Two Arabidopsis Loci Encode Novel Eukaryotic Initiation Factor 4E Isoforms That Are Functionally Distinct from the Conserved Plant Eukaryotic Initiation Factor 4E<sup>1</sup><sup>[W]</sup><sup>[OPEN]</sup>

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Canonical translation initiation in eukaryotes begins with the Eukaryotic Initiation Factor 4F (eIF4F) complex, made up of eIF4E, which recognizes the 7-methylguanosine cap of messenger RNA, and eIF4G, which serves as a scaffold to recruit other translation initiation factors that ultimately assemble the 80S ribosome. Many eukaryotes have secondary eIF4E genes with divergent properties. The model plant Arabidopsis (Arabidopsis thaliana) encodes two such genes in tandem loci on chromosome 1, eIF4E1B (At1g29550) and eIF4E1C (At1g29590). This work identifies eIF4E1B/eIF4E1C-type genes as a Brassicaceae-specific diverged form of eIF4E. There is little evidence for eIF4E1C gene expression; however, the eIF4E1B gene appears to be expressed at low levels in most tissues, though microarray and RNA Sequencing data support enrichment in reproductive tissue. Purified recombinant eIF4E1B and eIF4E1C proteins retain cap-binding ability and form functional complexes in vitro with eIF4G. The eIF4E1B/eIF4E1C-type proteins support translation in yeast (Saccharomyces cerevisiae) but promote translation initiation in vitro at a lower rate compared with eIF4E. Findings from surface plasmon resonance studies indicate that eIF4E1B and eIF4E1C are unlikely to bind eIF4G in vivo when in competition with eIF4E. This study concludes that eIF4E1B/eIF4E1C-type proteins, although bona fide cap-binding proteins, have divergent properties and, based on apparent limited tissue distribution in Arabidopsis, should be considered functionally distinct from the canonical plant eIF4E involved in translation initiation.

Cap-dependent translation in eukaryotes begins with recognition of the 7-methylguanosine cap at the 5′ end of an mRNA by the translation initiation factor eIF4E, which forms the eIF4F complex with the scaffolding protein eIF4G. The binding of the RNA helicase eIF4A along with eIF4B promotes unwinding of mRNA secondary structure (Aitken and Lorsch, 2012). The eIF4E complex then serves to circularize mRNA by interaction of eIF4G with poly(A) binding protein and recruit the preinitiation complex through binding of eIF4G to eIF3 and eIF5, ultimately leading to the assembly of the 80S ribosome (Aitken and Lorsch, 2012). eIF4E is an attractive target for global regulation of translational activity through its position at the earliest step, mRNA cap recognition. In many organisms, eIF4E availability is regulated by 4E-binding proteins as well as phosphorylation and sumoylation (Jackson et al., 2010; Xu et al., 2010). However, plants appear to lack 4E-binding proteins, and the role of phosphorylation of eIF4E in translational control is less clear (Pierre et al., 2007).

The eIF4E proteins generally thought to be involved in translation initiation are Class I eIF4E proteins (Joshi et al., 2005), of which two exist in flowering plants: eIF4E, which pairs with eIF4G to form the eIF4F complex, and the plant-specific isoform eIFiso4E, which pairs with eIFiso4G to form eIFiso4F (Mayberry et al., 2011; Patrick and Browning, 2012). Class I eIF4E family members have conserved Trp residues at positions equivalent to Trp-43 and Trp-56 of Homo sapiens eIF4E (Joshi et al., 2005), and the canonical members of this class, such as plant eIF4E and eIFiso4E, have the ability to promote translation through binding of mRNA cap structure and eIF4G (or eIFiso4G).

In some organisms, however, secondary Class I isoforms exist with expression patterns and functions divergent from the conserved eIF4E (Rhoads, 2009). Caenorhabditis elegans has four isoforms involved in differentiation between mono- and trimethylated mRNA caps (Keiper et al., 2000) and have specialized roles for regulation of certain sets of mRNAs, particularly in the germline (Amiri et al., 2001; Song et al., 2010). Trypanosoma brucei has four isoforms with varying ability to bind cap analog and eIF4G isoforms (Freire et al., 2011). Schizosaccharomyces pombe has a second eIF4E isoform, eIF4E2, which is nonessential under normal growth conditions, but accumulates in response to high temperatures (Ptushkina et al., 2001). It cannot, however, complement deletion of EIF4E1, and while it can bind capped mRNA and promote translation in vitro, it has reduced ability to bind an eIF4G-derived peptide.

