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ORIGINAL PAPER

Non-synonymous single nucleotide polymorphisms in the watermelon eIF4E gene are closely associated with resistance to *Zucchini yellow mosaic virus*

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Abstract *Zucchini yellow mosaic virus* (ZYMV) is one of the most economically important potyviruses infecting cucurbit crops worldwide. Using a candidate gene approach, we cloned and sequenced eIF4E and eIF(iso)4E gene segments in watermelon. Analysis of the nucleotide sequences between the ZYMV-resistant watermelon plant introduction PI 595203 (*Citrullus lanatus* var. *lanatus*) and the ZYMV-susceptible watermelon cultivar 'New Hampshire Midget' ('NHM') showed the presence of single nucleotide polymorphisms (SNPs). Initial analysis of the identified SNPs in association studies indicated that SNPs in the eIF4E, but not eIF(iso)4E, were closely associated to the phenotype of ZYMV-resistance in 70 F_2 and 114 BC_{1R} progenies. Subsequently, we focused our efforts in obtaining the entire genomic sequence of watermelon eIF4E. Three SNPs were identified between PI 595203 and NHM. One of the SNPs (A241C) was in exon 1 and the other two SNPs (C309A and T554G) were in the first intron of the gene. SNP241 which resulted in an amino acid substitution (proline to threonine) was shown to be located in the critical cap recognition and binding area, similar to that of several plant species resistance to potyviruses. Analysis of a cleaved amplified polymorphism sequence (CAPS) marker derived from this SNP in F_2 and BC_{1R} populations demonstrated a cosegregation between the CAPS-2 marker and their ZYMV resistance or susceptibility phenotype. When we investigated whether such SNP mutation in the eIF4E was also conserved in several other PIs of *C. lanatus* var. *citroides*, we identified a different SNP (A171G) resulting in another amino acid substitution (D71G) from four ZYMV-resistant *C. lanatus* var. *citroides* (PI 244018, PI 482261, PI 482299, and PI 482322). Additional CAPS markers were also identified. Availability of all these CAPS markers will enable marker-aided breeding of watermelon for ZYMV resistance.

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Introduction

Watermelon (*Citrullus lanatus* [Thunb.] Matsum. & Nakai var. *lanatus*) is an important vegetable fruit crop worldwide (FAO 2007). Several aphid-borne potyviruses, including *Zucchini yellow mosaic virus* (ZYMV), *Watermelon mosaic virus* (WMV), and *Papaya ringspot virus* (PRSV), spread rapidly in watermelon fields, are difficult to control, and cause up to 50% yield loss (Lisa and Lecoq 1984; Provvidenti et al. 1984; Provvidenti 1996; Xu et al. 2004; Ma et al. 2005). Late summer and fall plantings are particularly vulnerable due to increased vector population and abundant inoculum sources from early season productions (Boyhan et al. 2007).

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The ZYMV is a member of the genus potyvirus in the family *Potyviridae*. In the United States, two major strains, ZYMV-CT and ZYMV-FL, have been identified (Provvidenti et al. 1984). The strain ZYMV-FL is the most widespread while ZYMV-CT is restricted to the north-eastern US. In the field, ZYMV is spread by several species of aphid as well as by mechanical inoculation. ZYMV-infected watermelon plants show symptoms of leaf distortion, yellow mosaic, plant stunting, and mottled fruits of reduced size.

In screening of the United States Department of Agriculture (USDA) watermelon germplasms, Provvidenti (1991) identified four accessions (PI 482261, PI 482299, PI 482308, and PI 482322) with resistance to ZYMV. Subsequently, he determined that a single recessive locus (*zym*) conferred resistance to ZYMV-FL in PI 482261 (Provvidenti 1991). Boyhan et al. (1992) identified cv. Egun (apparently PI 595203) resistance to ZYMV-FL. Guner (2004) re-evaluated the entire USDA plant introduction (PI) collection of watermelon germplasm (1,613 PIs) and identified 16 additional PIs with ZYMV-resistance, including PI 595203. Xu et al. (2004) determined that resistance to the ZYMV-China strain in PI 595203 is conferred by a single recessive locus (*zym*-CH). Resistance to ZYMV-FL in PI 595203 was also controlled by a single recessive locus, *zym*-FL. The discovery of resistance to such geographically distant ZYMV isolates indicates that PI 595203 may also be resistant to other ZYMV isolates, including ZYMV-CT.

Resistances to plant viruses, specifically potyviruses, are often inherited recessively (Provvidenti and Hampton 1992). To introgress virus resistance, plants need to be self-pollinated after one or two generations of backcrossing, followed by inoculation with the virus to identify resistance, which increases the time to introduce the recessive resistance allele into a breeding line. Marker-assisted selection (MAS) will improve breeding efficiency in the development of ZYMV resistant watermelons.

Mutations in the eukaryotic translation initiation factors [i.e., eIF4E, eIF(iso)4E, eIF4G or eIF(iso)4G] have resulted in resistance to specific RNA viruses in a number of plant systems (for reviews, see Kang et al. 2005b; Robaglia and Caranta 2006; Maule et al. 2007), including the model plant *Arabidopsis thaliana* (Duprat et al. 2002; Lellis et al. 2002; Yoshii et al. 2004; Sato et al. 2005), monocots (Kanyuka et al. 2005; Stein et al. 2005; Albar et al. 2006), and other dicots (Ruffel et al. 2002, 2005, 2006; Nicaise et al. 2003; Gao et al. 2004; Kang et al. 2005a; Yeam et al. 2005, 2007; Nieto et al. 2006; Charron et al. 2008). In melon (*Cucumis melo*), a SNP in the 3′ terminal portion of the eIF4E gene was responsible for resistance to *Melon necrotic spot virus* (MNSV), a Carmovirus (Nieto et al. 2006). However, in cucumber (*Cucumis sativus*), neither eIF4E nor eIF(iso)4E was associated with ZYMV resistance (Meyer et al. 2008). In this study, we demonstrate that eIF4E is closely associated with ZYMV resistance in watermelon.

