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Eukaryotic translation initiation factor 2B-beta (*eIF2Bβ*), a new class of plant virus resistance gene

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SUMMARY

Recessive resistances to plant viruses in the *Potyvirus* genus have been found to be based on mutations in the plant eukaryotic translation initiation factors, *eIF4E* and *eIF4G* or their isoforms. Here we report that natural, monogenic recessive resistance to the potyvirus Turnip mosaic virus (TuMV) has been found in a number of mustard (*Brassica juncea*) accessions. Bulked segregant analysis and sequencing of resistant and susceptible plant lines indicated the resistance is controlled by a single recessive gene, *recessive TuMV resistance 03* (*retr03*), an allele of the *eukaryotic translation initiation factor 2B-beta* (*eIF2Bβ*). Silencing of *eIF2Bβ* in a TuMV-susceptible mustard plant line and expression of *eIF2Bβ* from a TuMV-susceptible line in a TuMV-resistant mustard plant line confirmed the new resistance mechanism. A functional copy of a specific allele of *eIF2Bβ* is required for efficient TuMV infection. *eIF2Bβ* represents a new class of virus resistance gene conferring resistance to any pathogen. eIF2B acts as a guanine nucleotide exchange factor (GEF) for its GTP-binding protein partner eIF2 via interaction with eIF2·GTP at an early step in translation initiation. Further genotyping indicated that a single non-synonymous substitution (A120G)

in the N-terminal region of *eIF2Bϐ* was responsible for the TuMV resistance. A reproducible marker has been developed, facilitating marker-assisted selection for TuMV resistance in *B. juncea*. Our findings provide a new target for seeking natural resistance to potyviruses and new opportunities for the control of potyviruses using genome editing techniques targeted on *eIF2Bϐ*.

INTRODUCTION

Turnip mosaic virus (TuMV) is a member of the *Potyvirus* genus and has a very broad host range of plant species, including not only many species in the *Brassicaceae*, but also legumes, ornamentals and weed plants (Edwardson and Christie, 1991). TuMV is a positive-sense RNA virus with a genome of approximately 9830 nucleotides encoding 11 functional proteins that facilitate its translation and amplification in host plants (Chung *et al.,* 2008; Ohshima *et al.,* 1996; Walsh and Jenner, 2002). The virus is transmitted by aphids in the non-persistent, stylet-borne manner and is the major virus infecting *Brassica* crops, causing significant economic losses worldwide (Walsh and Jenner, 2002).

The development and use of plant varieties that are resistant to TuMV is the preferred strategy to tackle the problems associated with TuMV infection. TuMV-resistant germplasm has been identified in several species (Hughes *et al.,* 2002; Robbins *et al.,* 1994; Walsh *et al.,* 1999). In *Brassica* species, a number of TuMV resistance genes have been mapped, mainly from *Brassica napus* and *Brassica rapa* (Chung *et al.,* 2014; Hughes *et al.,* 2003; Jin *et al.,* 2014; Qian *et al.,* 2013; Rusholme *et al.,* 2007; Walsh *et al.,* 1999). Recently, great advances have been made in understanding of the molecular basis of plant resistance to pathogens,

including the mechanisms of dominant resistance genes, recessive resistance genes, as well as viral defence based on RNA silencing (Nicaise, 2014; Revers and Nicaise, 2014). Recessive virus resistance is more common for potyviruses than for viruses of other families; 63.8% of the examples of recessive resistance to plant viruses listed in one review corresponded to potyviruses (Diaz-Pendon *et al.,* 2004).

