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ORIGINAL PAPER

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Identification of markers tightly linked to *sbm* recessive genes for resistance to *Pea seed-borne mosaic virus*

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Abstract Two virus resistance loci on linkage groups II and VI have provided the only sources of natural resistance against *Pea seed-borne mosaic virus* (PSbMV, Potyviridae) in the important crop plant *Pisum sativum* L. A combination of parallel approaches was used to collate linked markers, particularly for *sbm-1* resistance on linkage group VI. We have identified sequences derived from the genes for the eukaryotic translation initiation factors *eIF4E* and *eIF(iso)4E* as being very tightly linked to the resistance gene clusters on linkage groups VI and II, respectively. In particular, no recombinants between *sbm-1* and *eIF4E* were found amongst 500 individuals of an F_2 cross between the BC_4 resistant line (JI1405) and its recurrent susceptible parent 'Scout'. In a different mapping population, the gene *eIF(iso)4E* was also shown to be linked to *sbm-2* on linkage group II. A parallel cDNA-AFLP comparison of pairs of resistant and susceptible lines also identified an expressed tag marker just 0.7 cM from *sbm-1*. *eIF4E* and *eIF(iso)4E* have been associated with resistance to related viruses in other hosts. This correlation strengthens the use of our markers as valuable tools to assist in breeding multiple virus resistances into peas, and identifies potential targets for resistance gene identification in pea.

Introduction

Pea seed borne mosaic virus (PSbMV), in the genus *Potyvirus* in the family Potyviridae, is a pathogen of peas, broad beans, lentils and chickpeas and causes major crop losses. The disease is currently managed largely through the elimination of transmitting insects from the crop and by identifying and discarding batches of infected seed (Khetarpal and Maury 1987). PSbMV is represented by a large number of isolates and strains. With respect to those that infect peas and lentils, these have been grouped on the basis of their reaction to pea indicator lines of varying susceptibility into pathotypes P1, P4 and L1 (sometimes referred to as P2).

In 1971, Hagedorn and Gritton identified two Ethiopian lines of pea (PI193586 and PI193835) that exhibited resistance to the common strains of PSbMV (pathotype P1). They also showed that this trait was conferred by a single recessive resistance gene (*sbm*) (Stevenson and Hagedorn 1971; Hagedorn and Gritton 1973). This locus was found to be linked to *wlo* and *p* on linkage group (LG) VI (Gritton and Hagedorn 1975; Hampton and Marx 1981). Later, Provvidenti and Alconero (1988) found that cultivar 'Bonnevillle' showed resistance to the lentil (L1) strain of PSbMV and that this was closely linked to *mo*, a gene conferring resistance to two other potyviruses, *Bean yellow mosaic virus* (BYMV) and *Watermelon mosaic virus* (WMV). They also showed that a second gene, linked to *sbm* on LG VI conferred resistance to PSbMV L1. Gene *mo* is also linked with *k* (winged keel). These and later studies (Provvidenti and Alconero 1988; Provvidenti and Muehlbauer 1990; Providenti et al. 1991) have provided a picture of potyvirus resistance in pea based upon two clusters of recessive resistance genes specifying resistance to a diverse range of potyviruses (reviewed in Provvidenti and Hampton 1991). The locus on LG VI gives resistance to PSbMV P1 (*sbm-1*), PSbMV L1 (*sbm-3*), PSbMV P4 (*sbm-4*), *Clover yellow vein virus* (ClYVV; *cyv-2*) and *White lupin mosaic virus* (*wlv*). The locus on LG II gives independent resistance to PSbMV L1 (*sbm-2*) and resistance to BYMV and WMV (*mo*), *Bean common mosaic virus* (*bcm*), ClYVV (*cyv-1*), and *Pea mosaic virus* (*pmv*).

Through linkage of these loci with morphological markers, the introgression of the resistances into commercial lines without virus testing was possible (Provvidenti et al. 1991). However, because of the genetic distances

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involved and the potential existence of a modifier-gene system, caution was expressed as to the reliability of this approach (Hampton 1980). Hence, many commercial varieties of pea remain susceptible to the more common strains (P1 and P4) of PSbMV, presumably reflecting problems in manipulating the LG VI locus in commercial breeding strategies.

In addition to the recessive resistance loci in pea, other plant species exhibit recessive resistance to potyvirus infection. In lentil, the *sbv* gene confers resistance to PSbMV (Haddad et al 1978). In *Capsicum* spp., a series of recessive genes (*pvr1*, $pvr2^1$, $pvr2^2$ and *pvr3*; reviewed in Kyle and Palloix 1997) provide resistance against *Potato virus Y* (PVY), *Tobacco etch virus* (TEV) and *Pepper mottle virus* (PepMoV). Also in the Solanaceae, the *va* gene in *Nicotiana tabacum* cv. TN 86 confers resistance to *Tobacco vein mottling virus* (TVMV) (Deom et al. 1997). In lettuce, the *mo1* gene in lettuce confers resistance to *Lettuce mosaic virus* (LMV) (references in Nicaise et al. 2003). In the case of *pvr* and *mo1*, these resistances have been identified as separate allelic series for the eukaryotic translation initiation factor *eIF4E* (Ruffel et al. 2002; Nicaise et al. 2003).