Materials and methods

Plant materials

Six USDA plant introductions (PIs) previously shown to be ZYMV resistant were used. These include PI 595203, an Egusi type (*C. lanatus* var. *lanatus*) that was originally collected in Nigeria (Boyhan et al. 1992; Guner 2004; Xu et al. 2004), and five PIs of *C. lanatus* var. *citroides* (PI 244018, PI 482261, PI 482299, PI 482308, and PI 482322) (Provvidenti 1991; Guner 2004). Seeds of these PIs were obtained from the USDA Southern Regional Plant Introduction Station (Griffin, GA, USA). Watermelon cv. 'Black Diamond', 'Charleston Gray', 'Crimson Sweet', 'Jubilee', and 'New Hampshire Midget' ('NHM') are susceptible to ZYMV. The F_1, F_2, and reciprocal BC_1 populations (BC_{1R}: backcrossing F_1 to PI 595203), derived from a cross between PI 595203 (ZYMV-resistant) and 'NHM' (ZYMV-susceptible), were developed at North Carolina State University (Guner 2004). Seeds were germinated in a commercial soil mix (MetroMix 360, Sun Gro Horticulture, USA) in a greenhouse with 20–30°C and 12–14 h natural lighting at the US Vegetable Laboratory in Charleston, South Carolina, USA. Routine measures were used to control insects.

Virus cultures and inoculation

ZYMV-FL (Provvidenti et al. 1984) was maintained on squash cv. 'Gray Zucchini' and transferred every 6–8 weeks. Inoculum was prepared by macerating virus-infected leaf tissue (1:5 w/v) in 0.01 M phosphate buffered saline, pH 7.0 with a mortar and pestle. After lightly dusting the seedlings with Carborundum (320 mesh grit powder, Fisher Scientific, USA), the virus inoculum was gently rubbed on leaves with a cotton swab. The Carborundum and excess inoculum solution were then rinsed off, and seedlings placed under shade for a few hours to minimize direct sunlight damage to the newly inoculated leaves. Double inoculations were performed in 3 to 4 days intervals to prevent escape in inoculation. Plants were monitored daily for the presence of viral symptoms and the final reading was carried out 4 weeks after the inoculation. Disease severity index was rated as: 0 no symptom, 1 slight mosaic on the inoculated leaves with normal appearance on the systemic leaves, 2 slight yellow mosaic on the systemic leaves, 3 slightly deformed apical leaves with yellow mosaic, 4 severely deformed apical leaves with mosaic appearance; and 5 extensive mosaic appearance and severe leaf deformation, or plant death.

Enzyme-linked immunosorbent assay (ELISA)

During symptom examination, leaf tissue from newly developed, non-inoculated leaves was also collected and used to evaluate for the presence of ZYMV. ELISA was performed according to the manufacturer's instructions (BioReba AG, Switzerland). Absorbance values at OD_{405nm} were measured with an ELISA reader (SpectraMax 384 Plus, Molecular Devices, USA). A sample with an absorbance value of at least twice that of the mean healthy control was considered positive.

In addition to using the disease severity index to determine test plants for ZYMV resistance, absorbance values in ELISA were also used to support the phenotype determination. Plants with no systemic symptoms (ratings 0 and 1) and low absorbance values ($OD_{405nm} < 0.400$) were considered ZYMV-resistant. The susceptible plants were those with higher disease severity indices (ratings 2–5) and stronger absorbance values ($OD_{405nm} > 0.500$). Even though the mechanism of resistance to ZYMV in PI 595203 has not been characterized, virus titers on the inoculated PI 595203 plants as determined by ELISA were extremely low (<0.200) to non-detectable.

Nucleic acids extraction

Total nucleic acid was extracted from young leaves (~200 mg) collected from each test plant. Leaf tissue was macerated thoroughly in a 2.0-ml plastic test tube with a FastPrep-24 system (MP Biomedicals, USA) in 1 ml CTAB extraction buffer (100 mM Tris–HCl, pH 8.0, 50 mM EDTA, 1.4 M NaCl, 1% PVP-40, and 2.5% Cetyltrimethylaminonium Bromide). Total plant DNA was further purified using a DNeasy plant kit (Qiagen, USA). The purified plant DNA was used for PCR amplification as described in the following. Similarly, total plant RNA was extracted from selected leaf tissue using an RNeasy kit for plants (Qiagen, USA).

Cloning and sequence analysis of the eIF4E and eIF(iso)4E genes

Two degenerate primers, eIF4E-dF and eIF4E-dR (Table 1), previously developed for the melon study (Nieto et al. 2006) were used to amplify the potential eIF4E or eIF(iso)4E sequences in watermelon. PCR amplification was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min. The final extension was 72°C for 10 min. Two amplicons with expected sizes, 1.9 kb for eIF4E and 500 bp for eIF(iso)4E, were produced from watermelon PI 595203 and 'NHM' DNA. Two watermelon-specific eIF4E primers, KL07-60 and KL07-61, were designed to allow the analysis of SNP mutations through direct sequencing of PCR products (Table 1). However, the sequence from this 1.9 kb PCR product lacked the 5′ and 3′ terminal regions of eIF4E. To obtain the 5′ and 3′ terminal regions of watermelon eIF4E, two additional primer sets, KL08-46/KL07-14 and KL08-42/KL08-45 (Table 1), were developed based on the consensus sequence from an alignment between melon (EF188258) and cucumber (EU196160) eIF4E genomic sequence region (data not shown). Individual amplicon from PI 595203 or 'NHM' was cloned into a pCR4 vector using the TOPO TA cloning system (Invitrogen, USA) and sequenced. Nucleotide sequencing was performed in-house using the GenomeLab™ DTCS-Quick Start Kit and CEQ8000 genetic analyzer (Beckman Coulter, USA). Sequence data generated in this study were deposited in the GenBank database with accession numbers FJ184033–FJ184038.

