Turnip mosaic virus (TuMV) Is Able to Use Alleles of Both *eIF4E* and *eIF(iso)4E* from Multiple Loci of the Diploid *Brassica rapa*

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Three copies of *eIF4E* and three copies of *eIF(iso)4E* have been identified and sequenced from a Turnip mosaic virus (TuMV)-susceptible, inbred, diploid *Brassica rapa* line, R-o-18. One of the copies of *eIF4E* lacked exons 2 and 3 and appeared to be a pseudogene. The two other copies of *eIF4E* and two of the three copies of *eIF(iso)4E* were isolated from a bacterial artificial chromosome library of R-o-18. Using an *Arabidopsis* line (Col-0::dSpm) with a transposon knock-out of the *eIF(iso)4E* gene which resulted in a change from complete susceptibility to complete resistance to TuMV, complementation experiments were carried out with the two versions of *eIF4E* and the two versions of *eIF(iso)4E*. When transformed into Col-0::dSpm, all four *Brassica* transgenes complemented the *Arabidopsis eIF(iso)4E* knock-out, conferring susceptibility to both mechanical and aphid challenge with TuMV. One of the copies of *eIF4E* did not appear to support viral replication as successfully as the other copy of *eIF4E* or the two copies of *eIF(iso)4E*. The results show that TuMV can use both *eIF4E* and *eIF(iso)4E* from *B. rapa* for replication and, for the first time, that a virus can use *eIF4E* and *eIF(iso)4E* from multiple loci of a single host plant.

A key step in plant mRNA translation involves the binding of the 5' cap to the 40S ribosomal subunit. This is achieved partly through the interaction of the cap with the eukaryotic initiation complex eIF4F, itself a combination of the cap-binding subunit eIF4E and the larger subunit eIF4G (Browning 1996). Isoforms of each component, eIF(iso)4E and eIF(iso)4G, also exist in plants (Allen et al. 1992). It is becoming clear that most eukaryotic organisms encode and express multiple eIF4E family members, some for general translation and others for specific functions, including control of translation (Rhoads 2009).

Arabidopsis thaliana possesses three genes encoding eIF4E (At4g18040, At1g29550, and At1g29590), one for eIF(iso)4E (At5g35620) and a further gene encoding a similar protein named novel cap-binding protein (nCBP) (At5g18110) (Ruud et al. 1998). The eIF4E and eIF(iso)4E proteins have distinctive functions. Whereas eIF4E is present in all tissues except the root specialization zones, eIF(iso)4E is particularly abundant in floral tissues and young tissue, and the proteins are

thought to have differing roles in plant metabolism and development (Rodriguez et al. 1998). Each is under different regulatory control pathways (Dinkova et al. 2000).

The first evidence that plant viruses interacted directly with the eukaryotic translation initiation complex of plants was provided when it was shown that the virus-encoded genome-linked protein (VPg) of Turnip mosaic virus (TuMV; genus *Potyvirus*), which binds to the 5' end of the viral RNA, bound to *A. thaliana* eIF(iso)4E in yeast two-hybrid binding assays (Wittmann et al. 1997). A recent comparison of TuMV VPg interactions with *A. thaliana* eIF(iso)4E, nCBP, and all three eIF4E proteins in the yeast two-hybrid system revealed that only eIF(iso)4E acted as a functional partner (Gallois et al. 2010). Furthermore, in an enzyme-linked immunosorbent assay (ELISA)-based system, VPg interacted with tagged *A. thaliana* eIF(iso)4E and, to a lesser extent, with tagged *A. thaliana* eIF4E (Léonard et al. 2000).

In contrast to the in vitro results, it is the *eIF(iso)4E* gene alone that is required for successful infection of *A. thaliana* by TuMV (Sato et al. 2005). Insertional mutagenesis of *At.eIF(iso)4E* using a defective maize transposon tagged with an herbicide resistance gene (*dSpm*) produced a plant line with no detectable mRNA or protein from the gene. This line is able to grow normally and is resistant to potyvirus infection, in particular, TuMV and *Lettuce mosaic virus* (Duprat et al. 2002). Additionally, an EMS-induced mutation of *A. thaliana* named *lsp1* also conferred loss of susceptibility to TuMV and other members of the *Potyviridae*; the mutation was found to be in *eIF(iso)4E* (Lellis et al. 2002). In contrast, TuMV is able to infect *A. thaliana* lines with T-DNA insertions in *eIF4E* (Sato et al. 2005), *eIF4G*, *eIF(iso)4G1*, or *eIF(iso)4G2* (Nicaise et al. 2007).

