Pyramiding resistances based on translation initiation factors in Arabidopsis is impaired by male gametophyte lethality

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In eukaryotes, eIF4E translation initiation factors are essential proteins encoded by a small multigene family. In plants, they are a source of host plant resistance to potyviruses that require specific 4E factors to infect cells. Combining mutations in different eIF4E genes could be a way of broadening the spectrum of plant resistance to viruses. We attempted to combine null mutations affecting the two main Arabidopsis thaliana 4E factors eIF4E1 and eIFiso4E but discovered that this combination is lethal. Transmission through the male gametophyte is completely abolished in the eif4e1 eifiso4e double mutant. This shows that eIF4E1 and eIFiso4E are essential for male gametophyte development and act redundantly. These results may have implications for eIF4E-based pyramiding strategies to improve crop resistance.

Eukaryotic translation is initiated by the interaction between the mRNA cap structure that is present at the 5' end of most mRNAs and the eIF4E protein. Other elf factors, including the large scaffold protein eIF4G, are involved in this process that results in the assembly of the 43S pre-initiation complex. Translation initiation is thought to be a fundamental process in cell development, and mutation of the Saccharomyces cerevisiae elf4E gene (or CDC33) shows that this gene is indeed essential for cell growth. Plants have two types of translation initiation complex. The elf4F complex is made up of the elf4E and elf4G proteins, and the elfiso4F complex is made up of their respective isoforms elfiso4E and elfiso4G. In Arabidopsis thaliana, elf4E proteins are encoded by a small multigene family of the three elf4E genes elf4E1 (At4g18040), elf4E2 (At1g29590) and elf4E3 (At1g29550), the isoform elfiso4E gene (At5g35620) and the atypical nCBP gene (At5g18110). The elf4G family members are elf4G (At3g60240) and the two isoform genes elfiso4G1 (At5g35870) and elfiso4G2 (At2g24050). All single Arabidopsis mutants affecting these translation initiation factor genes are viable, suggesting a high degree of redundancy among the respective elf4E and elf4G genes. However, an elfiso4g1 elfiso4g2 double mutant displays pleiotropic developmental defaults including dwarfism and reduced fertility. Likewise, silencing of several elf4E genes in Nicotiana tabacum or Lycopersicon esculentum led to dwarf plants.

In plants, the 4E and 4G translation initiation factors have also been shown to be central in plant resistance to RNA viruses, especially members of the Potyvirus genus of single-stranded, positive sense RNA viruses. In Arabidopsis, systematic studies of mutants affecting translation initiation factors showed that distinct potyviruses can recruit different specific elf4F isoforms. For example, the Turnip Mosaic Virus (TuMV) requires elfiso4E to infect Arabidopsis whereas the Clover Yellow Vein Virus (CIYVV) uses elf4E1. If mutations affecting both these host genes were combined it may be possible to enlarge the resistance spectrum of the host and possibly counter overcoming viral strains that manage to bypass specific elf4E-mediated resistance.

To implement such a strategy, KO mutations affecting elf4E1 and elfiso4E were combined. An F1 population was obtained by crossing plants homozygous for the elfiso4e mutation caused by a d5pm element insertion with plants homozygous for the elf4e1 mutation caused by a T-DNA insertion in the first intron of elf4E1 (SALK_145583). The F1 plants were genotyped to ensure that they were heterozygous at both loci (elf4e1;elf4e1; elfiso4e;elfiso4e) (Fig. 1) and were allowed to self. F2 plants were genotyped to search for elf4e1 elfiso4e double mutants but no plants homozygous for both mutations were isolated (n > 500 F2 plants, resulting from 3 independent selfed F1 parents). This shows clear segregation bias in the F2 progenies.

Besides the diploid sporophytic phase, the higher plant life cycle includes a postmeiotic haploid phase that takes place in male and female reproductive organs. Given the effect of mutations affecting elf4E in haploid yeast, we hypothesized that the haploid lethality of the gametophytes harboring mutations in both elf4E1 and elfiso4E could be the reason for the segregation bias observed in the F2 Arabidopsis plants.

