]. In germinating pollen grains of *NeIF-4A8*-GUS plants, the GUS activity remained at the same high level over more than 30 h of *in vitro* germination (data not shown), as it was determined for mature pollen grains. Expression of GUS activity could also be measured after transient introduction of the *NeIF-4A8*-GUS construct into pollen via bombardment and subsequent pollen germination (data not shown). In this second scenario, NeIF-4A8 would be especially important during the early phase of pollen-pistil interaction.

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References

1. Aguilar F, Montandon P-E, Stutz E: Two genes encoding the soybean translation elongation factor eEF-1 are transcribed in seedling leaves. Plant Mol Biol 17: 351–360 (1991).
2. Audet RG, Goodchild J, Richter JD: Eukaryotic initiation factor 4A stimulates translation in microinjected *Xenopus* oocytes. Devel Biol 121: 58–68 (1987).
3. Bashe D, Mascarenhas JP: Changes in potassium ion concentrations during pollen dehydration and germination in relation to protein synthesis. Plant Sci Lett 35: 55–60 (1984).
4. Brander KA, Mandel T, Owttrim GW, Kuhlemeier C: Highly conserved genes coding for eucaryotic translation initiation factor eIF-4A of tobacco have specific alterations in functional motifs. Biochim Biophys Acta, in press (1995).
5. Bucher M, Kuhlemeier C: Long-term anoxia tolerance. Multi-level regulation of gene expression in the amphibious plant *Acorus calamus L.* Plant Physiol 103: 441–448 (1993).
6. Capkova-Balatkova V, Hrabetova E, Tupy J: Effects of cycloheximide on pollen of *Nicotiana tabacum* in culture. Biochem Physiol Pflanzen 175: 412–420 (1980).
7. Chapman GP: The tapetum. In: Giles KL, Prakash J (eds) International Review of Cytology, pp. 111–125. Academic Press, London (1987).
8. Curie C, Liboz T, Bardet C, Gander E, Medal C, Axelos M, Lescure B: *Cis* and *trans*-acting elements involved in the activation of *Arabidopsis thaliana* A1 gene encoding the translation elongation factor EF-1. Nucl Acids Res 19: 1305–1310 (1991).
9. Curie C, Axelos M, Bardet C, Atanassova R, Chaubet N, Lescure B: Modular organization and developmental activity of an *Arabidopsis thaliana* EF-1 gene promoter. Mol Gen Genet 238: 428–436 (1993).
10. De Block M, Debrouwer D: In-situ enzyme histochemistry on plastic-embedded plant material. The development of an artefact-free β-glucuronidase assay. Plant J 2: 261–266 (1992).
11. Draper J, Scott R, Armitage P, Walden R: Plant Genetic Transformation and Gene Expression. Blackwell Scientific Publications, Oxford (1988).