As part of the larger goal of *sbm-1* characterisation from pea, we aimed to identify new linked markers that would assist in the breeding of virus resistance and in gene identification. By taking a candidate gene approach, we showed that *eIF4E* and *eIF(iso)4E* were tightly linked to *sbm-1* and *mo*, respectively. Our markers for *sbm-1* have been converted into PCR primers that can be conveniently used to assist in the breeding of virus resistance in pea.

Materials and methods

Plant and virus materials

Genetic mapping of *Pisum sativum* L. (pea) was carried out using two pairs of PSbMV P1-resistant or -susceptible lines. Resistant (*sbm-1*) JI1405 is a BC_4 line with the recurrent susceptible (*Sbm-1*) parent JI2009. These lines were originally received from Dr. F. Muehlbauer as X78122 (registered as PI595954) and 'Scout' (registered as W6 17534), respectively (Muehlbauer. 1983). Resistant (*sbm-1*) line 835 and its recurrent susceptible (*Sbm-1*) backcross parent, line 744 (also referred to as RCG1402), were obtained from Dr. R. Cousin, INRA, Versailles. The infection phenotype of these lines was confirmed. Segregation of markers with respect to *Sbm-1* and other known markers was assessed using the progeny of an F_2 cross between JI1405 and JI2009 and a series of recombinant inbred lines (RILs) generated from cross between pea lines JI15 and JI399. Markers mapping to LG II were analysed using an RI family (16 lines) generated from a cross between JI281 and JI399. All of the RILs were susceptible to PSbMV P1. Plants were grown in a glasshouse with a 14-h photoperiod and a temperature of 18–25°C.

PSbMV infection of susceptible peas leads to a systemic chlorosis and reduced growth and seed set. This is sometimes associated with virus seed transmission so contaminating subsequent generations (Maule and Wang 1996). To avoid this problem, an alternative approach was developed based upon the visualisation of a colorimetric reporter of infection in detached leaves. Virus infections were carried out using a recombinant PSbMV isolate DPD1 (pathotype P1) expressing β-glucuronidase from within the viral genome (PSbMV-GUS, Borgstrom and Johansen 2001). The inoculum was in the form of a DNA vector containing the PSbMV-GUS cDNA cloned between the CaMV 35S promoter and the nopaline synthase termination signal. The *GUS* gene is positioned between the P1 and helper-component-protease (*HC-Pro*) genes of the PSbMV polycistronic genome. The GUS protein is released from the viral polyprotein during the early stages of virus replication by excision by viral encoded protease activities. This recombinant virus is unchanged from the wild type isolate in its response to *sbm-1*. The plasmid DNA was coated onto gold particles (0.97-μm diameter, Christou et al. 1991) and used to inoculate the second and third leaves of pea seedlings using a hand-held microprojectile bombardment gun, essentially as described by Gal-On et al. (1997). After inoculation, leaves were removed and maintained in moistened petri dishes. Infection was visualised at 3 days post-inoculation (dpi) by incubating detached leaves in the β-glucuronidase substrate, X-Gluc (0.25 mg/ml in assay solution at 37°C overnight) [assay solution**:** 100 mM $NaPO_4$buffer, pH 7.0, 0.1% Triton X-100, 10 mM EDTA, 1% H_2O_2(30% solution)].

cDNA-AFLP

cDNA-AFLP analysis was carried out essentially as described in Bachem et al. (1996). Total RNA was extracted from young pea leaves using TRI reagent (Sigma), and mRNA was purified using the

Table 1 PCR primers

Primers		*T* (°C)	PCR products
eIF4E Med5′	5′CCGAAAGAGAGAGAACAAGC3′	60.7	*Medicago truncatula eIF4E* cDNA fragment, 745 bp
eIF4E Med3′	5′CATCTCTCATGCACTTGCTACC3′	63.6	
eIFexpress5′	5′ATGGTTGTAGAAGAAACCCC3′	82.0	Pea*eIF4E* cDNA fragment, 690 bp
eIFexpress3′	5′CGCTATACAGTTCATACAAC3′	74.2	
peaeIF4E5′	5′ACTGTTGAAGAGTTTTGGAGCATTTACAAT3′	67.8	Pea*eIF4E* genomic DNA fragment 1,870 bp
eIF4E3pea3′	5′CGCAATACAGTTCACACAACAT3′	61.7	
eIF-1	5′AAGAAAGTGGAGTTTCTGGTTCGAT3′	65.8	Pea*eIF(iso)4E* cDNA fragment, 306 bp
iso4E23′	5′AATTGCTCTCCAATCAAAGCCATCAA3′	70.7	
ZG1035′	5′ATGCTTGTTGATGGGTTTAGACGGTG3′	71.0	Dominant allele of ZG10, 147 bp
ZG10SNP	5′CTCAGCAAACGGAAAAGGGCAGGTC3′	74.6	
ZG1035′	5′ATGCTTGTTGATGGGTTTAGACGGTG3′	71.0	Recessive allele of ZG10, 147 bp
ZG10SNP2	5′CTCAGCAAACGGAAAAGGGCAGGTT3′	73.0	
4Egenomic5′	5′GAGCTAGATGGTTGTTATGATGTTTATCAG3′	65.3	Two alleles of pea *eIF4E*, 425 bp and 535 bp
4Egenomic3′	5′ATTCTCGATCACACTAGCCCCCTCC3′	71.7	