Since these original discoveries, there have been many reports of recessive resistance to *Potyvirus* spp. and members of certain other virus groups (*Cucumovirus*, *Carmovirus*, and *Bymovirus*) resulting from mutations in *eIF4E*, *eIF(iso)4E*, or the other components of the eukaryotic translation initiation complex, *eIF4G* or *eIF(iso)4G* (Robaglia and Caranta 2006). For example, the alleles *pvr1/2* (*Capsicum* spp.) confer resistance to *Potato virus Y* (Kang et al. 2005), *rym4/5/6* (barley) to *Barley yellow mosaic virus* (Kanyuka et al. 2005), *mo1* (lettuce) to *Lettuce mosaic virus* (Nicaise et al. 2003), *sbm1/wlv/cyv-2* (pea) to various viruses (Andrade et al. 2009; Bruun-Rasmussen et al. 2007; Gao et al. 2004), and *bc-3* (bean) to *Bean common mosaic virus* (Naderpour et al. 2010), and all correspond to *eIF4E*. Pea *sbm2*, acting against *Pea seed-borne mosaic virus*, is linked to *eIF(iso)4E* (Gao et al. 2004), *Capsicum pvr6* is a null allele of pepper *eIF(iso)4E* (Ruffel et al. 2002) and *retr01* in *Brassica rapa* has been suggested to be *eIF(iso)4E*

Nucleotide sequence data is available in the GenBank database under accession numbers HM131206 to HM131211.

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(Rusholme et al. 2007). Mutations in *eIF4G* and *eIF(iso)4G* have been identified as the resistance genes *cum1* and *rymv1*, respectively (Albar et al. 2006).

Here, we describe the identification and cloning of multiple copies of *eIF4E* and *eIF(iso)4E* from *B. rapa*. Using a *dSpm* transposon knock-out line of *Arabidopsis* (Duprat et al. 2002) we have been able to show, by complementation, that TuMV can utilize at least two copies of both *eIF4E* and *eIF(iso)4E*. This may help to explain why recessive resistance to TuMV in *Brassica* spp. is less common than recessive resistance to *Potyvirus* spp. in some other plant species.

RESULTS

Identification of *B. rapa* *eIF4E* and *eIF(iso)4E* genes.

The *B. rapa* line R-o-18 is highly inbred and uniformly susceptible to TuMV. Three copies of *eIF4E* and three copies of *eIF(iso)4E* were identified by polymerase chain reaction (PCR) on this line using primers designed to *Brassica* Genome Sequence Survey (GSS) and expressed sequence tag (EST) sequences. The PCR products were then used to probe a bacterial artificial chromosome (BAC) library of this line (Rana et al. 2004) to obtain the full-length genomic sequences. The structures of the genes were inferred from homology to *A. thaliana* genes and to *B. napus* EST sequences. Each gene consisted of five exons and four introns, with the exception of *BraA.eIF4E.b*, which lacked exons 2 and 3 and appeared to be a pseudogene (Fig. 1). Most of the variation between the *eIF4E* genes was due to size differences of intron 1 whereas the *eIF(iso)4E* genes differed markedly in the length of intron 3. Mapping has shown that *BraA.eIF4E.a*, *BraA.eIF4E.c*, *BraA.eIF(iso)4E.a*, and *BraA.eIF(iso)4E.c* are at different loci (Rusholme et al. 2007) (data not shown).

The sequences of the predicted proteins are shown in Figure 2. *BraA.eIF(iso)4E.a* and *BraA.eIF(iso)4E.c* were 91.0% identical to each other and 90.8 and 86.7% identical to *At.eIF(iso)4E*, respectively. *BraA.eIF4E.a* and *BraA.eIF4E.c* had 87.8% identity to each other but only 51.8 and 52.8% identity, respectively, to *At.eIF(iso)4E*.

B. rapa *eIF4E* or *eIF(iso)4E* genes complement an *eIF(iso)4E* knock-out in *A. thaliana* plants for TuMV infection.

In order to determine which of the *B. rapa* genes could be used by TuMV during the infection process, copies of the genes were transformed into a Sainsbury Laboratory *Arabidopsis* transposant line (SLAT) possessing a nonfunctional *At.eIF(iso)4E* gene (Col-0::dSpm). Because TuMV is unable to infect this line (Duprat et al. 2002), any transformed lines challenged with TuMV that were susceptible would indicate functional complementation by the *Brassica* transgene. *A. thaliana* lines were transformed with *BraA.eIF4E.a*, *BraA.eIF4E.c*, *BraA.eIF(iso)4E.a*, and *BraA.eIF(iso)4E.c*. *BraA.eIF4E.b* was a pseudogene and *BraA.eIF(iso)4E.b* was not found in the BAC library; therefore, they were not included.

Col-0 plants developed severe symptoms and were stunted within 2 weeks of challenge with TuMV CDN 1, whereas Col-0::dSpm plants remained symptomless, with no virus detected throughout the period of the test (Fig. 3). Infected plants were prone to secondary fungal infections and frequently rotted within 5 weeks of virus challenge. All mock- and virus-inoculated Col-0::dSpm plants remained symptomless and free of virus as determined by ELISA at 3 weeks postinoculation.