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Vertebrates encode a novel Class I isoform called E1F4E1B with oocyte-specific expression and functions (Evšikov and Marín de Evšikova, 2009). Zebrfish (Danio rerio) E1F4E1B, with expression limited to muscle and reproductive tissue, has conserved residues identified as necessary for binding cap analog and eIF4G, yet fails to bind either and cannot functionally complement deletion of yeast (Saccharomyces cerevisiae) eIF4E (Rosalino et al., 2004). In Xenopus spp. oocytes, the eIF4E1B protein was found to bind eIF4E transporter and cytoplasmic polyadenylation element binding protein to form a translation-repressing complex (Minshall et al., 2007). Drosophila species have undergone extensive expansion of E1F4E-encoding loci to as many as seven different Class I eIF4E isoforms (Tettweiler et al., 2012). The seven E1F4E isoforms of Drosophila melanogaster are differentially expressed, with only five able to bind to eIF4G and complement deletion of yeast eIF4E (Hernández et al., 2005). The eIF4E-3 isoform of D. melanogaster was recently described as having a specific role in spermatogenesis (Hernández et al., 2012).

Upon completion of sequencing of the Arabidopsis (Arabidopsis thaliana) genome (Rhee et al., 2003), it was discovered that in addition to the conserved plant E1F4E (At4g18040) and E1FISO4E (At5g35620), there existed a tandem pair of genes of high sequence similarity on chromosome 1 that also encoded Class I eIF4E family proteins, E1F4E1B (At1g29550, also known as E1F4E3) and E1F4E1C (At1g29590, also known as E1F4E2). Published microarray and RNA Sequencing (RNA-Seq) data indicate little to no E1F4E1C gene expression; however, the E1F4E1B gene appears to be expressed at low levels in most tissues and enriched in tissues involved in reproduction. The protein sequences contain the residues predicted to be involved in regular eIF4E function but also showed some divergence at highly conserved residues of the canonical plant eIF4E. Genome sequencing data indicate that these genes are part of a divergent eIF4E clade specific to Brassicaceae.

The biochemical properties of the eIF4E1B and eIF4E1C proteins were investigated in this work, and it was found that while they can bind mRNA cap analog and eIF4G and support translation in yeast lacking eIF4E, their eIF4G-binding and translation initiation enhancing capabilities in vitro were less robust when compared with the conserved Arabidopsis eIF4E. In addition, it appears that these E1F4E1B-type genes cannot substitute for E1F4E or E1FISO4E in planta because deletion of both of these genes appears to be lethal. Taken together, these findings indicate the E1F4E1B-type genes represent a divergent eIF4E whose roles should be considered separately from the canonical eIF4E in plant translation initiation.

RESULTS AND DISCUSSION

In Silico Analysis

BLAST searches of available genomic and EST data using National Center for Biotechnology Information and Phytozone (Benson et al., 2012; Goodstein et al., 2012) find that E1F4E1B-type genes are present in close Arabidopsis relatives, including Capsella rubella and Brassica, Raphanus, and Thellungiella spp. However, there is no evidence of these genes outside of Brassicaceae, including the closest relative species sequenced, Carica papaya. It therefore appears that the E1F4E1B-type genes are the result of a Brassicaceae-specific gene duplication and specialization. The genomes of Eucalyptus grandis and Fragaria vesca also encode predicted divergent eIF4E protein forms (E1F4E1BL genes in Fig. 1), though it remains to be determined whether these genes are expressed or conserved in other related species.

The genomes of Arabidopsis, Arabidopsis lyrata, C. rubella, and Brassica rapa encode two E1F4E1B-type loci, called E1F4E1B and E1F4E1C, while Thellungiella halophila and Thellungiella parulana only have evidence for one copy of the gene. Alignment and phylogenetic tree construction of eIF4E and eIFISO4E sequences (Fig. 1) show that the eIF4E1B-type protein sequences cluster together, separately from the conserved eIF4E of flowering plants and from eIFISO4E, which diverged from eIF4E early in the flowering plant lineage (Patrick and Browning, 2012). In addition to completed and draft genomes, there is EST evidence of E1F4E1B and/or E1F4E1C in Brassica oleracea, Brassica napus, Raphanus raphanistrum, and Raphanus sativus, as well as Genome Survey Sequence support for the presence of an E1F4E1B-type gene in Sisymbrium irio (Supplemental Fig. S1).

Interestingly, the E1F4E1B and E1F4E1C genes of Arabidopsis and C. rubella are more closely conserved at the sequence level to each other than to E1F4E1B and E1F4E1C of Brassica spp. (Fig. 1), indicating that there has been recent duplication of E1F4E1B separately in each lineage. This is supported by the fact that while Arabidopsis and C. rubella have E1F4E1B and E1F4E1C as a tandem duplication on one chromosome, B. rapa has E1F4E1B and E1F4E1C genes on separate chromosomes. Thellungiella spp., meanwhile, have only one copy of the gene.