Dynabeads mRNA purification kit (Dynal AS, Oslo, Norway). First- and second-strand cDNA synthesis was carried out according to standard protocols (Sambrook and Russell 2001) using Superscript II reverse transcriptase (Invitrogen), RNase H and DNA polymerase I. First-strand cDNA synthesis was primed with a degenerate poly-T primer (GTCGAC*CTGCAG*GCGT$_{19}$V; V=A, G, C), carrying a *Pst*I site. This provided a *Pst*I site 3′to the poly A of synthesised cDNAs. The template for cDNA-AFLP was prepared using *Pst*I and *Mse*I. The sequences of primers and adapters used for AFLP reactions are as follows (N can be any nucleotide): *Pst*I adapter: top strand 5′ CTCGTAGACTGCGTACATGCA3′, bottom strand 5′TGTACG-CAGTCTAC3′; *Mse*I adapter: top strand 5′GACGATGAGTCCT-GAG3′, bottom strand 5′TACTCAGGACTCAT3′; *Pst*I pre-amplification primer: 5′GACTGCGTACATGCAG3′; *Mse*I pre-amplification primer: 5′GATGAGTCCTGAGTAA3′; *Pst*I selective amplification primer (*Pst*A-*Pst*Q): 5′GACTGCGTACATGCAGNN3′; *Mse*I selective amplification primer (*Mse*I-*Mse*XIV): 5′GATGAGTCCT-GAGTAANN3′.

Amplification products were separated on a 5% polyacrylamide gel run at 100 W until the bromophenol blue reached the bottom of the gel. Gels were dried and exposed to Kodak Biomax film (Sigma). Polymorphic bands were cut out from the dried gel after alignment with the autoradiograph. The DNA fragments were eluted from the excised gel pieces and re-amplified following the same PCR conditions and primer combinations.

One polymorphic cDNA-AFLP band (ZG10) was used as a marker for mapping. The sequence of this cDNA has been submitted to GenBank as accession AY423376.

Isolation of *eIF4E* and *eIF(iso)4E* cDNA fragments

To PCR-amplify a DNA product for *eIF4E*, two pairs of primers were designed from the mRNA sequence for *eIF4E* from *Medicago truncatula* (TC71850, http://www.tigr.org). These and other primers used for marker identification are listed in Table 1. Primers eIF4E Med5′and eIF4E Med3′were used to amplify a partial cDNA from *Medicago*. Primers eIFexpress5′and eIFexpress3′were used to amplify the homologous pea cDNA. Pea-specific primers peaeIF4E5′and eIF4E3pea3′, corresponding to nucleotides (nt) 308–337 and nt 725–747 on the *Medicago* coding sequence, were used to amplify pea genomic DNA at the *eIF4E* locus.

To amplify *eIF(iso)4E* cDNA, two primers (eIF-1 and iso4E23′) were designed from an alignment of several mRNA sequences from *Arabidopsis thaliana* (accession AF538308, U62044, AY086315, NM_122953, AY054630, Y10547). The amplified cDNA fragments were cloned into pGEM-T Easy vector (Promega, Madison, Wis.) and sequenced in both orientations. The sequence of the pea cDNA has been submitted to GenBank as accession AY423377.

Molecular markers and mapping

An F_2 population from a cross between JI1405 and JI2009 was generated. Individual F_2 plants were scored for their infection phenotype on excised leaves, and the plants were left to grow to maturity for the collection of F_3 seeds. Sixteen RILs each from crosses between JI281 × JI399 and JI 15 × JI399 were used for mapping new markers. Pea genomic DNA was prepared (Ellis 1994) and used either in southern analyses or as templates for genomic PCR assays.

Markers for *sbm-1*

For the JI15 × JI399 RILs, the ZG10 cDNA was used in an RFLP analysis of segregation. For the analysis of the JI1405 × JI2009 F_2 population and to develop a practical marker for marker-assisted selection of resistant lines, PCR primers were designed from the sequence of the cDNA-AFLP fragment, ZG10, and for *eIF4E*. For ZG10, the design was based on a single nucleotide polymorphism (SNP) found between homologous cDNAs from JI1405 and JI2009. Primers ZG1035′and ZG10SNP identified the dominant allele, whereas ZG1035′and ZG10SNP2 identified the recessive allele. Segregation for the JI1405 × JI2009 F_2 population was scored after visualising the PCR products on stained agarose gels. *eIF4E* cDNA provided a RFLP marker. This was also converted into a co-dominant PCR-based marker using primers 4Egenomic5′and 4Egenomic3′and used to score segregation in the same F_2 population.