PCR tests on two to six plants per transformation confirmed the presence of wild-type *At.eIF(iso)4E* in Col-0 lines and the continued presence of the *dSpm* insertion in *At.eIF(iso)4E* in

Fig. 1. Structure of *Brassica rapa* *eIF4E* and *eIF(iso)4E* genes indicating positions of exons, introns, and generic primers used for probe production. Exons are indicated by black boxes and introns are thin lines. Sequences were determined outside the generic primer range using BACs or genome-walking.

all Col-0::*dSpm* plants tested. The T₂ plants segregated for the presence of the transgenes, and the presence of the selection marker (*bar* or *nptII*) was associated with the presence of the appropriate *Brassica* transgene. The absence of the selection marker corresponded with the absence of the *Brassica* transgene. Sequence checks confirmed there were no alterations in the transgenes.

Following TuMV challenge, plant symptoms were assessed visually and virus titers were estimated by ELISA followed by transformation for residual maximum likelihood (REML) analyses. Plants with symptoms were found to have significantly higher virus titers (ELISA optical density [OD] values) than plants with no symptoms ($P < 0.001$) (Table 1). Col-0 T₂ plants with *Brassica* transgenes appeared identical to the parental Col-

Fig. 2. Comparison of the *Arabidopsis thaliana* eIF(iso)4E protein sequence with predicted proteins from *Brassica rapa* line R-o-18 eIF(iso)4E and eIF4E genes. **A**, eIF(iso)4E proteins. Differences from the protein sequence At.eIF(iso)4E are indicated by shading. **B**, eIF4E proteins. Differences from the protein sequence BraA.eIF4E.c are indicated by shading.

Fig. 3. Complementation of *Arabidopsis thaliana* plants with *Brassica rapa* eukaryotic translation initiation factor genes, following challenge by mechanical inoculation with Turnip mosaic virus 20 days postinoculation. **A**, Col-0 mock inoculated; **B**, Col-0; **C**, Col-0::*dSpm*; **D**, Col-0 with transgenes; **E**, Col-0::*dSpm* with transgenes; **i**, BraA.eIF4E.a; **ii**, BraA.eIF4E.c; **iii**, BraA.eIF(iso)4E.a; and **iv**, BraA.eIF(iso)4E.c. The *dSpm* transposon insertion is in At5g35620, inactivating the gene At.eIF(iso)4E.

0 both visually (Fig. 3) and by ELISA (Table 1), indicating no effect of the transgene in this background on virus replication. Col-0::dSpm T₂ plants lacking *Brassica* transgenes remained uninfected, like the parental Col-0::dSpm line. T₂ Col-0::dSpm plants with *Brassica* transgenes produced symptoms as severe as the Col-0 line, with the exception of Col-0::dSpm transformed with *BraA.eIF4E.a*, where plants had notably milder symptoms. There were significant ($P < 0.001$) differences in the amount of virus detected between lines with symptoms (Table 1, occasion no. 1), although limited numbers of plants were analyzed (both as individual families and by pooling families per transgene). Only Col-0::dSpm families transformed with *BraA.eIF4E.a* had a significantly ($P < 0.05$) lower virus titer than infected Col-0 plants. When larger numbers of plants from one family per transgene were tested (Table 1, occasion no. 2), results confirmed that the mild symptoms seen on Col-0::dSpm plants with the *BraA.eIF4E.a* transgene related to a significantly ($P < 0.05$) lower virus titer than the other infected lines.

Plants challenged with TuMV using aphids were less efficiently infected (all potentially susceptible controls were not infected), and produced milder symptoms than those challenged mechanically. The virus titer as measured by ELISA was also significantly reduced ($P < 0.001$) (Table 2). The lower levels of symptom severity and virus titer achieved by aphid transmission are likely to reflect a lack of TuMV acquisition by aphids or a smaller viral inoculum load.

The symptoms were remarkable in the consistency of visual severity between plants within any one T₂ family (derived from a T₁ plant with a *Brassica* transgene); plants appeared either stunted to equal degrees or totally healthy. There was

also consistency in visual symptom severity between plants of different families containing the same *Brassica* transgene. If the T₁ plants had possessed a single copy of the transgene, the T₂ families would be expected to segregate 3:1 for the presence of the transgene, with a quarter of plants having two copies of the transgene, half having one copy (heterozygotes), and one quarter having no copies. The consistency of infection levels (visual or ELISA) suggested that homozygous and heterozygous transgenic plants could not be distinguished on the basis of degree of virus susceptibility.

DISCUSSION

It has been shown that very few *Potyvirus* spp. use eIF4E in one host and eIF(iso)4E in another host for protein translation; for example, *Tobacco etch virus* (TEV) uses eIF4E in pepper (Kang et al. 2005) and tomato (Ruffel et al. 2005) but eIF(iso)4E in *A. thaliana* (Lellis et al. 2002). Also, it is very rare that a *Potyvirus* sp. uses both eIF4E and eIF(iso)4E in the same host; for example, *Pepper vein mottle virus* (PVMV) uses both in pepper (Ruffel et al. 2006). Because TuMV infection of *A. thaliana* requires functional eIF(iso)4E (Duprat et al. 2002; Lellis et al. 2002), we asked whether the same preference would be shown for genes from *B. rapa*, an economically important host. Additionally, because *B. rapa* possesses multiple loci of *eIF4E* and *eIF(iso)4E*, we investigated the ability of TuMV to use the genes at the different loci. A requirement for a particular gene would be an excellent target for the basis of recessive resistance, either natural or engineered via gene silencing (Zhang et al. 2006). Alternatively, it would be impor-