Fifteen residues within the protein have been identified as 90% conserved in flowering plant canonical eIF4E while consistently altered in eIF4E1B-type proteins; many of these residues are conserved as a specific divergent amino acid in eIF4E1B and eIF4E1C (Fig. 2; Supplemental Fig. S1). Residues that have been identified as being involved in cap binding from crystal structures of wheat (Triticum aestivum; Morzingo et al., 2007) and pea (Pisum sativum) eIF4E (Ashby et al., 2011) or by mutational analysis (Yeam et al., 2007; German-Retana et al., 2008) are well conserved in eIF4E1B-type proteins. One exception is the conserved positively charged residue at K78, which is predicted in the wheat eIF4E crystal structure to stabilize the negatively charged phosphate backbone of the cap structure. In eIF4E1B/ eIF4E1C-type proteins, this residue is changed to Asn, which may weaken this interaction. However, mutation of this position in pea eIF4E had no effect on the ability to promote translation in yeast (Ashby et al., 2011).
Figure 1. Cladogram of Brassicaceae eIF4E1b-like proteins in relation to the conserved eIF4E and elfiso4E proteins of flowering plants. The Phylogeny.fr pipeline (Dereeper et al., 2008) was used for alignment and phylogenetic tree generation with alignment by MUSCLE and tree construction by PhyML using 500 bootstrap replicates.
Several mutations in eIF4E1b-type proteins occur at locations that are both well conserved between eIF4E of plants and mammals and predicted to be involved in eIF4E binding to eIF4G from a cocrystal structure of yeast eIF4E with a fragment of eIF4G (Gross et al., 2003). eIF4E residues P61, L62, and D149 (Fig. 2) are all predicted to be part of the eIF4G binding interface and are altered in eIF4E1b-type proteins. These changes appear to have altered the ability of eIF4E1b/eIF4E1c to interact with eIF4G compared with eIF4E (see below). While mutations in eIF4E that confer viral resistance are naturally occurring (Robaglia and Caranta, 2006) and directed mutagenesis has further identified residues conferring virus resistance (German-Retana et al., 2008; Ashby et al., 2011), the 15 conserved flowering plant eIF4E residues differing in eIF4E1b-type proteins do not overlap with these residues, with the exception of a K78 mutation, which confers virus resistance in pea (Ashby et al., 2011). Interestingly, transfer DNA (T-DNA) insertion mutants for EIF4E1B or EIF4E1C do not have any effect on turnip mosaic virus infection in Arabidopsis (Gallois et al., 2010). To date, neither EIF4E1B nor EIF4E1C has been reported to be a virus resistance gene in contrast to numerous reports of virus resistance attributed to EIF4E and EIFISO4E alleles (Wang and Krishnaswamy, 2012).

Analysis of homozygous T-DNA insertion lines for EIF4E1B (GK-874C07) or EIF4E1C (GK-361E12) do not show an obvious phenotype. Due to their close proximity on chromosome 1, it was not possible to obtain a double mutant even after screening more than 5,000 plants from a cross (data not shown).

**EIF4E1B/EIF4E1C Expression**

Due to their sequence similarity, EIF4E1B and EIF4E1C share a spot on many commonly used micro-arrays, limiting data as to whether either or both are expressed. However, RNA-Seq data can distinguish between the two genes and indicate that in flower tissue (Jiao and Meyerowitz, 2010; Niederhuth et al., 2013), shoot apical meristem (Torti et al., 2012), developing embryos (Nodine and Bartel, 2012), and the central cell of the female gamete (Schmid et al., 2012), EIF4E1B mRNA is expressed and associates with polysomes in flowers (Jiao and Meyerowitz, 2010); however, EIF4E1C mRNA was at much lower to undetectable levels in these tissues. In an analysis of 80 genomes released by the 1001 Genomes Project, EIF4E1C was predicted to be spontaneously deleted in 12 strains, suggesting that it is likely not providing any advantage to promote its retention in the genome (Cao et al., 2011).