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Mutations that are gametophytic lethal can be readily inferred from the non-Mendelian segregation of alleles.\textsuperscript{18} If a gene is essential for haploid gametophyte development, either male or female, gametes harboring this mutation will not develop properly or be transmitted to the progeny. As a result, the transmission of the mutation to the progeny will be skewed.\textsuperscript{19} If two genes are essential for gametophyte development but act redundantly, a single mutation affecting one of those genes will be transmitted normally through the gametes, but combined mutations affecting both genes will not be transmitted.\textsuperscript{20}

Large genomic deletions have been shown to correlate with gametophytic lethality\textsuperscript{19} so it was checked that there was no deletion at the insertion site of either \textit{eif4e1} nor \textit{eifiso4e} mutations.\textsuperscript{8} We then tested whether single mutations affecting \textit{eIF4E1} or \textit{eIFiso4E} had any effect on gametophyte development. Reciprocal crosses were performed between heterozygous \textit{eif4e1/eIF4E1} \textit{F1} plants and wild-type Columbia (Col). The progenies were sown and the percentage of plants harboring the \textit{eif4e1} allele was assessed by genotyping. This percentage reflects the proportion of viable gametophytes (male or female) carrying the \textit{eif4e1} mutation that have been transmitted successfully. If the mutation has no effect on the gametophyte development, it is expected that 50% of the plants will be heterozygous for the \textit{eif4e1} mutation. No segregation bias was found for the null \textit{eif4e1} allele, either through the male gametophyte (48\% male transmission of \textit{eif4e1}, \textit{n} = 56) or through the female gametophyte (52\% female transmission of \textit{eif4e1}, \textit{n} = 44). Similar reciprocal crosses were performed with \textit{eifiso4e} and again, no male nor female transmission defect was associated with this mutant allele (61\% male transmission of \textit{eifiso4e}, \textit{n} = 56; 44\% female transmission of \textit{eifiso4e}, \textit{n} = 56). These experiments show that single mutations affecting \textit{eIF4E1} or \textit{eIFiso4E} do not affect either male or female transmission and therefore have no significant effect on gametophyte development.

To assess the effect of a loss of function of both \textit{eIF4E1} and \textit{eIFiso4E} in gametophytes, \textit{F1} plants heterozygous for both mutations (\textit{eif4e1/eIF4E1}; \textit{eifiso4e/eIFiso4E}) were crossed to wild-type Col. Progenies were genotyped to determine the transmitted gamete genotypes (Table 1). When the \textit{eif4e1/eIF4E1}; \textit{eifiso4e/eIFiso4E} plants were used as the female parent, both single and double mutations segregated as expected, showing that loss of function of \textit{eIF4E1} and \textit{eIFiso4E} has no effect on female gametophyte development in \textit{Arabidopsis}.

| mutant     | oligo name | sequence                |
|------------|------------|-------------------------|
| \textit{eif4e1} | LBb1       | ATTTTGCGATTTCCGAAC       |
|            | Z2014      | TTCCATTGTTTCCAAATGCTC    |
|            | Z2015      | GAAACCAACCTCTGAGGGAAG    |
| \textit{eifiso4e} | dspm1     | CTTATTTCAGTAAGAGTGCTGAGGGTTTGG |
|            | Z2835      | AAGAAGATTTAAATGCTCTGATGGAC |
|            | Z2836      | CTCATCTGCTTCAATGCTCT     |

Figure 1. Genotyping of the plants used in this study. (A) Oligonucleotides used for genotyping. For \textit{eIF4E1}, wild-type and mutant alleles were genotyped with Z2014-Z2015 and LBb1-Z2015, respectively. For \textit{eIFiso4E}, wild-type and mutant alleles were genotyped with Z2835-Z2836 and dspm1-Z2835, respectively. (B) Wild-type plants (Col), plant homozygous for the \textit{eif4e1} mutation or for the \textit{eifiso4e} mutation (\textit{iso}) and \textit{F1} plants were genotyped for the T-DNA or transposon insertion in \textit{eIF4E1} (\textit{e1}) or \textit{eIFiso4E} (\textit{iso}), respectively.
However, when *eif4e1/eIF4E1; eifiso4e/eIFiso4E* plants were used as the male parent, a strong segregation bias was detected in progeny. No plant carrying both the *eif4e1* and *eifiso4e* mutations was obtained out of 211 plants tested from the progeny (expected number of double heterozygous plants if unbiased segregation, 53). This result shows that eIF4E1 and eIFiso4E are essential for male gametophyte development. These results highlight the functional redundancy between eIF4E1 and eIFiso4E as the male gametophyte develops normally when either one of the genes is functional. This redundancy implies that both eIF4E1 and eIFiso4E are expressed similarly during the male gametophyte development. We looked at transcriptomes from different stages of male gametophyte development from data collected by microarray analysis. The level of expression of eIF4E1 and eIFiso4E are very similar at the different stages of pollen development (Table 2). However, we noted that two other genes encoding 4E initiation factors, eIF4E3 (At1g29550) and nCBP (At5g18110), are expressed in male gametophytes, albeit at a lower level. The genetic results indicate that neither eIF4E3 nor nCBP can replace eIF4E1 and eIFiso4E during male gametophyte development. This might be due to their low expression level or to differential functions during translation initiation. The latter explanation is very likely for nCBP, which encodes a non-canonical eIF4E protein. Future experiments based on promoter swaps between eIF4E homologs could help to confirm this.