Recessive resistance is usually achieved through the absence of factors that are required for the virus to complete its life cycle in its host. Over the past decade, a number of recessive resistance genes have been shown to encode eukaryotic translation initiation factors (eIFs), mostly eIF4E and eIF4G and their isoforms, eIF(iso)4E and eIF(iso)4G (Nicaise, 2014; Revers and Nicaise, 2014). *In vitro* assays have shown that the viral protein genome-linked (VPg) of potyviruses interacts with plant eIF(iso)4E (Wittmann *et al.,* 1997), indicating the mechanism might relate to recruitment of the translation initiation complex to the viral RNA (Robaglia and Caranta, 2006). However, it has also been suggested that the mechanism might relate to virus cell-to-cell movement (Gao *et al.,* 2004). Mutations in *eIF4E* or *eIF(iso)4E* in a range of plant species have been shown to confer resistance to some potyviruses (Duprat *et al.,* 2002; Lellis *et al.,* 2002; Robaglia and Caranta, 2006) and mis-splicing of *eIF(iso)4E* in *B. rapa* has been shown to result in recessive resistance to TuMV (Nellist *et al.,* 2014). A core set of eIFs is conserved to facilitate the assembly of a translation-competent ribosome at the initiation codon of an mRNA (Hinnebusch and Lorsch, 2012; Sonenberg and Dever, 2003). The recruitment of the host plant eukaryotic

translation machinery for virus translation initiation in some plant-virus interactions appears to be essential for viral survival; a block in any step of this translation initiation process can result in virus resistance.

B. juncea (AABB) is an allopolyploid/amphidiploid species originating from hybridisation and chromosome doubling between *B*. *rapa* (AA) and *Brassica nigra* (BB) (U, 1935). In allopolyploid species, virus resistance becomes more complicated because of the presence of multiple copies of genes resulting from genome duplication, particularly in the case of recessive resistance. *B. juncea* plant lines with resistance to TuMV were identified. Bulked segregant analysis by resequencing was used to map the resistance to TuMV in *B*. *juncea*. This identified one copy of *eIF2Bϐ* as the gene involved in the resistance. Silencing of *eIF2Bϐ* in a TuMV-susceptible plant line and expression of *eIF2Bϐ* from a TuMV-susceptible line in a TuMV-resistant mustard plant line confirmed the new resistance mechanism. *eIF2Bϐ* is one of the sub-units of eIF2B, hence mutations in *eIF2Bϐ* might affect eIF2B's role in guanine nucleotide exchange for its GTP-binding protein partner eIF2 and hence regulation of protein synthesis. Further genotyping indicated that a single non-synonymous substitution (A120G) in the N-terminal region of *eIF2Bϐ* was responsible for the TuMV resistance and this provided the basis for a reproducible marker for future marker-assisted selection for TuMV resistance in *B. juncea*.

RESULTS

Identification of TuMV-resistant *B. juncea* germplasm

A TuMV isolate (ZJ) from Zhejiang Province of China with close *Coat Protein* (*CP*) nucleotide homology to the CHN4 and CHN3 TuMV isolates (Ohshima *et al.,* 2002) (Supplementary Figure S1) provided inoculum for experiments. Following inoculation of plants with TuMV ZJ, visual assessments, RT-qPCR detection of the *CP* gene and ELISA (enzyme-linked immunosorbent assay) were performed to assess 35 *B. juncea* accessions for resistance. Visual assessments (Supplementary Figure S2) indicated that ten accessions were resistant, Quantitative reverse transcription polymerase chain reaction (RT-qPCR) (Supplementary Figure S3) indicated eight were resistant and ELISA (Supplementary Figure S4) indicated nine were resistant. Seven of the *B. juncea* accessions showed resistance based on all three of the tests (Table 1).

TuMV resistance is controlled by a recessive gene in *B. juncea*

The TuMV-resistant line (VC029) was crossed with two TuMV-susceptible lines (STZ and WZD) (Figure 1 and Table 2) to develop segregating populations. Following challenge with TuMV ZJ, 20 F_1 plants from both crosses showed stunting and chlorotic symptoms typical of TuMV infection; high *TuMV-CP* gene expression and high virus titres were detected by RT-qPCR and ELISA. Of 226 F_2 plants derived from a cross between VC029 and STZ, 55 were resistant and 171 susceptible (Table 2). Of 124 F_2 plant derived from a cross between VC029

and WZD, 30 were resistant and 94 susceptible (Table 2). Of 80 BC_1 plants from the VC029 backcross [(VC029 × WZD) × VC029], 44 were resistant and 36 susceptible (Table 2). These results are not significantly different from a 1:3 ratio (χ^2 = 0.05 and P = 0.82 for the VC029 × STZ cross and χ^2 = 0.04 and P = 0.84 for the VC029 × WZD cross) for the F_2 populations and a 1:1 ratio (χ^2 = 0.8 and P = 0.37) for the BC_1 population, indicating monogenic recessive resistance.