Bin mapping *eIF(iso)4E*

The cloned *eIF(iso)4E* cDNA fragment was used as an RFLP marker following *Hin*dIII digestion of pea genomic DNA. The marker was mapped onto 16 RILs from JI281 × JI399. When a genetic map has been created for a mapping population (in this case an RI population), only a limited number of segregants is required to correlate a new marker with a known one showing identical segregation. The number of segregants can be determined empirically from the marker data by determining for a given set of x RILs how many unlinked markers have the same scores.

In general, where m is the number of lines with identical scores, and n is the number of lines with differing scores, the likelihood of the data under linkage (L_l) given a linkage value of r is given by:

$$L_l = (1-r)^m \, r^n \tag{1}$$

From Haldane and Waddington (1931), we have $r=R/2(1-R)$, where $R=n/(m+n)$. The likelihood of the data under no linkage (L_u) is the value of L_l where $r=0.5$. We can calculate the LOD score as $\mathrm{Log}_{10}(L_l/L_u)$, so the maximum possible LOD score is where $n=0$ and $r=0$.

$$L_{l(\max)} = 1 \tag{2}$$

$$L_u = 0.5^{(m+n)} \tag{3}$$

$$\begin{aligned}\mathrm{LOD}_{(\max)} &= \mathrm{Log}_{10}(L_l/L_u) = \mathrm{Log}_{10}\left(1/0.5^{(m+n)}\right)\\ &= \mathrm{Log}_{10}2^{(m+n)}\end{aligned} \tag{4}$$

Where $(m+n)=10$, the maximum LOD score is just over 3. So, from series of known markers scored for these lines, linkage and location of a new marker can be obtained with as few as ten RILs. If the data have a single mismatch, then for 16 RILs the LOD score is 3.12. Thus, for 16 RILs it is possible to position markers where the map is not recombination limited (i.e. a new marker may lie within an interval between two crossover events in the subpopulation in which there is no mapped marker). This procedure places markers with respect to an existing genetic map, but does not improve the genetic map. In essence, the procedure assigns markers to 'recombination bins'. These are sets of markers of identical scores and where the bin size is determined by the number and location of the crossover events in the selected lines.

For *sbm-1*, ZG10, *eIF4E* and *eIF(iso)4E*, segregation data were converted to map positions using the programme JoinMap (Stam 1993; Stam and van Ooijen 1995).

Results

Phenotypic analysis using a colourimetric reporter

Using PSbMV-GUS to assess the virus infection phenotype, the amount and distribution of GUS activity after 3 dpi clearly differentiated between susceptible and resistant

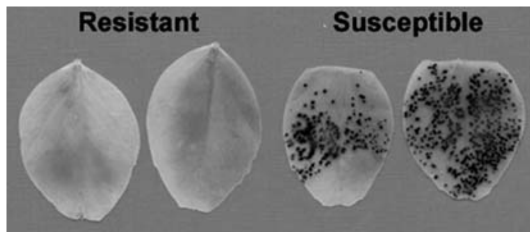

Fig. 1 Phenotypic assay for resistance and susceptibility based upon replication and expression of PSbMV DPD1-GUS. GUS staining at 3 days post-inoculation identifies distinct foci of infection not present in resistant leaves

leaves. The former showed large spreading lesions, whereas on resistant leaves no blue staining was visible (Fig. 1). All of the parental lines for the mapping populations were confirmed for their phenotype following infection with PSbMV P1. Lines JI2009, JI281, JI15, JI399 and 744 were susceptible; lines JI1405 and 835 were resistant.

cDNA-AFLP analysis of expressed genes in resistant and susceptible peas

Because of the large size of the pea genome and the abundance of repetitive DNA (Murray et al. 1978), we decided to concentrate upon expressed genes in the search for polymorphic markers. The strategy utilised *Pst*I and *Mse*I digestions and adapted primers for cDNA amplification. *Pst*I was selected since it is relatively abundant in the small set of known pea gene sequences in the EMBL database. A *Pst*I site was also included in the preamplification cDNA primer. Hence, all cDNAs had the potential to be recognised by the *Pst* series amplification primers and should have been identified as a discrete band if there was an internal *Mse*I site. A total of 256 amplifications had the potential to give complete genome coverage.

To assist in the identification of resistance gene linked cDNAs, we compared two resistant lines (JI1405 and 835) with two susceptible lines (JI2009 and 744) with each differential pair coming from different breeding lineages. A 'complete' genome survey identified only two polymorphic bands (Fig. 2a), detected with primer combinations *Pst*O/*Mse*II and *Pst*O/*Mse*V, which showed consistent segregation with resistance or susceptibility. The cDNAs were excised, reamplified and sequenced. The sequences showed that the two bands were alleles of the same gene. The nucleotide and amino acid sequences showed 88% and 78% identities with a galactosyltransferase gene from chromosome 4 of *A. thaliana* (accession NP_193838). The cloned fragments were used in a hybridisation analysis of pea genomic DNA. Only a single genomic band was detected after separate digestion with *Eco*RI, *Eco*RV and *Hin*dIII, indicating that the cDNA called ZG10 corresponded to a single gene in pea (data not shown). Based upon the nucleotide sequences of ZG10 from the two alleles, SNPs were identified and used to design allele-specific PCR primers (Fig. 2b). Segregation