Table 1. Phenotypes of transgenic *Arabidopsis thaliana* plants possessing *Brassica rapa* eukaryotic translation initiation factor genes following mechanical inoculation with *Turnip mosaic virus* (visual symptoms and enzyme-linked immunosorbent assay [ELISA])^a

Brassica transgene	T ₂ family	No. of plants with symptoms ^b				Mean ELISA optical density (no. of plants) ^c			
		Col-0		Col-0::dSpm		Col-0		Col-0::dSpm	
		0	SM	0	SM	0	SM	0	SM
Occasion no. 1									
None	...	0	12	12	0	–	1.77 (3)	–0.03 (12)	–
BraA.eIF4E.a	i	0	12	4	8	–	1.58 (3)	0.00 (2)	0.33 (2)
	ii	0	12	1	11	–	1.65 (3)	0.01 (1)	0.28 (3)
	iii	0	12	4	8	–	1.74 (3)	0.00 (2)	0.40 (3)
	iv	0	12	7	5	–	1.73 (3)	0.02 (2)	0.28 (2)
	Total	0	48	16	32	–	1.68 (12)	0.01*** (7)	0.32* (10)
BraA.eIF4E.c	i	0	12	5	7	–	1.64 (3)	0.02 (1)	1.13 (3)
	ii	0	12	2	10	–	1.57 (3)	0.03 (1)	1.21 (3)
	iii	0	12	2	10	–	1.30 (3)	0.00 (1)	0.75 (3)
	iv	0	12	1	11	–	1.70 (3)	0.03 (1)	0.62 (3)
	Total	0	48	10	38	–	1.55 (12)	0.02*** (4)	0.93 (12)
BraA.eIF(iso)4E.a	i	0	12	2	10	–	1.54 (3)	0.04 (1)	1.53 (3)
	ii	0	12	0	12	–	1.60 (3)	–	0.53 (4)
	iii	0	12	3	9	–	1.31 (3)	0.01 (1)	1.01(3)
	iv	0	12	3	9	–	1.40 (3)	0.01 (1)	0.81 (3)
	Total	0	48	8	40	–	1.50 (12)	0.02*** (3)	0.94 (13)
BraA.eIF(iso)4E.c	i	0	12	7	5	–	1.70 (3)	0.01 (2)	0.25 (2)
	ii	0	12	5	7	–	1.60 (3)	0.02 (2)	1.15 (2)
	iii	–	–	0	12	–	–	–	0.58 (4)
	iv	–	–	1	11	–	–	0.02 (1)	0.92 (3)
	Total	0	24	13	25	–	1.65 (6)	0.02*** (5)	0.77 (11)
Occasion no. 2									
None	...	0	12	12	0	–	1.14 (12)	0.00*** (12)	–
BraA.eIF4E.a	i	0	12	6	6	–	1.18 (12)	–0.01*** (6)	0.17* (6)
BraA.eIF4E.c	i	0	12	3	9	–	1.17 (12)	–0.01*** (3)	1.23 (9)
BraA.eIF(iso)4E.a	i	0	12	0	12	–	1.20 (12)	–	1.21 (12)
BraA.eIF(iso)4E.c	i	0	12	11	1	–	1.26 (12)	–0.03*** (11)	1.41 (1)

^a dSpm transposon insertion is in At5g35620, inactivating the gene *At.eIF(iso)4E*. Symptoms observed: 0 = no symptoms, SM = systemic mosaic with stunting and leaf deformation, – = not applicable.

^b Twelve plants inoculated per T₂ family. Families have not been selected for transgene homozygosity and are segregating for the presence of the transgene.

^c ELISA optical density measured at 405 nm after 2 h. Statistically significant differences (by residual maximum likelihood) compared with Col-0 plants with no transgene are indicated by * and *** ($P < 0.05$ and 0.001 , respectively).

tant to know whether TuMV is able to use different copies of *eIF4E* or *eIF(iso)4E* or whether it has a requirement for more than one copy, in order to deploy such resistance in *B. rapa*.

Our results are unusual in that TuMV exhibits the ability to use four different translation initiation factor proteins to counteract the absence of At.eIF(iso)4E in the knock-out *A. thaliana* line. This is the first example of a virus being able to use different copies of *eIF4E* and *eIF(iso)4E* from multiple loci of the same plant. The use of simultaneous knock-outs of At.eIF(iso)4G1 and At.eIF(iso)4G2 has been reported to prevent TuMV infection of *A. thaliana*, whereas plants with only one of the two genes inactivated are susceptible (Nicaise et al. 2007). However, the ability to use four isoforms (two each of BraA.eIF4E and BraA.eIF(iso)4E) from one host species is remarkable. A slightly similar situation occurs for *Chilli veinal mottle virus* (ChiVMV), whose VPg interacts (in yeast two-hybrid studies) with both *Capsicum* eIF4E and eIF(iso)4E (Hwang et al. 2009). Mutations or gene silencing of both these genes together (also known as *pvr1/2* and *pvr6*) confers resistance to ChiVMV and PVMV (Ruffel et al. 2006), although it is unclear whether *Cap-sicum annuum* possesses other copies of either gene.