Bulked segregant analysis by whole-genome resequencing identifies *eIF2Bβ* as the candidate resistance gene

The TuMV-resistance gene was mapped by whole-genome resequencing of F_2 resistant and susceptible bulks (49 resistant and 49 susceptible plants) from the VC029 x STZ cross. In total, approximately 181 and 190 million clean reads were generated for susceptible and resistant pools respectively from Illumina Hiseq2500 (Supplementary Table S1). The resequencing data showed a stochastic distribution on all chromosomes (Supplementary Figure S5). Referring to the reference genome, we identified more than 3 million single nucleotide polymorphisms (SNPs) for each susceptible and resistant pool, including transitions and transversions (Supplementary Table S2). Over 3 million SNPs were collected between susceptible and resistant pools and located on the *B. rapa* A genome. Of these, 147,000 were non-synonymous (Supplementary Table S3). Using association analysis based on SNP index, fifteen regions associated with the resistance were identified on chromosomes 1, 2, 4 and 14 (Figure 2a and Supplementary Table S4). After annotation and

enrichment analysis of the genes in the associated regions using nr, Swissprot, GO (Gene Ontology) and COG (Cluster of orthologous groups) (Supplementary Table S5 and Supplementary Figure S6 and S7), we identified a eukaryotic translation initiation factor family protein (*BjuA006209*) on chromosome 1 of the A genome of *B. juncea* as the candidate resistance gene (Figure 2b). In the gene coding and intergenic regions of *BjuA006209*, 84 SNPs showed the expected segregation between susceptible and resistant pools (Figure 2b). *BjuA006209* has 93% amino acid homology with the *eukaryotic translation initiation factor 2B-beta* (*eIF2Bβ*) from Arabidopsis (*At3G07300*). There were 30 SNPs in the coding sequence of susceptible (STZ) and resistant (VC029) plants, of which only one SNP (A120G) was non-synonymous (Supplementary Figure S8 and S9).

eIF2Bβ genotyping analysis in _B. juncea_ and evolution in _Brassicaceae_

Orthologues of *eIF2Bβ* from *B. rapa*, *B. nigra*, *B. juncea* and *Arabidopsis* spp. were identified and a phylogenetic tree constructed (Figure 3a). Following genome triplication in the evolution of the diploid brassica species, two orthologues of *Arabidopsis* (*At3G07300*) *eIF2Bβ* were identified in *B. rapa* (*Bra040371* and *Bra029637*) and two in *B. nigra* (*BniB042510* and *BniB018993*). A third copy could not be identified in *B. rapa*, or *B. nigra*, suggesting paralogous loss (Figure 3a and 3b). Three orthologues of *eIF2Bβ At3G07300* were identified in the allopolyploid *B. juncea* genome, two of which were in the A sub-genome (*BjuA006209* and *BjuA020776*) and one in the B sub-genome (*BjuB006522*) (Figure 3a and 3b). Genotyping *eIF2Bβ* (*BjuA006209*) from seven resistant and seven susceptible lines of *B.*

juncea revealed that all seven susceptible lines (STZ, WZD, MF, 03C0804, 03C0909, VB109 and VA003) had the amino acid Ala at the non-synonymous SNP (A120G). Of the seven resistant plant lines genotyped, four (VC029, VC028, VC024, and WT-28) had the amino acid Gly at this non-synonymous substitution and three (03B0103, 03D0102 and 4D101) had the amino acid Trp at this position (Figure 3c).

BjuA006209 (*eIF2Bβ*) and *BjuB006522* appear to be newly formed homologues in the allopolyploid *B. juncea* genome, and their amino acid homology was 99% (Figure 3a). In the susceptible line STZ, both of these *eIF2Bβ* homologues have the amino acid Ala at the non-synonymous substitution SNP (A120G). In the resistant line VC029, the candidate resistance gene (*BjuA006209*) has Gly and the homologue (*BjuB006522*) has Ala (Figure 3d).