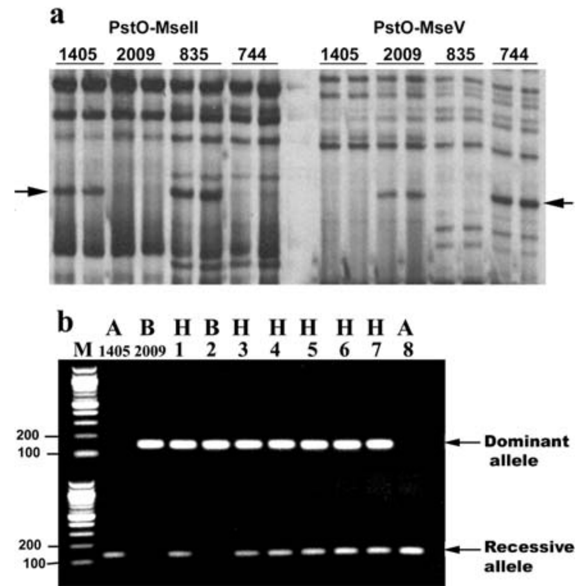

Fig. 2a, b Polymorphic marker ZG10 segregates in resistant and susceptible pea lines. **a** cDNA AFLP comparison between resistant (JI1405 and 835) and susceptible (JI2009 and 744) lines identified single polymorphic bands with each of two primer combinations, *Pst*0/*Mse*V and *Pst*0/*Mse*II. **b** Conversion of the ZG10 cDNA into a PCR-based co-dominant polymorphic marker. Segregation within the segregating F_2 population identified resistant (*A*), susceptible (*B*) and heterozygous lines (*H*)

of ZG10 in the F_2 population identified a map position at 0.7 cM away from *sbm-1*.

eIF4E co-localises with *sbm-1* on LG VI

Prompted by the genetic and functional association between *eIF4E* and resistance to potyvirus infection (Wittmann et al. 1997; Duprat et al. 2002; Lellis et al. 2002; Ruffel et al. 2002; Nicaise et al. 2003), we also followed a candidate gene approach to the identification of markers linked to *sbm-1*. Because *Medicago* is a close relative of pea, two primers, eIF4E Med5′and eIF4E Med3′, were designed from the *Medicago eIF4E* mRNA sequence. A 745-bp cDNA fragment was amplified from *Medicago*; these primers failed to amplify pea cDNA. However, using the fragment as a probe for Southern hybridisation of restriction-digested pea DNA, a hybridising region of pea DNA was identified. Restriction analysis indicated that this was a single-copy gene (Fig. 3a). *Hin*dIII digestion also identified a polymorphism between resistant and susceptible lines (Fig. 3a). The *Medicago* cDNA sequence contains two *Hin*dIII sites. To identify the origin of the polymorphic bands in pea, the three fragments from the *Hin*dIII digestion of the *Medicago* cDNA PCR fragment were used separately to probe replicate blots. Only the central *Hin*dIII fragment identified the smaller polymorphic bands (data not shown). Another pair of primers, eIFexpress5′and eIFexpress3′, was successful in amplifying a 658 bp *eIF4E* cDNA from pea line JI2009. The pea cDNA sequence,

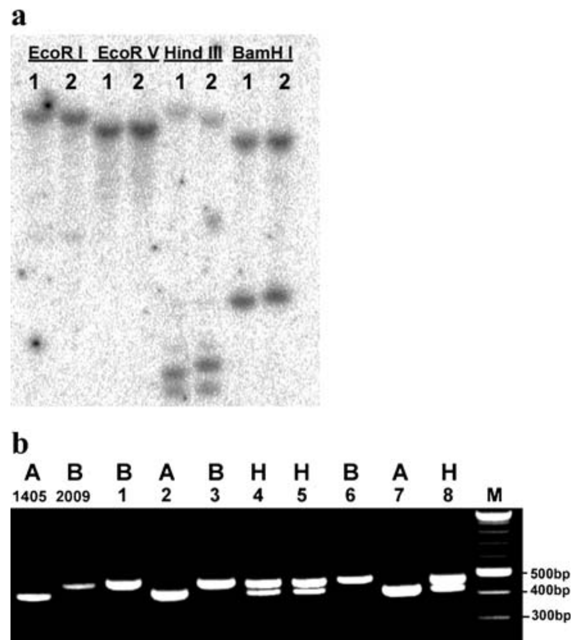

Fig. 3a, b Marker *eIF4E* shows tight linkage with *sbm-1*. **a** Southern hybridisation of pea genomic DNA from resistant line JI1405 (*1*) and susceptible line JI2009 (*2*) after digestion with *Eco*RI, *Eco*RV, *Hin*dIII or *Bam*HI, using the *Medicago eIF4E* fragment cDNA fragment as a probe. **b** Conversion of *eIF4E* genomic DNA into a PCR-based co-dominant polymorphic marker. Segregation within the segregating F_2 population identified resistant (*A*), susceptible (*B*) and heterozygous lines (*H*)