Microarray data from the Arabidopsis eFP Browser (Winter et al., 2007) suggest that EIF4E1B is most highly expressed in developing flowers, while Genevestigator
Zimmermann et al., 2004) supports expression in shoots and reproductive tissue. EIF4E1B was identified as a gene up-regulated during pollen tube growth (Wang et al., 2008). Additionally, EIF4E1B was significantly enriched in pollen tubes grown by a semi in vivo method (Qin et al., 2009). EIF4E1B was identified as a sperm-enriched gene, while EIF4E was sperm depleted (Borges et al., 2008). In a microarray experiment investigating developing embryos, EIF4E1B was found to be expressed at high levels at the zygote stage of development relative to EIF4E, while EIF4E1C levels were near background levels (Xiang et al., 2011). Taken together, these findings may indicate a role for EIF4E1B in reproduction in Brassicaceae similar to that in Drosophila spp. or zebrafish.

eIFiso4E or eIF4E Is Required for Viability

The T-DNA knockout line for EIFISO4E (iso4e-1, Duprat et al., 2002) and the nonsense mutant for EIF4E (cucumber virus multiplication1 [cum1]; Yoshii et al., 2004) are viable and do not exhibit major developmental phenotypes individually. However, extensive attempts have been made by this laboratory to isolate an iso4e-1/cum1 double mutant without success. iso4e-1 plants heterozygous for cum1 are viable, but self-fertilized plants do not yield viable double mutants in the ratio expected for normal progeny (Table I). The defect appears to be embryo lethal, as nearly all planted seeds germinated and were successfully screened. Similarly, cum1 plants heterozygous for iso4E-1 do not yield viable double mutants (Supplemental Table S1). A lethal phenotype has also been reported with the iso4e/4e1 genotype, preventing the recovery of a double homozygous mutant (Callot and Gallois, 2014). These results suggest that EIF4E1B and EIF4E1C gene products are not sufficient to fulfill the necessary role for a canonical Class I eIF4E protein, either due to low or localized expression or loss of properties that contribute to translation initiation, such as binding eIF4G and/or mRNA cap structure.

Table 1. Screening of iso4e-1 cum1/EIF4E progeny from self-fertilization

One hundred seven seeds were planted on Murashige and Skoog agar plates, with 105 germinating and 103 successfully transplanted and screened. Recovery of iso4e-1 cum1/EIF4E was lower than expected (35%), and double homozygous mutant plants were not recovered.

| Genotype            | No. with Genotype | Percentage of Total | Expected Mendelianb |
|---------------------|-------------------|--------------------|--------------------|
| iso4e-1 cum1/cum1   | 0/107             | 0                  | 25%                |
| iso4e-1 cum1/EIF4E  | 37/107            | 35                 | 50%                |
| iso4e-1 EIF4E/EIF4E | 66/107            | 62                 | 25%                |
| Not screeneda       | 4/107             | 4                  | Not Applicable     |

aSeed did not germinate or died before screening. bExpected amounts if normal Mendelian inheritance.

Figure 3. PAGE analysis of purified proteins. A, Purified Arabidopsis eIF4G and eIF4E isoforms. Lane 1, eIF4G1_1-1727 (3 μg); lane 2, eIF4E (1.5 μg); lane 3, eIF4E1b (1.5 μg); and lane 4, eIF4E1c (1.5 μg), were separated by 12.5% SDS-PAGE and stained with Coomassie brilliant blue. B, Recombinant Arabidopsis eIF4F complexes from dicistronic constructs were expressed in E. coli and purified by m’GTP-Sepharose affinity and phosphocellulose chromatography. Lane 1, Wheat eIF4F (3 μg); lane 2, Arabidopsis eIF4G1_1-1727/eIF4E (3 μg); lane 3, eIF4G1_1-1727/eIF4E1c (3 μg); lane 4, eIF4G1_1-1727/eIF4E1b (3 μg). Proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Note that the order of protein loading is different in A and B.
Because *EIF4E1B* and *EIF4E1C* genes are products of a Brassicaceae-specific gene duplication, they may have specialized functions and/or lost the ability to function as cap-binding proteins. We sought to determine if elf4E1b and elf4E1c proteins are biochemically capable of performing the functions of cap-binding proteins in vitro, as well as in vivo using a yeast complementation system.

**elf4E1b and elf4E1c Bind to m^7^GTP and Form Complexes with elf4G**

Arabidopsis elf4E, elf4E1b, and elf4E1c were expressed in *Escherichia coli* and purified by affinity chromatography using 7-methyl guanosine triphosphate (m^7^GTP)-Sepharose (Fig. 3A). Both elf4E1b and elf4E1c were found to bind and elute from m^7^GTP-Sepharose in comparable yields to elf4E (data not shown). elf4E1b and elf4E1c therefore seem to have biologically relevant cap-binding ability.