12. Hu C, Chee PP, Chesney RH, Zhou JH, Miller PD, O'Brien WT: Intrinsic GUS-like activities in seed plants. Plant Cell Rep 9: 1–5 (1990).
13. Jefferson RA, Kavanagh TA, Bevan MW: GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6: 3901–3907 (1987).
14. Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB: Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224 (1990).
15. Lasko PF, Ashburner M: The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature 335: 611–617 (1988).
16. Leroy P, Alzari P, Sassoon D, Wolgemuth D, Fellous M: The protein encoded by a murine male germ line-specific transcript is a putative ATP-dependent RNA helicase. Cell 57: 549–559 (1989).
17. Martin T, Wöhner RV, Hummel S, Willmitzer L, Frommer WB: The GUS reporter system as a tool to study plant gene expression. In: Gallagher SR (ed) GUS Protocols, pp. 23–43. Academic Press, San Diego (1992).
18. Mascarenhas JP: The biochemistry of angiosperm pollen development. Bot Rev 41: 260–314 (1975).
19. Mascarenhas JP, Mermelstein J: Messenger RNAs: their utilization and degradation during pollen germination and tube growth. Acta Soc Bot Pol 50: 13–20 (1981).
20. Mascarenhas JP: The male gametophyte of flowering plants. Plant Cell 1: 657–664 (1989).
21. Mascarenhas JP: Gene activity during pollen development. Ann Rev Physiol Plant Mol Biol 41: 317–338 (1990).
22. Mascarenhas JP, Hamilton DA: Artefacts in the localization of GUS activity in anthers of petunia transformed with a CaMV 35S-GUS construct. Plant J 2: 405–408 (1992).
23. Mascarenhas JP: Anther- and pollen-expressed genes. In: Verma DPS, Goldberg RB (eds) Temporal and Spatial Regulation of Plant Genes, pp. 97–115. Springer-Verlag, Wien (1988).
24. Mascarenhas NT, Bashe D, Eisenberg A, Willing RP, Xiao CM, Mascarenhas JP: Messenger RNAs in corn pollen and protein synthesis during germination and pollen tube growth. Theor Appl Genet 68: 323–326 (1984).
25. McCormick S: Male gametophyte development. Plant Cell 5: 1265–1275 (1993).
26. Merrick WC: Mechanisms and regulation of eukaryotic protein synthesis. Microbiol Rev 56: 291–315 (1992).
27. Nielsen PJ, McMaster GK, Trachsel H: Cloning of eucaryotic protein synthesis initiation factor genes: isolation and characterization of cDNA clones encoding factor eIF-4A. Nucl Acids Res 13: 6867–6880 (1985).
28. Owttrim GW, Hofmann S, Kuhlemeier C: Divergent genes for translation initiation factor eIF-4A are coordinately expressed in tobacco. Nucl Acids Res 19: 5491–5496 (1991).
29. Owttrim GW, Mandel T, Trachsel H, Thomas AAM, Kuhlemeier C: Characterization of the tobacco eIF-4A gene family. Plant Mol Biol, in press (1994).
30. Plegt L, Bino RJ: β-Glucuronidase activity during development of the male gametophyte from transgenic and non-transgenic plants. Mol Gen Genet 216: 321–327 (1989).
31. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
32. Schmid SR, Linder P: D-E-A-D protein family of putative RNA helicases. Mol Microbiol 6: 283–292 (1992).
33. Schrauwen JAM, de Groot PFM, van Herpen MMA, van der Lee T, Reynen WH, Weterings KAP, Wullems GJ: Stage-related expression of mRNAs during pollen development in lily and tobacco. Planta 182: 298–304 (1990).
34. Sonenberg N, Guertin D, Lee KAW: Capped mRNAs with reduced secondary structure can function in extracts from poliovirus-infected cells. Mol Cell Biol 2: 1633–1638 (1982).
35. Sood PP: Histoenzymological compartementation of β-glucuronidase in the germinating pollen grains of *Protulaca grandiflora*. Biol Plant 22: 124–127 (1980).
36. Stinson JR, Eisenberg AJ, Willing RP, Pe ME, Hanson DD, Mascarenhas JP: Genes expressed in the male gametophyte of flowering plants and their isolation. Plant Physiol 83: 442–447 (1987).
37. Twell D, Klein TM, Fromm ME, McCormick S: Transient expression of chimeric genes delivered into pollen by microprojectile bombardment. Plant Physiol 91: 1270–1274 (1989).
38. Twell D, Yamaguchi J, Wing RA, Ushiba J, McCormick S: Promoter analysis of genes that are coordinately expressed during pollen development reveals pollen-specific enhancer sequences and shared regulatory elements. Genes Devel 5: 496–507 (1991).
39. Twell D: The diversity and regulation of gene expression in the pathway of male gametophyte development. In: Scott R, Stead T (eds) Molecular and Cellular Basis of Plant Reproduction. Company of Biologists, Cambridge, in press (1994).
40. Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, Ryals J: Regulation of pathogenesis-related protein-1a gene expression in tobacco. Plant Cell 5: 159–169 (1993).
41. Webster C, Gaut RL, Browning KS, Ravel JM, Roberts JKM: Hypoxia enhances phosphorylation of eukaryotic initiation factor 4A in maize root tips. J Biol Chem 266: 23341–23346 (1991).
42. Zarsky V, Capkova V, Hrabetova E, Tupy J: Protein changes during pollen development in *Nicotiana tabacum L.* Biol Plant 27: 438–444 (1985).