Of the four translation initiation factor genes, TuMV replication was least supported by BraA.eIF4E.a when expressed in *A. thaliana*. Further data would be needed to understand this; for example, whether the underlying cause is due to different levels of transcription or translation (and whether this is also the case in *B. rapa*) or due to protein structure. However, comparison of the protein sequences with the resolved structure of wheat eIF4E (Monzingo et al. 2007) revealed that the four *Brassica* proteins possess all the conserved residues noted as important in cap-binding and for stabilizing the structure of the protein, with the notable exception of a key tryptophan residue lacking in BraA.eIF4E.a (residue 145, replaced by phenylalanine).

It is likely that the viral protein interacting with the *B. rapa* eIF4E/eIF(iso)4E proteins is the VPg. A single amino acid substitution in TuMV VPg that can abolish the interaction with *Arabidopsis* eIF(iso)4E in yeast two-hybrid experiments simultaneously renders the virus unable to infect *B. perviridis* (a synonym for *B. rapa* var. *perviridis*) (Léonard et al. 2000). TuMV VPg has been reported to interact with one version of eIF(iso)4E from *B. rapa* in the yeast two-hybrid system (Hwang et al. 2009), although the authors did not describe different versions of the gene. Co-purification studies from *B. perviridis* have confirmed that TuMV VPg (as a precursor protein with or without the adjacent 6K protein and Pro protease sections) does interact in planta with eIF(iso)4E/eIF4E (Léonard et al. 2004). Infection by TuMV alters the profile of isomers found; Léonard and associates (2004) reported finding eIF(iso)4E in mock-inoculated and TuMV-infected plants but detectable levels of eIF4E in infected plants only. The two proteins appear to have different subcellular locations in *Nico-*

tiana benthamiana, eIF(iso)4E being primarily associated with rough endoplasmic reticulum and eIF4E with free ribosomes (Beauchemin et al. 2007). Because different forms of VPg are found in different cellular locations, Beauchemin and associates (2007) suggested that, in *N. benthamiana* at least, the 6K-VPg-Pro is the most likely form to be involved in viral translation (with eIF(iso)4E) and the VPg-Pro form may be involved in other cellular perturbations. TuMV VPg can bind to, but does not disrupt, the eIF(iso)4E-eIF(iso)4G complex, and it is believed to compete with capped cellular mRNAs for the translation machinery (Miyoshi et al. 2006; Plante et al. 2004). However, it should be noted that other potyviral proteins have associations with eIF4E, such as NIa interacting with eIF4E in yeast two-hybrid tests (Schaad et al. 2000) or CI being involved in eIF4E-based resistance breaking (Abdul-Razzak et al. 2009).

Kang and associates (2007) found that transforming tomato with *eIF4E* from the *pvr1* locus in pepper resulted in dominant resistance to several TEV strains and other *Potyvirus* spp. In our experiments, wild-type Col-0 *A. thaliana* plants transformed with *eIF4E*, or *eIF(iso)4E* from *B. rapa*, were as susceptible to TuMV as untransformed wild-type plants.

Of the known plant virus resistances where heritability has been characterized, recessive resistances are more common for *Potyvirus* spp. than for viruses of other families; 64% of the examples listed by Díaz-Pendón and associates (2004) corresponded to *Potyvirus* spp. In their review of sources of resistance to *Potyvirus* spp., Provvidenti and Hampton (1992) showed that, where heritability had been determined, 60 genes were dominant and 39 were recessive. They listed two examples of recessive resistance, compared with three examples of dominant resistance in *Brassica* spp. A review of resistances to TuMV listed four dominant and three recessive resistances (Shattuck 1992). Despite a large amount of screening over a prolonged period, we have found that recessive resistances to TuMV are relatively scarce in *Brassica* spp.; we have mapped six dominant resistance genes and one recessive gene (Walsh and Jenner 2002) and it took a screen of more than 3,000 lines of the diploid *B. rapa* (Liu et al. 1996) to find the latter. This recessive resistance was effective against a range of TuMV isolates from different parts of the world and representing different serotypes, pathotypes, and genetic groups (Walsh et al. 2002) and, thus far, has not been broken. Two loci controlling the resistance were mapped; the first gene, *retr01*, was recessive and the second, *ConTR01*, was dominant (Rusholme et al. 2007). We also showed that the A genome of *B. napus* (derived from *B. rapa*) appears to have three *eIF4E* loci and three *eIF(iso)4E* loci and that *retr01* appeared to be coincident with one of the *eIF(iso)4E* loci and *ConTR01* appeared to be coincident with one of the loci of *eIF4E*, or possibly one of the other loci of *eIF(iso)4E* (Rusholme et al. 2007). A possible explanation for the relative scarcity of recessive resistances to TuMV in *Brassica* spp. is