The initial selection of the coding sequence of Arabidopsis elf4G (elf4G_{1–1727}) to express was made based on protein similarity to the N terminus of wheat elf4G (Mayberry et al., 2007). However, subsequent peptide sequence data in the pep2pro database (http://fgcz-pep2pro.uzh.ch/index.php; Baerenfaller et al., 2011) suggested that upstream initiation codons are utilized. Because the precise initiation codon (or if there are multiple start sites, as occurs for mammalian elf4G; [Coldwell et al., 2012]) is not known for Arabidopsis, the codon selected by The Arabidopsis Information Resource was also used to generate an expression construct for elf4G_{1–1727}, which includes all the peptides identified in the pep2pro database.

elf4E, elf4E1b, and elf4E1c were coexpressed with Arabidopsis elf4G_{1–1727}, which retains the elf4E binding site, to form elf4F complexes that were purified by m^7^GTP-Sepharose affinity chromatography (Fig. 3B). elf4E1b copurified with elf4G_{122–1727} to form the elf4F1b complex, and elf4E1c copurified with elf4G_{322–1727} to form the elf4F1c complex. Both elf4F1b and elf4F1c purified with comparable yield to elf4E. Confirming that elf4E1b and elf4E1c were able to bind elf4G and form a stable complex.

Purified elf4E, elf4E1b, and elf4E1c were assayed by surface plasmon resonance (SPR) for their binding affinity to purified elf4G_{122–1727} (Table II). The dissociation constant (K_D) for elf4E binding to elf4G_{122–1727} was extremely tight, at 0.275 ± 0.002 nm. This finding is consistent with the measurement of the wheat elf4G and elf4E binding K_D of 0.181 ± 0.002 nm (Mayberry et al., 2011). Surprisingly, elf4E1b binding to elf4G_{322–1727} was 1,640-fold weaker than elf4E (451 ± 2 nm), while elf4E1c binding was weaker still (970 ± 10 nm).

The elf4E1b/elf4E1c binding affinity to elf4G_{322–1727} is lower than was observed in the wheat system for a mixed complex of elfiso4E binding to elf4G (14.3 ± 0.2 nm; Mayberry et al., 2011). It was previously shown that elfiso4E was displaced from a mixed complex with wheat elf4G by elf4E, thus the correct binding partner is selectively favored (Mayberry et al., 2011). Based on the observed lower binding affinity, despite the ability of elf4E1b or elf4E1c to copurify with elf4G_{322–1727} in vitro, it is unlikely either could form a complex with elf4G in vivo in Arabidopsis unless elf4E is absent (see Fig. 5 below).

### Table II. The binding affinity of purified Arabidopsis cap-binding proteins to elf4G_{122–1727}

| Cap-Binding Protein | K_D (nM) |
|---------------------|----------|
| elf4E1              | 0.275 ± 0.002 |
| elf4E1b             | 451 ± 2   |
| elf4E1c             | 970 ± 10  |

**elf4E1b and elf4E1c Have Translation-Enhancing Activity in Vitro But Are Displaced by elf4E**

To measure the ability of elf4E1b and elf4E1c to function in the initiation of translation, in vitro translation assays in wheat germ S30 depleted of cap-binding complexes were carried out. Recombinant Arabidopsis elf4G_{1–1727} was mixed with equimolar amounts of cap-binding proteins to form elf4F complexes, and these were tested for their ability to translate mRNA compared to recombinant wheat elf4F (Fig. 4). Arabidopsis elf4G alone provided little stimulation of translation, while the conserved Arabidopsis elf4F complex of elf4G with elf4E performed similarly to wheat elf4F. elf4G paired with either elf4E1b or elf4E1c showed similar activity but
required significantly higher concentrations of the complexes (approximately 5- to 10-fold) to approach the extent of stimulation of the elf4F complex from either wheat or Arabidopsis.

The contribution of elf4E1b to in vitro translation was further examined with an assay placing elf4E1b in competition with elf4E for elf4G to observe changes in activity of in vitro translation (Fig. 5). The complex of elf4E/elf4G was challenged with either additional elf4E or elf4E1b, and there was no significant change in the translational activity observed in either case (Fig. 5A); however, a complex of elf4E1b/elf4G was presented with increasing amounts of elf4E to determine if elf4E1b could be displaced by elf4E and form the more active elf4E/elf4E1b complex. The reaction mixture contained 4 pmol of barley α-amylase mRNA and 15 μL of a wheat germ S30 extract that had been depleted of elf4F and elfiso4F by passage over a m7GTP Sepharose column as described in “Materials and Methods.” White triangle, Two picomoles of elf4G titrated with elf4E1b as indicated; black triangle, 2 pmol of elf4G/elf4E1b titrated with additional elf4E1b as indicated; black circle, 2 pmol of elf4G/elf4E1b titrated with additional elf4E as indicated. Experiments were done in triplicate and averaged. The incorporation of [14C]Leu in the absence of any added factor was 10 pmol.