Table 1 Primers used for sequencing of watermelon eIF4E or CAPS markers

Primer designation	Sequence	Genomic location (sequence orientation)
eIF4E-dF[a]	5′-TGG ACI TTY TGG TTY GAY AA	196–215 (F)
eIF4E-dR[a]	5′-GGR TCY TCC CAY TTI GGY TC	2,113–2,132 (R)
KL07-14	5′-TCG CCT GCG CAG CTT CAT	2,444–2,464 (R)
KL07-60	5′-GCG TCT ATT CGA CCG ATC TA	250–269 (F)
KL07-61	5′-CCC ATT TAG GCT CAA TTT TA	2,106–2,126 (R)
KL07-75	5′-CCA ACA GCA AGA ACC GAA AG	368–349 (R)
KL07-76	5′-TTT GGT TCG ATA ACC CAT CC	203–222 (F)
KL08-03	5′-AAA GCT ACA TCC ACG GAA GA	22–41 (F)
KL08-04	5′-CTC CAA AAC TCC TCA ACA GTA G	299–278 (R)
KL08-42	5′-TTC CAA CAG CTG CTT GCT AT	2,336–2,355 (F)
KL08-45	5′-GGG ACR TGA ACT CAG ACC AAG CTA	3,169–3,192 (R)
KL08-46	5′-ATG GTA GTT GAA GAG ACG	1–18 (F)
KL08-47	5′-AGC ATG CTT TGC ACG AAT AC	2,899–2,918 (R)

[a] Primers were developed by Nieto et al. (2006)

Two watermelon eIF4E specific primers, KL08-46 and KL08-47 (Table 1), were designed to amplify the entire eIF4E gene in six PI accessions and five watermelon cultivars. Reverse transcription PCR (RT–PCR) was conducted with total plant RNA purified with RNeasy in a Superscript one-step RT–PCR system for long template (Invitrogen, USA). Subsequently, these amplicons were also cloned and sequenced as described above.

The three-dimensional (3D) structures of eIF4E were generated using the Geno3D server (http://geno3d-pbil.ibcp.fr). The visualization was carried out using the Chimera server (http://www.cgl.ucsf.edu/chimera). A model of the 3D structure of watermelon eIF4E was generated using a superposition of the wheat eIF4E structure that was determined by X-ray crystallography (Monzingo et al. 2007).

Identification of single nucleotide polymorphism (SNP) mutations in eIF4E and the development of CAPS markers

To analyze the presence of SNP mutations, nucleotide and amino acid sequences of eIF4E between the ZYMV-resistant PI 595203 and the five ZYMV-susceptible watermelon cultivars were compared using the multiple sequence alignment program (Clustal-X) in the DNAMAN program package (Lynnon BioSoft, Canada). Sequences surrounding SNPs were evaluated for restriction digestion polymorphisms to produce cleaved amplified polymorphism sequence (CAPS) markers for PI 595203 and 'NHM'. The segregation of the CAPS markers and ZYMV resistance were evaluated using two parental lines 'NHM' and PI 595203, and progenies of F_1, F_2, and BC_{1R}. For CAPS-1, an aliquot of KL07-75/KL07-76 amplicon (10 µl) was digested with *Mse*I (NEB, Ipswich, MA, USA). Similarly for CAPS-2, 10 µl of KL08-03/KL08-04 amplicon was treated with *Pas*I (Fermentas, USA) and analyzed by electrophoresis on a 2% agarose gel.

Amplification and mapping of the CAPS-1 marker in a testcross population

Using primer sets that were developed for CAPS analysis, size polymorphisms were detected when primers KL07-75 and KL07-76 were used to analyze the three previously described testcross mapping parents, Griffin 14113, 'NHM', and PI 386015 (Levi et al. 2002, 2006). PCR amplicons generated from these three lines showed natural size polymorphisms (187 bp, 185 bp, and 173 bp for Griffin 14113, 'NHM', and PI 386015, respectively). PCR products were M13 labeled with fluorophore D4 and analyzed on a CEQ8800 genetic analyzer (Beckman-Coulter, USA). As many as 88 testcross progenies were scored for their association of the CAPS-1 marker with other genetic markers previously developed (Levi et al. 2002, 2006). Linkage analysis was performed using the JoinMap version 3.0 software (Van Ooijen and Voorrips 2001) using a minimum LOD score of 10.0 for grouping.

Results

Cloning and sequence analysis of watermelon eIF4E and eIF(iso)4E

Two degenerate primers (eIF4E-dF and eIF4E-dR) produced amplicons of the expected sizes (~1.9 kb and 500 bp) from PI 595203 and 'NHM'. BLASTN searches to Genbank (http://blast.ncbi.nlm.nih.gov/) using these sequences revealed significant similarities to eIF4E and eIF(iso)4E in other plants (data not shown). However, these watermelon sequences covered only the internal portions of the eIF4E and eIF(iso)4E genes, lacking the 5′ and 3′ regions. Initial analysis through direct sequencing of amplicons in 81 F_2 and 114 BC_{1R} progenies showed that the SNP mutation in the eIF4E (C309A located in intron 1), but not the SNP mutation (G40A) in the eIF(iso)4E, was closely linked to resistance in PI 595203 by ZYMV-FL infection. We subsequently focused our efforts in obtaining sequences to complete the watermelon eIF4E gene. The entire eIF4E gene was obtained by using two additional primer sets, KL08-46/KL07-14 and KL08-42/KL08-45 (Table 1). Primers KL08-46 and KL07-14 extended the 5′ portion of the eIF4E sequence (positions 1–2,464), and KL08-42 and KL08-45 the 3′ portion (positions 2,336–3,192). Overall, the sequenced genomic region contained an entire eIF4E homolog sequence (2,852 bp) starting from the translation initiation codon for PI 595203 (GenBank accession FJ184033) and 'NHM' (FJ184034). Similar to the eIF4E in melon (Nieto et al. 2006) or cucumber (Meyer et al. 2008), watermelon eIF4E genomic region included five exons (299 bp, 166 bp, 126 bp, 66 bp, and 51 bp) and four introns (1,740 bp, 131 bp, 193 bp, and 79 bp), respectively (Fig. 1a).