Table 2. Comparison of mechanical and aphid inoculation on degree of infection by Turnip mosaic virus of transgenic *Arabidopsis thaliana* plants possessing *Brassica rapa* eukaryotic translation initiation factor *eIF(iso)4Ea*

Brassica transgene	Inoculation method	No. of plants with symptoms ^b				Mean ELISA optical density ^c			
		Col-0		Col-0::dSpm		Col-0		Col-0::dSpm	
		0	SM	0	SM	0	SM	0	SM
None	Mechanical	0	5	5	0	—	2.67	0.15***	—
	Aphid	2	3	5	0	0.05***	0.52***	0.04***	—
BraA.eIF(iso)4E.a	Mechanical	0	12	4	8	—	2.72	0.11***	2.58
	Aphid	6	6	7	5	—	0.48***	0.04***	0.41***

^a dSpm transposon insertion is in At5g35620, inactivating the gene *At.eIF(iso)4E*. Symptoms observed: 0 = no symptoms, SM = systemic mosaic with stunting and leaf deformation, and — = not applicable.

^b Twelve plants inoculated per T₂ family. Families have not been selected for transgene homozygosity and are segregating for the presence of the transgene.

^c Enzyme-linked immunosorbent assay (ELISA) optical density measured at 405 nm after 1.5 h, all plants were tested. Statistically significant differences (by residual maximum likelihood) compared with Col-0 mechanically-inoculated plants are indicated by *** (*P* < 0.001).

that, for a plant to be resistant, all copies of *eIF4E* and *eIF(iso)4E* would have to be nonfunctional for TuMV. In amphidiploid *Brassica* spp. (*B. napus*, *B. juncea*, and *B. carinata*), recessive resistance based on *eIF4E* or *eIF(iso)4E* is potentially less likely, because there appear to be six loci of each gene present (three of each in the A genome and three of each in the C genome) (Rusholme et al. 2007).

MATERIALS AND METHODS

TuMV.

TuMV CDN 1 is a pathotype 4 isolate (Walsh 1989) able to overcome several dominant *Brassica* resistance genes. It was maintained in *B. juncea* cv. Tendergreen by mechanical inoculation as previously described (Walsh 1989). Aphid transmission was performed using *Myzus persicae* cultures raised on *B. napus* cv. Mikado. Aphids were starved for approximately 2 h, then allowed to feed on infected *B. juncea* leaves for a few minutes before they were transferred onto test plants (five per plant) for 2 days and, finally, killed using Aphox (Syngenta, Cambridge) insecticide.

Plant lines.

B. rapa subsp. *trilocularis* (yellow sarson) line R-o-18 is an inbred line highly susceptible to TuMV (Hughes et al. 2002). A BAC library of genomic DNA of R-o-18 (JBr, using vector pBIBAC2, average insert size 128 kb) was made by I. Bancroft, John Innes Centre (Norwich, U.K.) (Rana et al. 2004).

A. thaliana Col-0 is highly susceptible to TuMV infection. Col-0::*dSpm* is a SLAT line that is homozygous for a transposon (*dSpm*) insertion in exon 2 of At5g35620 (*At.eIF(iso)4E*) (Duprat et al. 2002). The transposon carries resistance to the herbicide glufosinate ammonium. The presence and location of the transposon was verified using the primer pairs *dspm1-4E2K01* and *dspm2-4E2K04* as described previously (Duprat et al. 2002). Col-0::*dSpm* is resistant to infection by TuMV CDN 1.

Identification of full-length *eIF* gene sequences from *B. rapa*.

Genomic DNA was extracted from young R-o-18 leaves using a DNeasy plant mini kit (Qiagen, Crawley, U.K.) followed by amplification using the GenomiPhi system (GE Healthcare, Little Chalfont, U.K.), both processes performed according to the manufacturers' recommendations.

Generic primers were designed using GSS and EST sequences with homology to known *eIF4E* and *eIF(iso)4E* genes to encompass the majority of the open reading frame (ORF) (Table 3). The sequences used for *eIF4E* were from *B. oleracea* (BH540903, BZ068573, and BZ449347), *B. rapa* (CX270207,

CX270456, and DY010188), and *B. napus* (CB686150, CD834916, CN733851, CX188254, CX189337, CX194455, CX195737, and CX280257); for *eIF(iso)4E*, sequences were from *B. oleracea* (BZ436642, BZ514129, EH413959, EH414327, EH425132, EH426021, and EH426034), *B. rapa* (CX266566 and CX268848), and *B. napus* (D. Lydiate and E. Higgins, personal communication).

PCR was performed on genomic DNA using standard protocols with *Taq* DNA polymerase (Invitrogen, Paisley, U.K.). Primer pair BR1-BR2 produced multiple products of differing sizes from which could be separated *BraA.eIF(iso)4E.a* (1,215 bp), *BraA.eIF(iso)4E.b* (1,123 bp), and *BraA.eIF(iso)4E.c* (995 bp). Primer pairs BR6-BR7 and BR20-BR21 amplified most of the coding region of *BraA.eIF4E.a* (1,158 bp) and *BraA.eIF4E.c* (1,435 bp), respectively. PCR products were cloned into pCR2.1 (Invitrogen) or pMOSBlue (GE Healthcare) prior to sequencing at least three colonies with the Big-Dye Terminator system (Applied Biosystems, Warrington, U.K.) with products run on an ABI Prism 3130xl Genetics Analyzer (Applied Biosystems).