eIF4E1b and eIF4E1c Can Complement elf4E Deletion in Yeast

Arabidopsis elf4E has previously been shown to be able to complement for an elf4E deletion in yeast (Rodriguez et al., 1998). The in vitro data suggest that elf4E1b and elf4E1c are functional in that they bind to m7GTP-Sepharose and elf4G. To further investigate the ability of elf4E1b and elf4E1c to function as bona fide cap-binding proteins, they were tested for their ability to complement elf4E deletion in yeast. As shown in Figure 6, both elf4E1b and elf4E1c are able to substitute for yeast elf4E gene deletion in vivo. This finding implies that elf4E1b and elf4E1c have biologically relevant ability to promote translation and, as shown in the in vitro experiments, retain sufficient cap-binding and elf4G-binding properties in spite of their sequence differences from elf4E. Thus, their inability to function in planta in a background lacking elf4E and elfiso4E suggests that their expression is highly localized or controlled.

A version of elf4E1c protein with an additional N-terminal sequence predicted by The Arabidopsis Information Resource (Rhee et al., 2003) was also tested as elf4E1c(long). This additional sequence is likely to be an artifact of gene assignment due to an incorrect prediction for the start site. The extra peptide sequence has no similarity to any known elf4E peptide sequences and would be unique among the elf4E1b-like genes as well as plant elf4E genes. The elf4E1c(long) gene was

Figure 5. Displacement of elf4E1b from complex with elf4G by elf4E as measured by in vitro translation activity. A, The complex of elf4G/elf4E was presented with increasing amounts of elf4E1b to determine if activity was reduced to the level of the elf4G/elf4E1b complex. White rectangle, Two picomoles of elf4G titrated with elf4E; black rectangle, 2 pmol of elf4G/elf4E titrated with additional elf4E as indicated; and white circle, 2 pmol of elf4G/elf4E titrated with additional elf4E1b as indicated. B, Alternatively, a mixed complex of elf4G/elf4E1b was presented with increasing amounts of elf4E to determine if elf4E1b could be displaced by elf4E and form the more active elf4G/elf4E1b complex. The reaction mixture contained 4 pmol of barley α-amylase mRNA and 15 μL of a wheat germ S30 extract that had been depleted of elf4F and elfiso4F by passage over a m7GTP Sepharose column as described in “Materials and Methods.” White triangle, Two picomoles of elf4G titrated with elf4E1b as indicated; black triangle, 2 pmol of elf4G/elf4E1b titrated with additional elf4E1b as indicated; black circle, 2 pmol of elf4G/elf4E1b titrated with additional elf4E as indicated. Experiments were done in triplicate and averaged. The incorporation of [14C]Leu in the absence of any added factor was 10 pmol.

Figure 6. The ability of Arabidopsis elf4E proteins to complement deletion of the elf4E gene in yeast. Complementation was tested by introducing pgC-1 plasmids for constitutive expression of Arabidopsis elf4E genes into a yeast strain (T93C; Altmann et al., 1989) with elf4E under control of a GAL promoter. Serial dilutions of midlog phase yeast were plated in 10-fold serial dilutions on SCM-Trp plates containing 2% Gal (A) or 2% Glc (B) and incubated at 30°C for 48 h. The experiment was performed in three biological replicates; representative results are shown.
not able to complement the deletion of *EIF4E* in yeast, indicating the additional N-terminal amino acid residues may interfere with either cap recognition or eIF4G binding, preventing productive translation initiation. This is consistent with observations by our laboratory that some N- or C-terminal fusions of plant cap-binding proteins are not viable in vivo in yeast or in Arabidopsis (E. Levins, C. Tseng, and K. Browning, unpublished data).