Amino acid substitutions in the eIF4E protein were identified between ZYMV-resistance accession and ZYMV-susceptible watermelon cultivars

When the genomic sequence of watermelon eIF4E was compared with the eIF4E genes in melon and cucumber, a higher percentage of sequence similarity was observed between the two *Cucumis* spp. (91%) with lower identity to watermelon (72–73%). However, when only coding sequences were compared, all three cucurbits shared high sequence identity (93% between watermelon and melon,

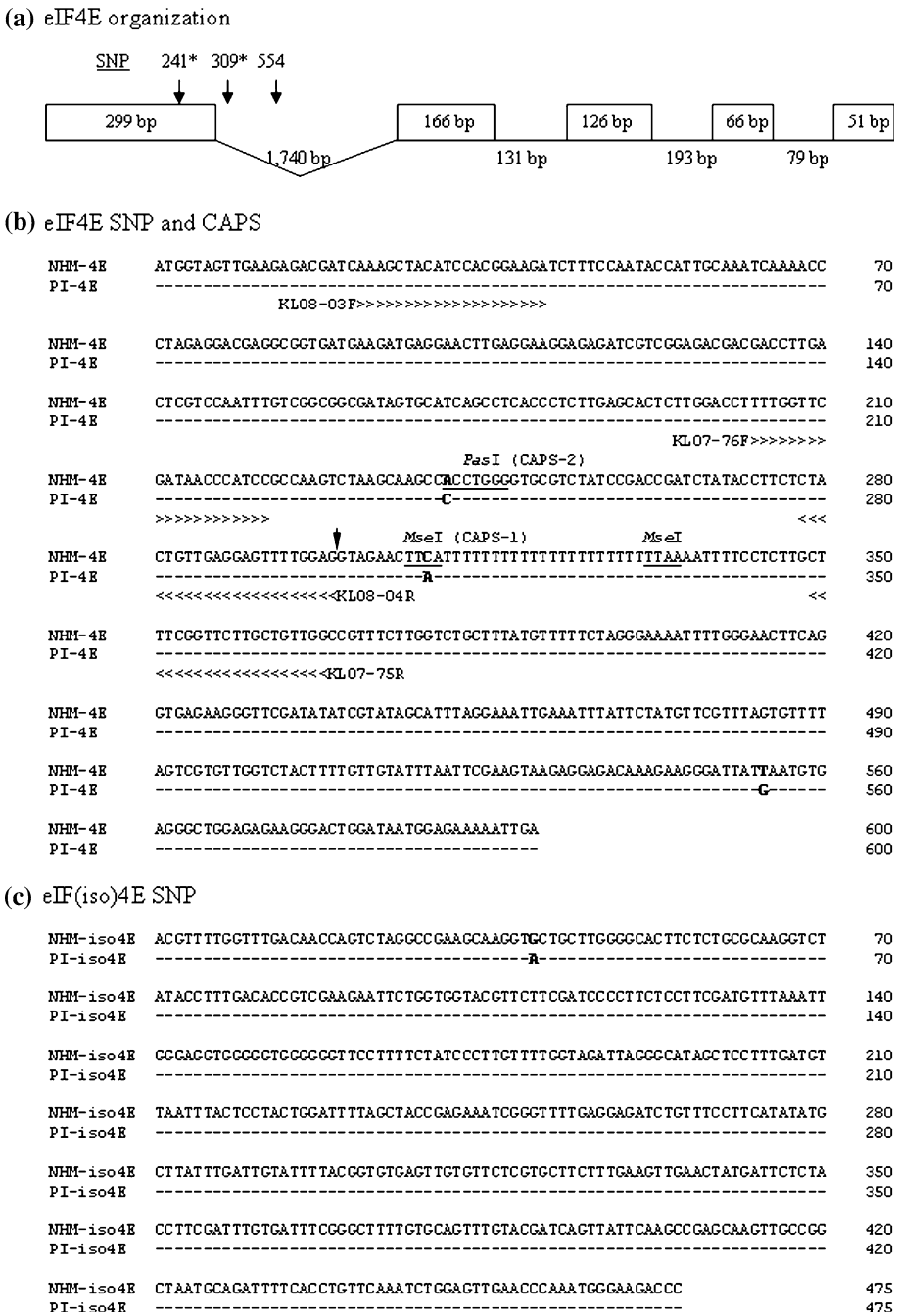

Fig. 1 **a** Schematic presentation of watermelon eIF4E gene organization and relative locations of single nucleotide polymorphism (SNP) mutations between the ZYMV-FL resistant PI 595203 (PI) and the ZYMV-FL susceptible cv. 'New Hampshire Midget' ('NHM'). *Boxes* represent exons and *lines* represent introns with nucleotide base pairs indicated. *Arrowheads* point to the SNP mutations. SNPs with *asterisk* were used for developing cleaved amplified polymorphism sequence (CAPS) markers. **b** A nucleotide sequence alignment of the 5′ terminal portion (600 bp) of eIF4E between PI 595203 (PI-4E) and cv. 'New Hampshire Midget' ('NHM'-4E). The identified SNP mutations (nt 241, 309, and 554) are highlighted in *bold* letters. The *arrow* points to the predicted splice site between the exon-1 and intron-1. **c** A nucleotide sequence alignment of the partial eIF(iso)4E region (475 bp) between PI 595203 (PI-iso4E) and cv. 'New Hampshire Midget' ('NHM'-iso4E). A single SNP mutation (A40G) was identified in the eIF(iso)4E sequenced region. Our initial analysis indicated that this SNP mutation was not associated with ZYMV resistance in PI 595203

94% between watermelon and cucumber, and 95% between melon and cucumber).