Functional analysis of *eIF2Bβ* confirms it as a new virus resistance gene

To further investigate the role of *eIF2Bβ* (*BjuA006209*) in the resistance / susceptibility of *B. juncea* to TuMV, Turnip yellow mosaic virus (TYMV)-induced gene silencing (VIGS) (Pflieger *et al.,* 2008) was used to silence *eIF2Bβ* (*BjuA006209*) in the TuMV-susceptible *B. juncea* line STZ. Plants treated with infection buffer only, the empty pTY-S silencing vector, or pTY-PDS-IR expressing the phytoene desaturase gene (Pflieger *et* al., 2008) served as mock, empty vector and positive controls, respectively. pTY-PDS-IR-treated STZ plants displayed a mosaic of white spots / patches on the leaves one week post challenge, indicating that the pTY-S vector was capable of infecting *B. juncea* systemically (Figure 4a and 4b). *eIF2Bβ* (*BjuA006209*) expression was significantly reduced in plants treated with the pTY-S vector

designed to silence *eIF2Bβ* (pTY-S/eS) (Figure 4c), whereas the expression of *BjuB006522* and *BjuA020776* were not significantly affected (Figure 4d). TuMV isolate ZJ was inoculated to Control, pTY-S- and pTY-S/eS-treated plants. Control and pTY-S-treated plants began to show symptoms of TuMV infection in their leaves 2 weeks post-inoculation of TuMV, whereas pTY-S/eS-treated plants grew normally (Figure 4a and 4b). Expression of *eIF2Bβ* (*BjuA006209*) was kept to a relatively low level in pTY-S/eS-treated plants (Figure 4e). Twelve pTY-S/eS-treated plants were resistant to TuMV and 6 plants showed TuMV symptoms (Supplementary Table S6). TuMV infection / susceptibility was related to *eIF2Bβ* (*BjuA006209*) expression levels in pTY-S/eS-treated plants; decreased expression of *eIF2Bβ* (*BjuA006209*) resulted in resistance to TuMV, whereas normal expression of *eIF2Bβ* (*BjuA006209*) resulted in susceptibility to TuMV (Supplementary Figure S11). No significant differences between the pTY-S- and pTY-S/eS-treated plants were seen, indicating that the pTY-S/eS treatment had no obvious / significant effects on the growth of plants (Figure 4a). *TuMV-CP* gene expression and TuMV titre were checked for all treatments by RT-qPCR and ELISA. *TuMV-CP* gene expression levels and TuMV titres agreed well with virus symptom expression (Figure 4f and 4g). The results demonstrated that *eIF2Bβ* (*BjuA006209*) was responsible for TuMV resistance in *B. juncea*; the resistance locus was named *retr03* (*recessive TuMV resistance locus 03*).

We used the VIGS vector pTY-S to generate pTY-S/eC to express *eIF2Bβ* from STZ in VC029 plants. VC029 plants were mock-inoculated with PBS buffer. Both mock- and empty pTY-S vector-inoculated plants were included as controls (Figure 5a and 5b). TuMV isolate ZJ was inoculated to VC029 plants and pTY-S and pTY-S/eC-inoculated VC029 plants. Buffer

inoculated control and pTY-S-inoculated plants grew normally, whereas pTY-S/eC-inoculated plants challenged with TuMV showed symptoms of TuMV infection in their leaves 2 weeks post-inoculation (Figure 5a and 5b). Expression of *eIF2Bβ* from STZ in VC029 was confirmed by sequencing of the *eIF2Bβ* fragment with the SNP variation (C/G) in pTY-S/eC-inoculated plants. Seven clones with C and three clones with G were observed in ten clones of two pTY-S/eC-treated plants (Figure 5c). Among the thirty pTY-S/eC-treated plants, twenty-five plants displayed TuMV symptoms of TuMV infection (Supplementary Table S6). Moreover, the pTY-S/eC plants were significantly accumulating TuMV as revealed by TuMV-CP expression and ELISA (Figure 5d and 5e). These results confirmed *eIF2Bβ* function using expression of *eIF2Bβ* from the susceptible line in the resistant plant line.