**CONCLUSION**

Arabidopsis is the best model system for plant translation initiation currently available due to the availability of knockout lines of many translation initiation factors for in vivo study as well as the successful purification of many recombinant proteins for these factors. Arabidopsis and other members of the Brassicaceae family have noncanonical *eIF4E* related genes present in their genomes. There is little evidence for *EIF4E1C* gene expression, and the *EIF4E1B* gene is expressed at low levels in most tissues, though microarray and RNA-Seq data support enrichment in reproductive tissue. Unfortunately, AtElF4E antibody cross reacts poorly with *eIF4E1B* and *eIF4E1C*, so it is not possible to confirm that these proteins are actually produced in vivo (data not shown). The *eIF4E1B* and *eIF4E1C* are bona fide cap-binding proteins sufficient to promote translation initiation in yeast and display translation initiation activity in vitro. However, due to their low binding affinity for *eIF4G* relative to *eIF4E* and their low level of expression, it seems unlikely that these genes contribute substantially to translation initiation in most plant tissues. Increasing numbers of noncanonical *eIF4E* family members have been described in eukaryotes (Rhoads, 2009). As more plant genomes are sequenced, other events similar to the *EIF4E* duplication and divergence in Brassicaceae may be observed. *E. grandis* and *F. vesca* both encode apparent divergent *EIF4E* genes, though there is not yet available data to tell whether they are expressed or if the genes are conserved among close relatives. Given the data for poor interaction with *eIF4G* and its identification as a sperm-enchanced gene, one might expect the role of *eIF4E1B* to be similar to what has been described in vertebrate oocytes: binding the 7-methylguanosine cap and excluding eIF4G binding to repress translation. The germline enrichment of Arabidopsis *EIF4E1B* seems in line with findings from vertebrates (Minshall et al., 2007), *C. elegans* (Amiri et al., 2001), and *Drosophila* spp. (Hernández et al., 2012) of specialized *eIF4E* isoforms with roles in reproductive tissue. However, the in vitro data from this work suggest that *eIF4E1B* does not contribute to translational repression in this manner, though mRNA-specific repression or interaction with other proteins cannot be ruled out. Arabidopsis *EIF4E1B* and *EIF4E1C* seem nonessential, as T-DNA insertion plants develop normally (data not shown); however, the strong conservation of the *EIF4E1B*-type genes within the Brassicaceae family implies that they provide some as-yet-unidentified contribution. In addition, crosses between *EIF4E* (cum1) and either *EIF4E1B* or *EIF4E1C* T-DNA lines do not have any observable phenotype or issues with production (data not shown). Thus, although *eIF4E1B* and *eIF4E1C* appear to be able to function as cap-binding proteins in vitro and in yeast, it remains to be determined if *EIF4E1C* is even expressed in plants, and the actual levels of *eIF4E1B* protein expression and localization remain to be determined. Based on the large difference (approximately 1,600-fold) in binding affinity of *eIF4E1B* for *eIF4G* relative to *eIF4E*, it is unlikely *eIF4E1B* plays any role in canonical translation, but perhaps there is a Brassicaceae-specific role in some tissues where *eIF4E* protein is not expressed and would allow *eIF4E1B* to interact with *eIF4G* or other proteins.

**MATERIALS AND METHODS**

**In Silico Analysis**

*eIF4E* and *eIFiso4E* gene sequences for alignment and analysis were collected from Phytozome (Goodstein et al., 2012) and BLAST searches to GenBank sequences (Benson et al., 2012). Alignment was performed by ClustalW2 (Larkin et al., 2007); residues defined as conserved in *eIF4E* were those with 90% or greater identity in the canonical coding from among the aligned sequences. The Phylology.fr pipeline (Dereeper et al., 2008) was used for alignment and phylogenetic tree generation with alignment by MUSCLE and tree construction by PhyML using 500 bootstrap replicates (see Supplemental Fig. S2).

**eIF4E and eIFiso4E Cross**

Mutant lines for *eIF4E* (cum1), a nonsense point mutation and *eIFiso4E* (iso4E-1, Sainsbury Laboratory Arabidopsis Transformants library) have been previously described (Duprat et al., 2002; Yoshii et al., 2004). Crosses between these two lines were performed in both directions and the T2 progeny screened by PCR to identify wild-type, heterozygous, or double homozygous lines. *soE-1* and *cum1* lines were screened with primers as described in Supplemental Table S2.

**Construction of eIF4E1, eIF4E1b, eIF4E1c, eIF4E1c(long), eIF4G322–1727, and eIF4F, eIF4F1b, and eIF4F1c Expression Constructs**

Initial attempts to express Arabidopsis (*Arabidopsis thaliana*) *eIF4G* protein from complementary DNA clones were unsuccessful. Using DNAWorks (Hoover and Lubkowski, 2002), Arabidopsis *eIF4G* (*iso4E-1*), *EIF4E1b*, *EIF4E1c*, and *EIF4E1c(long)* were designed with codon optimization for expression in Escherichia coli and assembled by overlap PCR of oligonucleotides (Supplemental Figs. S3–S8; Supplemental Tables S3–S7; Horton et al., 1989). Initial cloning of *eIF4G*322–1727 was into pCR-Blunt-II-TOPO (Invitrogen), followed by subcloning into pSB1AC3 (Shetty et al., 2008) and pET22b vectors (Novagen). *eIF4E* genes were cloned in one step. *eIF4G*322–1727 was cloned into pCR-Blunt-II-TOPO in four sections and then assembled in pSB1AC3. Full-length *eIF4G*322–1727 was created by cloning a synthetic DNA sequence (Geronscript) to provide the missing N-terminal sequence to *eIF4G*322–1727; the restriction site used to ligate the synthetic DNA was then altered to match wild-type protein sequence by site-directed mutagenesis (Mutagenex). The pET22b *eIF4G*322–1727 vector was used to clone *eIF4E1*, *eIF4E1b*, and *eIF4E1c* genes at a site 3′ of the *eIF4G* coding region to create dicistronic plasmids for expression of *eIF4F*, *eIF4F1b*, and *eIF4F1c* complexes.