Multiple alignments of watermelon eIF4E genomic sequences between ZYMV resistant PI 595203 and ZYMV-susceptible 'NHM' revealed three SNPs (Fig. 1a). While two SNPs were in intron-1, one SNP (A241C) located in exon-1 resulted in a non-synonymous amino acid change from threonine to proline in PI 595203 (Fig. 1b). This SNP mutation resulting in T81P substitution appeared to be unique for ZYMV-resistant PI 595203. The T81 was preserved not only in five ZYMV-susceptible watermelon cultivars ('NHM', 'Black Diamond', 'Charleston Gray', 'Crimson Sweet', and 'Jubilee'), but also in five *C. lanatus* var. *citroides* accessions (Fig. 2) or in melon and cucumber lines (Fig. 3a).

The predicted 3D structure of the watermelon eIF4E protein overlaid perfectly to the wheat eIF4E crystal structure (Fig. 3b). The amino acid substitution (T81P) in PI 595203 was located within the critical area for cap recognition and binding (Fig. 3a), similar to non-synonymous mutations identified in other plants with resistance to potyviruses (Fig. 3c, d).

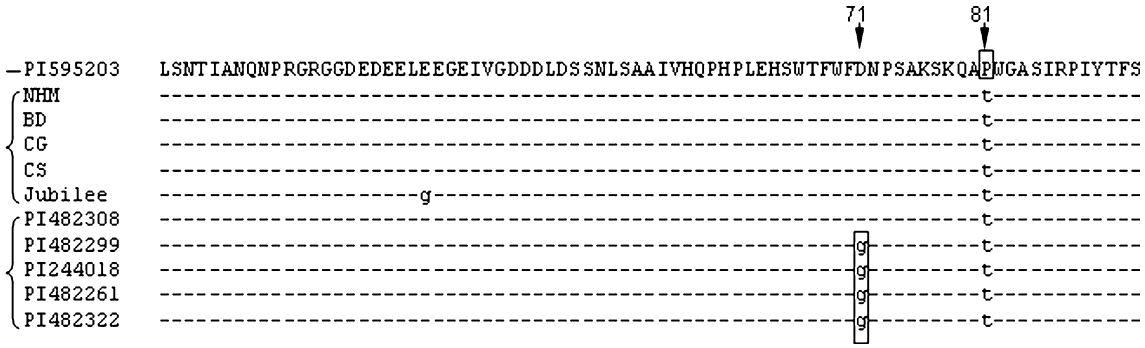

Fig. 2 Amino acid sequence alignment in a short segment of eIF4E exon-1 region (15-93 aa) among the ZYMV-FL resistant PI 595203 (*Citrullus lanatus* var. *lanatus*), five ZYMV-FL susceptible watermelon cultivars ('BD'—'Black Diamond', 'CG'—'Charleston Gray', 'CS'—'Crimson Sweet', 'Jubilee', and 'NHM'—'New Hampshire Midget'), four ZYMV-FL resistant *C. lanatus* var. *citroides* accessions (PI 244018, PI 482261, PI 482299, and PI 482322) and PI 482308 with heterogeneity for ZYMV resistance. The non-synonymous SNP mutation at amino acid residue 81 (T81P) was unique for ZYMV-resistant PI 595203. However, another non-synonymous SNP mutation (D71G) was identified in four other *C. lanatus* var. *citroides* accessions

To investigate whether a different SNP and amino acid change in the eIF4E coding sequences may be responsible for ZYMV resistance in *C. lanatus* var. *citroides*, a multiple sequence alignment in the exon-1 region of eIF4E was generated to include five *C. lanatus* var. *citroides* PIs (PI 244018, PI 482261, PI 482299, PI 482308, and PI 482322) (Fig. 2). Interestingly, a different non-synonymous SNP (A171G) resulting in aspartic acid to glycine substitution (D71G) was identified in four ZYMV-resistant *C. lanatus* var. *citroides* accessions (PI 244018, PI 482261, PI 482299, and PI 482322). The exception in PI 482308 may be due to some heterogeneity of seeds (obtained directly from the PI station) used in this study.

Development of CAPS markers and their close association to the *zym*-FL locus in watermelon

To differentiate between the ZYMV-resistant and susceptible plants based on the SNP309 (C309A) mutation in intron-1, the CAPS-1 marker was used with the restriction enzyme *Mse*I. Two *Mse*I sites (TTAA) were present in the amplicon from the ZYMV resistant PI 595203, resulting in three fragments (106 bp, 35 bp, and 25 bp). Digestion of the amplicon from ZYMV susceptible 'NHM' produced two products (131 bp and 35 bp) due to the A309C substitution abolished one *Mse*I recognition site (Fig. 1b). The two larger products (106 bp and 131 bp) were used to genotype F_2 progenies (Fig. 4a).

To differentiate between the ZYMV-resistant and susceptible plants based on the SNP241 (A241C) mutation located within the exon-1 of eIF4E gene, the CAPS-2 marker was used with the restriction enzyme *Pas*I. Presence of a *Pas*I recognition site (CCCTGGG) in PI 595203 and the absence of such sequence due to the A241C substitution in 'NHM' created a 57 bp size polymorphism between these two genotypes (Fig. 1b). When analyzed on an agarose gel, two products (211 bp and 57 bp) for PI 595203 and a single product (268 bp) for 'NHM' were observed (Fig. 4b). The two larger products, 211 bp and 268 bp, were easily resolved on the agarose gel upon electrophoresis.