The PCR products were labeled with ³²P dCTP using the RediPrimeII DNA labeling system (GE Healthcare) and standard protocols. The radiolabeled probes were hybridized to filters printed with 36,864 colonies of the JBr BAC library. Potential bacterial clones of interest were identified, purchased, and subjected to a further PCR using the original primers to verify the genes' presence. Between one and three BAC per gene were thus identified (e.g., *BraA.eIF4E.a* in JBr034F07, *BraA.eIF4E.c* in JBr039L10, *BraA.eIF(iso)4E.a* in JBr043O19, and *BraA.eIF(iso)4E.c* in JBr040N12), although no BAC with *BraA.eIF(iso)4E.b* was found. Two BAC (JBr042K14 and JBr043K20) were found to contain an *eIF4E*-like gene that lacked exons 2 and 3 and had stop codons in the normal reading frame of exons 1 and 4. This defective gene was designated *BraA.eIF4E.b*.

Primers matching the PCR product sequences were designed to extend the known sequence into the BAC DNA. Sequence was determined upstream and downstream of the genes in a stepwise manner for approximately 2 kb in each direction beyond the start and stop codons of the ORF (Supplementary Table 1).

Construction of binary vectors.

Having identified the start and stop codons of the ORF, primers BR57 to BR64 (Table 3) were used to amplify the *Brassica* genes together with likely promoters (200 to 600 bp upstream of the start codon). In the case of *BraA.eIF4E.a*, the upstream primer location was restricted by the need to avoid an upstream ORF discovered in close proximity. Downstream regions were chosen to include likely sites of polyA tail attach-

Table 3. Primers used for the identification and cloning of *Brassica rapa* *eIF4E* and *eIF(iso)4E* genes

Primer	Sequence (5'–3')	Genes	Location	Direction
BR1	ATGGCGACAGAGGATGTGAACG	<i>BraA.eIF(iso)4E.a, b, c</i>	Exon 1	Forward
BR2	TCTCCTTCCACTTCTTCCCAATAC	<i>BraA.eIF(iso)4E.a, b, c</i>	Exon 4	Reverse
BR6	CACTCTCAAGCCTAATGTCGC	<i>BraA.eIF4E.a</i>	Exon 1	Forward
BR7	AGAGGTTGTAGGTTTCAGGCAGTG	<i>BraA.eIF4E.a</i>	Exon 5	Reverse
BR20	CAATGGCGGTAGAAGACACTT	<i>BraA.eIF4E.b, c</i>	Exon 1	Forward
BR21	CATGGATGATGAAACCAATGT	<i>BraA.eIF4E.b, c</i>	Exon 4	Reverse
BR57	AAAAAGCAGGCTTTTGGTCTGCAGTTATGTTATTAG	<i>BraA.eIF4E.a</i>	Upstream	Forward
BR58	AGAAAGCTGGGTAAAAAGGCTTGCGAGTCA	<i>BraA.eIF4E.a</i>	Downstream	Reverse
BR59	AAAAAGCAGGCTTAGGACAAATGATATGGGGAGAGT	<i>BraA.eIF4E.c</i>	Upstream	Forward
BR60	AGAAAGCTGGGTAGCTTGGCGACCTTTTGA	<i>BraA.eIF4E.c</i>	Downstream	Reverse
BR61	AAAAAGCAGGCTACCCCTTAGTATCAATTAATTTCAGAAC	<i>BraA.eIF(iso)4E.a</i>	Upstream	Forward
BR62	AGAAAGCTGGGTTCTGGTCGAAATCAATAAGA	<i>BraA.eIF(iso)4E.a</i>	Downstream	Reverse
BR63	AAAAAGCAGGCTTTTTTAAAGATGGAGGGAGTAT	<i>BraA.eIF(iso)4E.c</i>	Upstream	Forward
BR64	AGAAAGCTGGGTGAAGCGCGGTCAAAAT	<i>BraA.eIF(iso)4E.c</i>	Downstream	Reverse

ment as found in comparable *Brassica* EST. The sizes of the cloned regions were *BraA.eIF4E.a*, 2,144 bp; *BraA.eIF4E.c*, 2,758 bp; *BraA.eIF(iso)4E.a*, 2,076 bp; and *BraA.eIF(iso)4E.c*, 1,818 bp.

The genes were amplified from the BAC using KOD Hotstart DNA polymerase (Merck, Nottingham, U.K.), and *attB* adapters were added by PCR with the same enzyme and Gateway primers *attB1* and *attB2* (Invitrogen). The products were cloned into the Gateway Entry plasmid pDONR221 using BP clonase (Invitrogen).