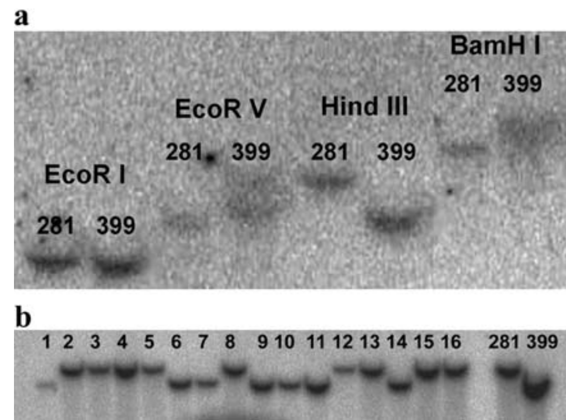

Fig. 4a, b Mapping of *eIF(iso)4E*. **a** Southern hybridisation of pea genomic DNA from pea lines JI281 and JI399 shows size polymorphism after digestion with *Eco*RV, *Hin*dIII or *Bam*HI. **b** RFLP analysis of 16 recombinant inbred (RI) lines from a cross between JI281 and JI399 identified a chromosomal location on linkage group (LG) II

which showed 84% identity with the *Medicago* sequence, failed to reveal any *Hin*dIII sites. Nevertheless, alignment of the pea and *Medicago* sequences permitted the identification of pea-specific primers (peaeIF4E5′and eIF4E3-pea3′) approximately flanking the central *Hin*dIII fragment in *Medicago* cDNA. When used in the amplification of pea genomic DNA, these primers identified a band approximately 1.8 kbp in size from lines JI2009 and JI1405. The sequence of these fragments identified 5′and 3′co-ordinates, corresponding to nt 308 and nt 747 on the *Medicago* cDNA sequence. This exon was split by three introns inserted at co-ordinates nt 492, 621 and 688. In JI2009, these introns were 89, 1,257 and 85 bp, whereas in JI1405 the introns were 89, 1,151 and 85 bp, i.e. 106 bp shorter in intron 2. This difference in size of 106 bp, and the presence of *Hin*dIII sites in intron 2 accounted for the polymorphic banding pattern in *Hin*dIII-digested genomic pea DNA. New primers (4Egenomic5′and 4Egenomic3′) were designed to convert the difference in size between the genomic DNA for JI1405 and JI2009 to a PCR-based polymorphic marker. This PCR marker produced PCR fragments of 425 bp and 535 bp from JI1405 and JI2009, respectively, and provided a co-dominant assay for the screen of the F_2 segregating progeny from the JI2009 × JI1405 cross (Fig. 3b).

When *eIF4E* was mapped relative to PSbMV resistance in a segregating F_2 population of 500 individuals, no recombinants were identified. This was confirmed in the F_3 generations. Hence, *eIF4E* appears to co-segregate with *sbm-1*.

eIF(iso)4E maps to a recessive resistance gene cluster on pea LG II

A multiple alignment of the nucleotide sequences of the *eIF(iso)4E* mRNA from *Arabidopsis* revealed several conserved regions in these proteins. Primers eIF-1 and iso4E23′were based upon these conserved sequences. These primers amplified a 306-bp fragment from pea cDNA. No difference was found between resistant and susceptible pea lines. However, when the amplified fragment was used as a hybridisation probe for restriction-digested pea genomic DNA, *eIF(iso)4E* was found to be a single-copy gene (Fig. 4a), which showed polymorphic behaviour between the parental lines JI281 and JI399. These lines have been used to generate an RI family (Ellis et al. 1992; Lacou et al. 1998) from which a subset of 16 RI lines were used for bin mapping, providing the opportunity to map *eIF(iso)4E* (Fig. 4b). The gene mapped close to several AFLP markers located in a region of LG II known to contain the *mo* locus and linked in turn to *sbm-2* (Ellis and Poyser 2002).

Discussion

Peas constitute an economically important crop in many temperate parts of the world. Many commercial pea lines (and lentil lines in the sub-tropical zones) are susceptible to PSbMV, and there is an urgent need to identify tightly linked markers that could facilitate the transfer of the recessive resistances within breeding programmes. The use of markers for recessive genes is particularly powerful as it removes the delays in breeding programmes associated with the restriction of the phenotypic analysis