The CAPS-2 marker showed a close linkage to the *zym*-FL resistance gene in 70 F_2 and 114 BC_{1R} plants. The CAPS-2 marker cosegregated with *zym*-FL in 70 F_2 plants (Table 2). Segregation data in the F_2 population fit that of the expected for a single recessive locus (Table 2). Similarly for the 114 BC_{1R} progenies analyzed, the CAPS-2 marker segregated closely with *zym*-FL. Discrepancies between the phenotype and genotype in 8 BC_{1R} lines (2 ZYMV-susceptible plants with P/P genotype and 6 ZYMV-resistant plants with P/N genotype) may not actually be recombinants, but be due to phenotyping errors (Table 3).

An additional CAPS marker was developed based on the identified SNP (A171G) in four ZYMV-resistant *C. lanatus var. citroides* PIs. Analysis of nucleotide sequence surrounding SNP171 revealed a restriction enzyme site for *Bst* EII that was capable of differentiating the ZYMV-resistant PIs of *C. lanatus var. citroides* (G/GTNACC, digested by *Bst* EII) from that of the ZYMV-susceptible watermelon cultivars (GATAACC, not to be digested by *Bst* EII).

Using the primers KL07-75/76 in PCR, eIF4E was mapped using 88 watermelon testcross progenies of Griffin 14113 (*C. lanatus* var. *citroides*) × 'NHM' (*C. lanatus* var. *lanatus*) × PI 386015 (*C. colocynthis*) (Levi et al. 2002, 2006). This eIF4E marker was assigned to the linkage group XIV, close to the randomly amplified polymorphic DNA (RAPD) marker U595-725c and the amplified fragment length polymorphism (AFLP) marker EGCAG-215 (Fig. 5). There are additional 11 RAPD, five sequence-related amplified polymorphism (SRAP), four inter-simple sequence repeat (ISSR), one AFLP and one simple sequence repeat (SSR) markers on this linkage group (Fig. 5, Levi et al. 2006).