Binary vectors pB2GW7 and pK2GW7 (Karimi et al. 2002) contain a 35S constitutive promoter, a 35S terminator, and glufosinate ammonium herbicide resistance (*bar*) or kanamycin resistance (*nptII*), respectively, for in planta selection. The *Brassica* genes were transferred from the Entry vector into the binary vectors using an LR clonase-mediated reaction (Invitrogen). Sequences were verified at all stages of the cloning.

Transformation of *B. rapa* genes into *Arabidopsis thaliana*.

Purified plasmid DNA of each construct was electroporated into *Agrobacterium tumefaciens* GV3101 pMP90 using standard techniques. The presence and stability of the transgenes was verified by PCR.

Arabidopsis thaliana Col-0 and Col-0::dSpm lines were grown and transformed with *Agrobacterium tumefaciens* containing the constructs using the floral dip method (Clough and Bent 1998). Constructs in pB2GW7 were used to transform Col-0; pK2GW7 was used to transform Col-0::dSpm. Each *Brassica* gene was transformed into each plant line (Table 4).

The primary transformed plants were allowed to set seed and the T₁ seed was harvested. For selection using glufosinate ammonium, seed was grown in trays on F2S seed compost (Levington, Suffolk, U.K.) that was drenched every 3 to 4 days with glufosinate ammonium (Harvest; Bayer Crop Science, Cambridge) at 150 mg/liter for 3 weeks. For selection using kanamycin, seed was grown in deep petri dishes on 0.5× Murashige and Skoog medium (Duchefa Biochemie, Melford Laboratories, Ipswich, U.K.) with kanamycin (Sigma-Aldrich, Gillingham, U.K.) at 50 µg/ml for 3 weeks. Genomic DNA from individual T₁ survivors was obtained as above and PCR was performed to check for the presence of the transgenes (*Brassica* genes and selection markers) prior to potting on the plants in compost without selection to allow the plants to flower and to set seed (T₂).

T₂ plants were grown and genomic DNA was extracted as above. PCR checks were performed using the *Brassica* primers used to generate the inserts for the binary clones with KOD Hotstart DNA polymerase. The products were sequenced to confirm that there were no errors in the final transgenes. The continued presence of dSpm in *At.eIF(iso)4E* was also checked in appropriate lines.

TuMV challenge of *A. thaliana* lines transformed with *B. rapa* genes.

For each transformed line, seed of up to four T₂ families (-1 to -4), derived from different T₂ individuals, were sown. Only

Table 4. Designations of lines of *Arabidopsis thaliana* transformed with *Brassica rapa* genes

Brassica gene	<i>A. thaliana</i>	
	Col-0	Col-0::dSpm ^a
<i>BraA.eIF4E.a</i>	TP085	AT25
<i>BraA.eIF4E.c</i>	TP082	AT22
<i>BraA.eIF(iso)4E.a</i>	TP083	AT23
<i>BraA.eIF(iso)4E.c</i>	TP084	AT24

^a Possesses dSpm transposon insertion in At5g35620, inactivating the gene *At.eIF(iso)4E*.

two families were produced from the transformation of Col-0 with *BraA.eIF(iso)4E.c*. Fifteen plants of each family were sown in F2S compost and grown at 20°C with a 9-h day length to maintain the vegetative state. After 33 days, four to six true leaves of 12 plants were mechanically inoculated using TuMV-infected *B. juncea* leaf sap and a cotton bud to hold the inoculum. The remaining plants were inoculated with buffer only.

Symptoms were assessed visually each week for 4 weeks. At 3 weeks postinoculation, leaf samples from two plants were collected for genomic DNA extraction. The presence of *Brassica* transgenes, selection marker genes, and the dSpm transposon in *At.eIF(iso)4E* were all checked by PCR. TuMV presence in three other plants was assayed by plate-trapped antigen ELISA using the mouse monoclonal antibody EMA67, essentially as previously described (Jenner et al. 1999), in order to confirm that symptoms were due to viral infection and not transgene presence. Leaf sap was extracted and diluted with 1 ml of water, then diluted one-third before loading on microtiter plates. OD values were measured after 2 h, or earlier if approaching the maximum measurable by the ELISA plate reader. Because infection of all the families containing the transgenes in the first experiment was unexpected, determination of virus titers in every individual plant was technically difficult. Therefore, the experiment was repeated for each of the four transgenes using mechanical inoculation, examining every individual plant of a single family per transgene (family i: TP085, TP082, TP083, TP084, AT25, AT22, AT23, and AT24) in order to determine the virus titer. A further experiment examined one family of lines possessing the *BraA.eIF(iso)4E.a* transgene (family iv: TP083 and AT23) in order to compare mechanical inoculation with aphid inoculation directly.

Due to the nature of the design of the experiments, data were analyzed using REML. REML allows estimation of variance components and treatment effects of a linear model with both fixed and random effects. It can be used, as in this case, to analyze unbalanced data sets. Data were transformed according to $\log_{10}(100 \times [\text{mean OD} + 0.05])$, then analyzed for each experimental occasion.

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