Pea linkage group VI

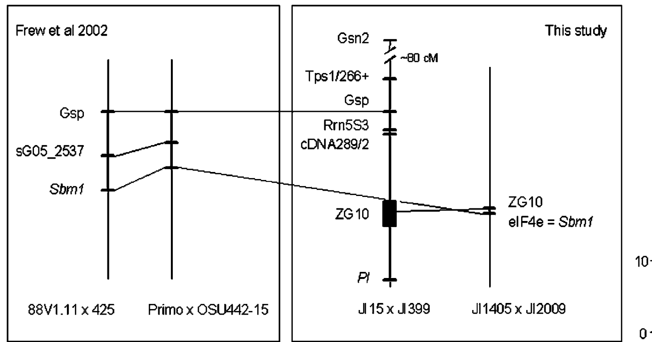

Fig. 5 Alignment of *eIF4E* and ZG10 relative to *sbm-1* and selected linked reference markers on LG VI. The relationship between ZG10, *sbm-1*/*eIF4E* and G05_2537 was determined by mapping ZG10 onto the JI15 × JI399 RI family for which the marker Gsp provided an anchor for comparisons with the crosses used by Frew et al. (2002). Because of the limited size of the RI family, the approximate region to which ZG10 was mapped is indicated by the *filled box*. The *scale* indicates distance in centiMorgans

to homozygous progeny. This, and inconsistencies in scoring infection phenotypes, has significantly delayed the introgression of PSbMV into elite pea lines. In peas, at least, the use of markers has the added attraction of assisting the introgression of further diverse potyvirus resistances genetically linked to *sbm*. Hence, *sbm-1*and *sbm-2* are associated with resistances to at least two and five other potyviruses, respectively.

Progress towards identifying markers very tightly linked to *sbm-1*/*-4* has been slow and may indicate a discrepancy between the physical and recombinational distances in this area. The previous closest markers to *sbm-1* were GS185 (Timmerman et al. 1993) and sG05_2537 (Frew et al. 2002) at 8 cM and ~4 cM, respectively, and our cDNA-AFLP search yielded only one closer marker, ZG10, at 0.7 cM. Alignment of our data and the published (Frew et al. 2002) maps shows the relative positions of ZG10, *eIF4E*/*sbm-1* and G05_2537 (Fig. 5). The determination of the orientation of *eIF4E*/*sbm-1* and ZG10 with respect to Gsp was assisted by the location of a microsatellite marker ('PSAC76a', proprietary information from the 'Agrogene Microsatellite Consortium') on the opposite side of *sbm-1* from ZG10 (data not shown).

Recessive resistance to potyviruses in lettuce and pepper and the identification of susceptibility factors in *Arabidopsis* have implicated the viral genome-linked protein (VPg) as a virulence factor interacting with eIF4E or eIF(iso)4E. Further, a direct physical interaction between *Turnip mosaic virus* (TuMV) VPg and eIF(iso)4E has been demonstrated (Wittmann et al. 1997). Similarly, the VPg from PSbMV has been identified as the viral resistance determinant for *sbm-1*and *sbm-4* on LG VI (Borgstrom and Johansen 2001). Interestingly, the avirulence determinant corresponding to *sbm-2* on LG II corresponds to the PSbMV P3 protein (Johansen et al. 2001), although *mo*-mediated resistance to BYMV, linked to *sbm-2* on LG II, does appear to involve VPg (Bruun-Rasmussen et al. 2003). It seems likely that resistances on LG II depend upon at least two functionally independent genes.

Using a segregating F_2 population for *sbm-1* resistance and a smaller RI family for mapping on LG II, we identified *eIF4E* and *eIF(iso)4E* as markers tightly linked to *sbm-1* and *mo*, respectively. In the case of *sbm-1*, the polymorphism between resistant and susceptible lines was based upon the presence of a small intron with a fortuitous *Hin*dIII site within the single *eIF4E* gene. From the analysis of the F_2, it appears that *eIF4E* was cosegregating with resistance. Our data does not show that *eIF4E* and *eIF(iso)4E* are functionally linked with these two resistance gene clusters, but they provide two useful tools for following the VPg-mediated resistances in breeding programmes and two strong potential candidates for the resistance gene products themselves. All of the *sbm*-linked sequences identified in this work have been converted to primers for PCR assays and are generally available for use.

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References

Bachem CW, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M, Visser RG (1996) Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 9:745–753

Borgstrom B, Johansen IE (2001) Mutations in *Pea seed-borne mosaic potyvirus* genome-linked protein VPg alter pathotype specific virulence in *Pisum sativum*. Mol Plant Microbe Interact 14:707–714

Bruun-Rasmussen M, Moller IS, Cordea M, Sundelin T, Johansen IE (2003) Molecular resistance determinants of the *Bean yellow mosaic virus* in *Pisum sativum*. EMBO Workshop – Genomic approaches in plant virology. Abstract, p 6

Christou P, Ford TL, Kofron M (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important *indica* and *japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio technology 9:957–962

Deom CM, Murphy JF, Paguio OR (1997) Resistance to *Tobacco etch virus* in *Capsicum annum*: inhibition of virus RNA accumulation. Mol Plant Microbe Interact 10:917-921

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

Ellis THN (1994) Approaches to the genetic mapping of pea. In: Modern methods of plant analysis, vol 16. Springer, Berlin Heidelberg New York, pp 117–160

Ellis THN, Poyser SJ (2002) An integrated and comparative view of pea genetic and cytogenetic maps. New Phytol 153:17–25

Ellis THN, Turner L, Hellens RP, Lee D, Harker CL, Enard C, Domoney C, Davies DR (1992) Linkage maps in pea. Genetics 130:649–663