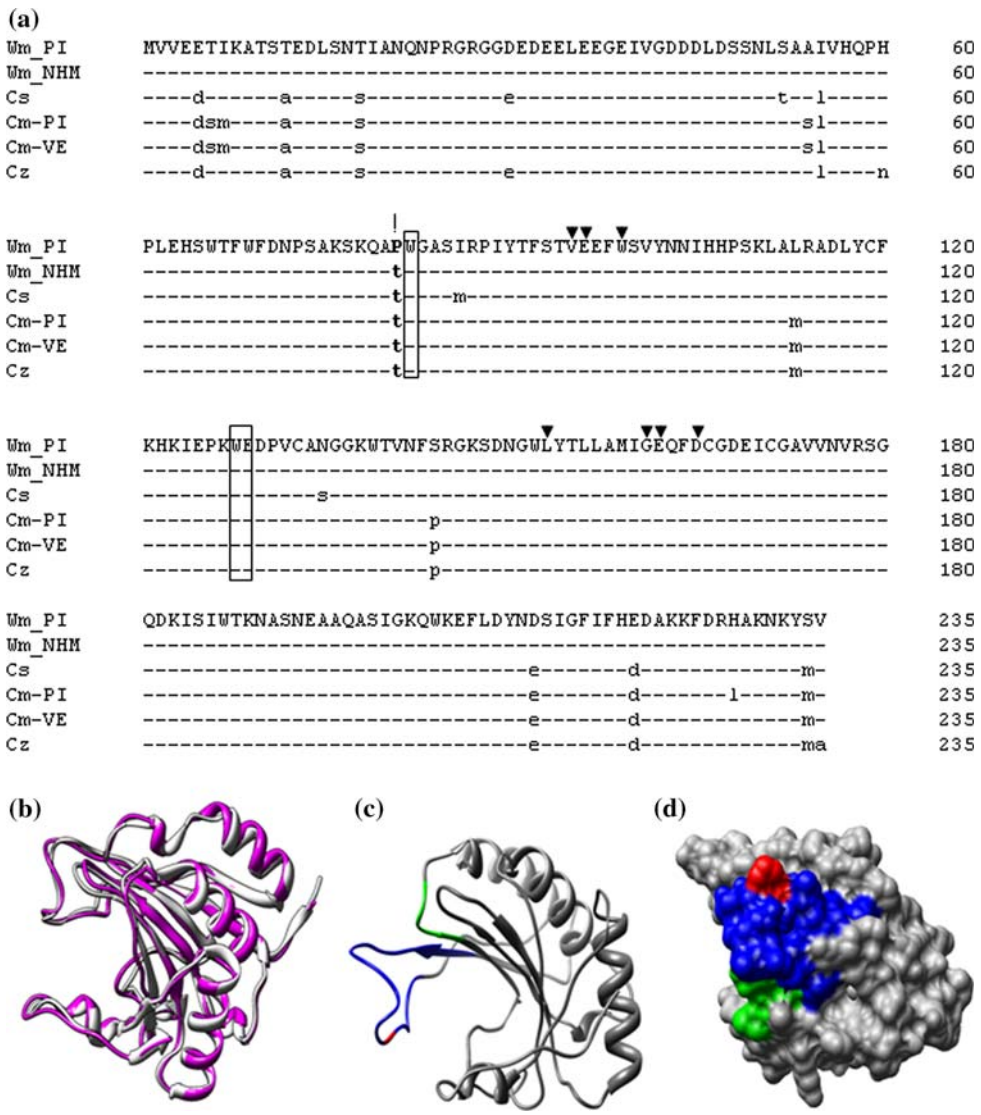

Fig. 3 **a** Amino acid sequence alignment of the eukaryotic translation initiation factor 4E among three cucurbit species (cucumber, melon, and watermelon). Alignment of predicted amino acids sequences from Wm_PI: PI 595203 (FJ184034), Wm_'NHM': cv. 'New Hampshire Midget' (FJ184033), Cs: *Cucumis sativus* (ABY56085), Cm-PI: *Cucumis melo* PI 161375 (ABD57969), Cm-VE: *Cucumis melo* 'Vedrantais' (ABD57970), Cz: *Cucumis zeyherii* (ABS18380). Amino acids in other species that were identical to that of the watermelon PI 595203 are presented with *dashed lines*. The amino acid substitution (threonine to proline) resulted from the SNP mutation (A241C) is highlighted in *bold* letter and identified with an *exclamation mark*. Amino acid residues implicated in the binding of the mRNA cap structure are marked by *arrowheads* and those in the recognition of eIF4E binding proteins are marked by *boxes* (Marcotrigiano et al. 1997; Nieto et al. 2006). **b** Predicted 3D structure of the eukaryotic translation initiation factor 4E protein for watermelon was constructed using the wheat eIF4E crystal structure (Monzingo et al. 2007). Superposition of the wheat eIF4E structure (indicated in *white*) and the model of watermelon eIF4E (indicated in *pink*) is shown. The three-dimensional structure was generated using the Geno3D server (http://geno3d-pbil.ibcp.fr) and the visualization was carried out using the Chimera server (http://www.cgl.ucsf.edu/chimera). **c** Model of watermelon eIF4E showing in *red* the T81P natural mutation associated with *Zucchini yellow mosaic virus* resistance; in *blue* and *green* are amino acids described in other plant systems to confer resistance to potyviruses (Robaglia and Caranta 2006). **d** As in (**b**) except the hydrophobicity surface is shown

Discussion

Several studies have demonstrated that components of eukaryotic translational initiation complex [i.e., eIF4E, eIF(iso)4E, eIF4G and eIF(iso)4G] are responsible in conferring resistance in plant systems to RNA viruses, particularly potyviruses (for reviews see, Kang et al. 2005b; Robaglia and Caranta 2006; Maule et al. 2007). We cloned eIF4E from watermelon and identified three SNPs in the eIF4E genomic region. One SNP (A241C) in exon 1 resulted in non-synonymous amino acid change (T81P) was unique for the ZYMV-resistant PI 595203. Another

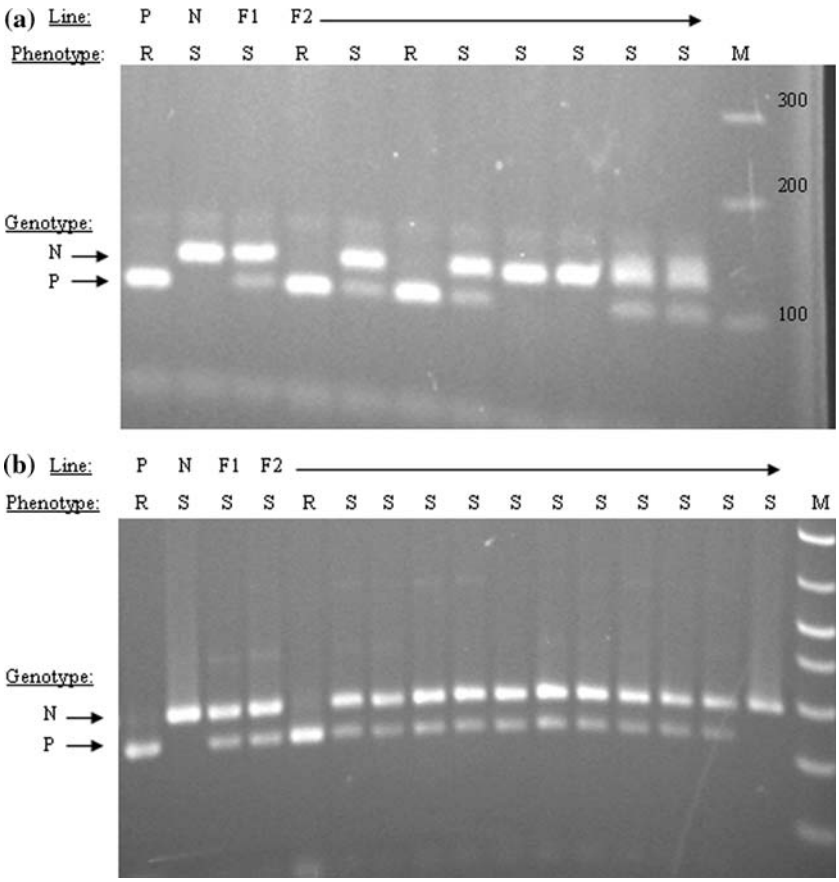

Fig. 4 **a** An example of restriction fragment length polymorphism generated using CAPS-1 marker on randomly selected F_2 progenies along with F1 and parental materials (P: PI 595203, N: New Hampshire Midget). Phenotype on virus susceptibility was determined through seedling inoculation with ZYMV-FL (R: resistance, S: Susceptible). Genotype was determined through comparisons to RFLP patterns in parents, P: PI 595203 (106 bp); N: New Hampshire Midget (131 bp). M is 1 kb plus molecular weight marker. **b** Similar to **a** but treated using CAPS-2 marker

Table 2 Segregation of the ZYMV-FL resistance gene and CAPS-2 in an F_2 population derived from 'New Hampshire Midget' and PI 595203

ZYMV phenotype	No. of plants[b]	Genotype at CAPS-2[a]		
		P/P[c]	P/N[c]	N/N[c]
Resistant	19	19	0	0
Susceptible	51	0	35	16