**Purification of Recombinant Proteins**

*eIF4E* proteins were expressed in BL21(DE3) *E. coli* and purified as previously described by m7GTP-Sepharose affinity chromatography (Mayberry et al., 2007). *eIF4F* complexes were expressed in Tuner(DE3) *E. coli* (Novagen).
and purified as previously described for wheat (Triticum aestivum) elf4iso4F (Mayberry et al., 2007, 2011), elf4G1, elf4G2, and elf4G3 were expressed in TunerDE3 E. coli and purified as previously described for wheat elf4G4 (Mayberry et al., 2007, 2011). Wheat elf4E and elf3 were purified as previously described (Lax et al., 1986; Mayberry et al., 2007).

In Vitro Translation Assay

Arabidopsis elf4G and cap-binding proteins were assayed in an in vitro translation assay using wheat germ S30 extract that had been depleted of cap-binding proteins and complexes. Three 4-mL portions of m7GTP-Sepharose (GE Biosciences) were equilibrated in 20 mM HEPES, pH 7.6, 120 mM KAc, 5 mM MgAc2, 10% (v/v) glycerol, and 5 mM 2-mercaptoethanol. A 2-mL aliquot of S30 extract was used to exchange the buffer from each of the three 4-mL portions of the m7GTP-Sepharose. Twenty-five milliliters of wheat germ S30 extract (Lax et al., 1986; Browning and Mayberry, 2006) was mixed for 15 min with 4 mL of m7GTP-Sepharose by rocking on ice; the supernatant was collected, and the process was repeated with the remaining two portions of 4 mL of m7GTP-Sepharose. The elf4G/elfiso4F-depleted S30 extract was aliquoted, flash frozen, and stored at −80°C.

The 50-μL translation assay reaction mixture contained 24 μM HEPES-KOH, pH 7.6, 2 μM MgAc2, 100 μM KAc, 30 μM KC1, 2.4 μM dithiothreitol, 0.1 μM spermine, 1 μM ATP, 0.2 μM GTP, 34 μM [3H]leucine, 50 μM 19 amino acids (–Leu), 7.8 μM creatine phosphate, 5 μM creatine kinase, 0.75 μM units of yeast (Saccharomyces cerevisiae) RNA15, 15 μL of depleted S30 extract, 4 to 5 pmol barley ( Hordeum vulgare) α-amylase mRNA, 10 μg of recombinant wheat elf4E, 0.5 μg of recombinant wheat elf4F, 6 μg of native wheat elf3, and the indicated amounts of elf4E, elf4F, and/or cap-binding proteins. Incubation was performed for 30 min at 27°C, and the amount of [3H]Leu incorporated into protein was determined as previously described (Lax et al., 1986; Browning and Mayberry, 2006; Mayberry et al., 2007).

SPR Analysis

SPR (Biacore) experiments were carried out as described previously (Mayberry et al., 2011) at Biosensor Tools by Dr. David Myszka. Briefly, protein binding was measured at 25°C using a Biacore 2000 optical biosensor equipped with a CM4 sensor chip in running buffer (20 mM HEPES, 100 mM KCl, 1.5 μM tris(2-carboxyethyl)phosphine 0.1 mM EDTA, 100 μM m7GTP, 5% glycerol, 0.01% Tween 20, and 0.1 mg mL−1 bovine serum albumin, pH 7.6). elf4G1, elf4G2, and elf4G3 were amine coupled at three surface densities (500, 1,370, and 4,430 resonance units). elf4E, elfiso4E, and elfiso4E1 proteins were tested for elf4G binding in 3-fold dilution series performed in triplicate. For elf4G1 and elf4G2, the highest concentration tested was 1.5 μM, and for elf4G3, it was 4.9 μM. Response data for each protein were determined as previously described (Lax et al., 1986; Mayberry et al., 2007).

Yeast Complementation of elf4E

The yeast strain T93C (Altmann et al., 1989), containing a chromosomal deletion of the elf4E gene and a plasmid under control of a Gal promoter (elf4E::LEU2 ura3 trp1 leu2 [pGai elf4E URAl]), was transformed with pG1 vectors (with an added Ncol site N terminus to BamHI in the cloning region) containing Arabidopsis elf4E constructs. pG1 provides constitutive gene expression in a tetracycline deletion in eukaryotes. Nat Struct Mol Biol 19: 568–576.

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