Frew TJ, Russell AC, Timmerman-Vaughan GM (2002) Sequence-tagged site markers linked to the *sbm1* gene for resistance to *Pea seed-borne mosaic virus*. Plant Breed 121:512–516

Gal-On A, Meiri E, Elman C, Gray DJ, Gaba V (1997) Simple hand-held devices for the efficient infection of plants with viral-encoding constructs by particle bombardment. J Virol Methods 64:103–110

Gritton ET, Hagedorn DJ (1975) Linkage of the genes *sbm* and *wlo* in peas. Crop Sci 15:447–448

Haddad NI, Muehlbauer FJ, Hampton RO (1978) Inheritance of resistance to *Pea seed-borne mosaic virus* in lentils. Crop Sci 18:613–615

Hagedorn DJ, Gritton ET (1971) Registration of Wisconsin 7105 and 7106 pea germplasm. Crop Sci 11:945–946

Hagedorn DJ, Gritton ET (1973) Inheritance of resistance to the *Pea seed-borne mosaic virus*. Phytopathology 63:1130–1133

Haldane JBS, Waddington CH (1931) Inbreeding and linkage. Genetics 16:357–374

Hampton RO (1980) *Pea seed-borne mosaic* symptoms variation among *Pisum* plant introduction accessions: expression & variation in symptom expression among different genotypes of *Pisum* and the pathological implications. Pisum Newsl 12:29–30

Hampton RO, Marx GA (1981) Immunity to *Pea seed-borne mosaic virus*: a re-assessment. Pisum Newsl 13:16–17

Johansen IE, Lund OS, Hjulsager CK, Laursen J (2001) Recessive resistance in *Pisum sativum* and potyvirus pathotype resolved in a gene-for-cistron correspondence between host and virus. J Virol 75:6609–6614

Khetarpal RK, Maury Y (1987) *Pea seed-borne mosaic virus*: a review. Agronomie 7:215–224

Kyle MM, Palloix A (1997) Proposed revision of nomenclature for potyvirus resistance genes in *Capsicum*. Euphytica 97:183–188

Lacou V, Haurogné K, Ellis N, Rameau C (1998) Genetic mapping in pea. 1- RAPD-based genetic linkage map of *Pisum sativum*. Theor Appl Genet 97:905–915

Lellis AD, Kasschau KD, Whitham SA, Carrington JC (2002) Loss-of-susceptibility mutants of *Arabidopsis thaliana* reveal an essential role for eIF(iso)4E during potyvirus Infection. Curr Biol 12:1046–1051

Maule AJ, Wang D (1996) Seed transmission of plant viruses: a lesson in biological complexity. Trends Microbiol 4:153–158

Muehlbauer FJ (1983) Eight germplasm lines of pea resistant to *Pea seed-borne mosaic virus*. Crop Sci 23:1019

Murray MG, Cuellar RE, Thompson WF (1978) DNA sequence organization in the pea genome. Biochemistry 17:5781-5790

Nicaise V, German-Retana S, Sanjuán R, Dubrana M-P, Mazier M, Maisonneuve B, Candresse T, Caranta C, LeGall O (2003) The eukaryotic translation initiation factor 4E controls lettuce susceptibility to the potyvirus *Lettuce mosaic virus*. Plant Physiol 132:1272–1282

Provvidenti R, Alconero R (1988) Inheritance of resistance to a lentil strain of *Pea seed-borne mosaic virus* in *Pisum sativum*. J Hered 79:45–47

Provvidenti R, Hampton RO (1991) Chromosomal distribution of gene for resistance to seven potyviruses in *Pisum sativum*. Pisum Genet 23:26–28

Provvidenti R, Muehlbauer FJ (1990) Evidence of a cluster of linked genes for resistance to *Pea seed-borne mosaic virus* and *Clover yellow vein virus* on chromosome 6. Pisum Newsl 22:43–45

Provvidenti R, Hampton RO, Muehlbauer FJ (1991) G-1000, Marx's pea breeding line for multiviral resistance. Pisum Genet 23:50–52

Ruffel S, Dussault MH, Palloix A, Moury B, Bendahmane A, Robaglia C, Caranta C (2002) A natural recessive resistance gene against *Potato virus Y* in pepper corresponds to the eukaryotic initiation factor 4E (*eIF4E*). Plant J 32:1067–1075

Sambrook J, Russell DW (2001) Molecular cloning—a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Springer Harbor, N.Y.

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. Plant J 3:739–744

Stam P, van Ooijen JW (1995) JoinMap, ver 2.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen, The Netherlands

Stevenson WR, Hagedorn DJ (1971) Reaction of *Pisum sativum* to the *Pea seed-borne mosaic virus*. Plant Dis Rep 55:408–409

Timmerman GM, Frew TJ, Miller AL, Weeden NF, Jermyn WA (1993) Linkage mapping of *sbm-1*, a gene conferring resistance to *Pea seed-borne mosaic virus*, using molecular markers in *Pisum sativum*. Theor Appl Genet 85:609–615

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