[a] P: CAPS-2 marker for PI 595203, N: CAPS-2 marker for 'New Hampshire Midget'

[b] Phenotype resistant:susceptible expected ratio (1:3); $\chi^2 = 0.171$, $P = 0.679$ ($df = 1$)

[c] Genotype P/P:P/N:N/N expected ratio (1:2:1); $\chi^2 = 0.257$, $P = 0.879$ ($df = 2$)

Table 3 Segregations of ZYMV-FL and CAPS-2 in a BC_{1R} population derived from 'New Hampshire Midget' and PI 595203

ZYMV phenotype	No. of plants[b]	Genotype at CAPS-2[a]	
		P/P[c]	P/N[c]
Resistant	53	47	6
Susceptible	61	2	59

[a] P: CAPS-2 marker for PI 595203, N: CAPS-2 marker for 'New Hampshire Midget'

[b] Phenotype resistant:susceptible expected ratio (1:1); $\chi^2 = 0.561$, $P = 0.454$ ($df = 1$)

[c] Genotype P/P:P/N expected ratio (1:1); $\chi^2 = 2.24$, $P = 0.134$ ($df = 1$)

non-synonymous amino acid substitution (D71G) in eIF4E was identified in four ZYMV-resistant *C. lanatus* var. *citroides* PIs (244018, 482261, 492299, and 482322) (Provvidenti 1991). Apparently, these two sources of ZYMV resistance were controlled by different allelic SNP mutations in the same *Citrullus* eIF4E. The occurrence of SNPs in different parts of the eIF4E gene resulting in allelic series of potyvirus resistance has already been observed in *Capsicum* and allele-specific CAPS markers were also developed based on the point mutations in the *Capsicum* eIF4E (Yeam et al. 2005). As it was identified earlier (Provvidenti 1991), PI 482261 has been broadly used by breeders in developing watermelon breeding lines for their resistance to ZYMV-FL infection. Thus, additional experiment in allelism test between these two sources of ZYMV resistance in *Citrullus* will be necessary.

Although most of SNP mutations in eIF4E conferring potyvirus resistance were located in the N-terminal region, a non-synonymous SNP mutation in the C-terminal region of eIF4E was responsible for resistance in melon (*C. melo*) to MNSV, a Carmovirus (Nieto et al. 2006). However,

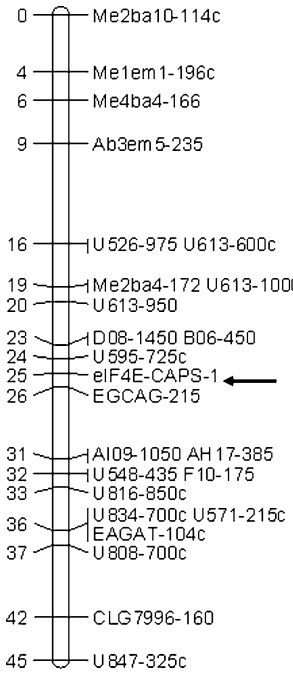

Fig. 5 Watermelon linkage groups XIV carrying the eukaryotic translation initiation factor eIF4E marker in relationship to other genetic markers that were generated by Levi et al. (2006). As determined in the present study, the eIF4E marker was co-segregating with the *zym*-FL locus in watermelon

neither eIF4E nor eIF(iso)4E in cucumber (*C. sativus*) was associated with ZYMV resistance (Meyer et al. 2008). We mapped the *Citrullus* eIF4E to XIV linkage group in watermelon between the RAPD marker U595-725c and the AFLP marker EGCAG-215 (Levi et al. 2002, 2006). As the CAPS markers and the ZYMV resistance gene cosegregate in watermelon, as well as the numerous examples of mutations in eIF4E conferring the potyvirus resistance in other plants (Kang et al. 2005b; Robaglia and Caranta 2006; Maule et al. 2007), we proposed here that the eIF4E is a candidate gene conferring ZYMV resistance in watermelon. Thus, the present study which identified a SNP mutation in exon-1 region of eIF4E that is associated with ZYMV-FL resistance in watermelon is another example of recessive viral resistance resulting from a mutation in the translational initiation complex. Using 3D modeling prediction, it was shown that the resulting amino acid mutation in watermelon eIF4E was located in the critical area in cap recognition and binding which is in agreement with several other plant systems for potyvirus resistance (Fig. 2) (Marcotrigiano et al. 1997; Michon et al. 2006; Monzingo et al. 2007). Despite the close association of eIF4E to ZYMV resistance in this study, functional complementation experiments are needed to demonstrate the actual role of eIF4E for ZYMV resistance in watermelon (Kang et al. 2005a; Nieto et al. 2006; Albar et al. 2006). It may also be possible to use TILLING technology to determine the function of eIF4E in watermelon responding to ZYMV infection. Furthermore, similar SNP mutation may also be identified through Eco-TILLING the USDA watermelon germplasm collection, currently with over 1,500 accessions. Recently, Kang et al. (2007) demonstrated eloquently that an ectopic expression of the recessive resistance gene from *Capsicum* (eIF4E) could generate a dominant potyvirus resistance in transgenic tomato plants. It may also be possible to exploit the finding in watermelon for application in melon and cucumber. Even though wide genetic crosses between watermelon and melon or cucumber are not likely, we may be able to use transgenic approach to introduce the identified watermelon eIF4E gene into melon or cucumber as well as to other cucurbits, such as squash, where sources of ZYMV resistance are scarce.

Traditional backcrossing to introgress a recessive resistance gene is slow due to the selfing step required to generate progenies segregating for the resistance allele. Marker-assisted selection using our CAPS markers developed in the present study would certainly accelerate the breeding of watermelon for ZYMV resistance.

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