The stage at which *eif4e1 eifiso4e* male gametophyte development is impaired remains to be determined. The analysis of pollen using viral pollen Alexander staining shows that pollen harvested from *eif4e1/eIF4E1; eifiso4e/eIFiso4E* F1 plants is 100% viable. This suggests that the lack of eIF4E1 and eIFiso4E affects later stages of pollen development. Interestingly, proteomics studies in *Oryza sativa* have pinpointed an upregulation of translational initiation factors eIF4G and eIF4A during pollen tube growth, consistent with an increased requirement for protein synthesis at that stage. In the *eiffie eifiso4e* gametophyte, we might predict that without such a boost in protein production, pollen tube germination and/or growth and/or ovule fertilization would be impaired. In contrast, the female gametophytic transcription was not affected by the combined *eif4el eifiso4e* mutations. This may be because the gene redundancy among genes encoding 4E proteins is different in the female gametophyte. Perhaps eIF4E2, eIF4E3 or nCBP are sufficient to initiate translation in the female gametophyte. Alternatively, the female gametophyte may contain enough 4E proteins to develop until fertilization.

These results also bring significant insights to the domain of plant-potyvirus interactions. Coevolution between plants and potyviruses has resulted in naturally occurring non-synonymous mutations in eIF4E genes. Often, these genes have retained their translation initiation properties but lost the capacity to interact with the viral proteins, hence resulting in the plant gaining resistance to the virus. This is exemplified by the *Capsicum annuum/Potato virus Y* and *Capsicum annuum/Tobacco etch Virus* pathosystems. Besides natural alleles, resistance to potyviruses can also be associated with *de novo* eIF4E mutants: in *Lycopersicon esculentum*, a *eIF4E1 KO* allele was obtained that made plants resistant to potyviruses. The results presented here highlight that in some cases the crop improvement strategy of pyramiding resistance using eIF4E KO alleles will be limited by gametophyte lethality. As eIF4E proteins are implicated in essential cell processes, it may be necessary to rely more on functional resistance alleles like the ones produced by natural variation to reinforce strategies to enlarge the resistance spectrum or to limit the spread of overcoming strains.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Table 1. Gametophytic transmission of eif4e1 and eifiso4e alleles in a backcross of eif4e1/eIF4E1; eifiso4e/eIFiso4E plants to wild-type Columbia plants**

| Gamete genotypes | eIF4E1 | eIF4E1 | eIF4E1 | eIF4E1 | eIF4E1 |
|------------------|--------|--------|--------|--------|--------|
| Unbiased         | 25%    | 25%    | 25%    | 25%    |        |
| Gametophytic lethal | 33%   | 33%    | 33%    | 0%     |        |
| Tmale            | 39%    | 30%    | 30%    | 0%     | 211    |
| Tfemale          | 25%    | 24%    | 29%    | 22%    | 249    |

*Predicted frequency of different allele combinations according to the type of segregation,* $^{a}$Tmale and $^{b}$Tfemale are respectively percentage of allele transmission in progenies of male and female backcrosses between double heterozygous mutant plants and wild type. *Total number of plants genotyped for each progeny.

**Table 2. Expression of the genes encoding translation initiation factors 4E in transcriptomes of the developing male gametophytes**

| AGI   | Gene Name | UNM | BCP | TCP | MPG |
|-------|-----------|-----|-----|-----|-----|
| At4g18041 | eif4e1   | 1770| 1807| 752 | 317 |
| At5g35620 | eifiso4e | 1327| 1338| 682 | 455 |
| At1g29550 | eif4e3   | 382 | 413 | 320 | 224 |
| At5g18110 | nCBP     | 658 | 564 | 293 | 236 |

Microarray data (gathered from Honys et al.) on relative gene expression during male gametophyte development. The development stages are: UNM, uninucleate microspores; BCP, bicellular pollen; TCP, tricellular pollen; and MPG, mature pollen grains.
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