TuMV resistance selection using an *eIF2Bβ*-based molecular marker in *B. juncea* During gene mapping, we identified 84 SNPs linked to TuMV resistance in the intergenic and gene coding regions of *eIF2Bβ* (*BjuA006209*). The *eIF2Bβ* gene comprises 9 exons and 8 introns. A 90 bp insertion was identified in the 3rd intron of the susceptible *B. juncea* line (STZ) compared to the resistant line (VC029) (Figure 6a, Supplementary Figure S10). Based on this variation, degenerate primers were designed to amplify PCR fragments of different lengths. Fragments of 799 bp and 709 bp were amplified from the susceptible and resistant lines, respectively (Figure 6b), providing a specific molecular marker (*BjTuR*) associated with TuMV resistance in *B. juncea*.

To check the molecular marker, it was used to genotype plants from the F_2 and BC_1 populations derived from the VC029 x WZD cross. In the F_2 population, all 55 resistant plants displayed the 709 bp fragment and 171 susceptible plants displayed both the 799 bp and 709 bp fragments (heterozygotes) or just the 799 bp fragment (Figure 6c). The marker was also consistent with TuMV resistance and susceptibility phenotypes in the BC_1 population (Figure 6d). The marker was then used to screen further resistant (VC029, VC028, VC024, 03D0102 and WT-28) or susceptible (STZ, WZD, VA003, 03C0909 and MF) *B. juncea* accessions. This revealed that all resistant accessions displayed the 709 bp fragment and all susceptible accessions displayed the 799 bp fragment indicating the marker may have wider use for identifying *B. juncea* lines with this form of resistance to TuMV (Figure 6e and Supplementary Figure S12).

DISCUSSION

A single recessive gene (*retr03*) conferring resistance in mustard (*B. juncea*) to an isolate of TuMV has been identified as one of the copies of *eIF2Bϐ* (*BjuA006209*) by using re-sequencing-based bulked segregant analysis, silencing of *eIF2Bϐ* in a TuMV-susceptible *B. juncea* line and expression of *eIF2Bϐ* from a susceptible *B. juncea* plant line in a resistant *B. juncea* plant line. The gene is on chromosome 1 of the A genome of *B. juncea*. Recessive resistance to TuMV has been identified in other *Brassica* species, primarily *B. rapa*. The best studied example is due to a single recessive gene, *retr01* (Rusholme *et al*., 2007; Nellist *et al*., 2014) in *B. rapa* var. *pekinensis* (Chinese cabbage; *Brassica* A genome) and provides

broad-spectrum resistance to TuMV. The mechanism involves the mis-splicing of the *eukaryotic initiation factor iso 4E* (*eIF(iso)4E*). *B. rapa* var. *pekinensis* has three copies of *eIF(iso)4E*, however results have clearly shown that only one copy (*BraA.eIF(iso)4E.a*) is involved in the life-cycle of TuMV (Nellist *et al.,* 2014). The TuMV-resistant plant line VC029 has three copies of *eIF2Bϐ*, two in the A sub-genome (*retr03*/*BjuA006209* on chromosome 1 and *BjuA020776* on chromosome 5) and one in the B sub-genome (*BjuB006522* on chromosome 11). Similar to the *eIF(iso)4E*-based resistance, it appears only one of the copies of *eIF2Bϐ* in the *B. juncea* lines analysed is involved in the life-cycle of TuMV, the suggestion being that *retr03*/*BjuA006209* is non-functional for TuMV in VC029 and results in the virus being unable to translate its RNA genome. The results indicate that the specific mutation of *eIF2Bϐ*, gene loss of one homologue gene and the inability of TuMV to use eukaryotic initiation complexes associated with the two other homologues of *eIF2Bϐ* are required for the recessive resistance in the allopolyploid *B. juncea* VC029 line.

Viruses recruit cellular translation factors to translate their RNA and regulate replication and systemic movement. In addition to the eukaryotic initiation factors that have been found to be involved in recessive resistance to plant viruses (*eIF4E* and *eIF4G* and their isoforms, *eIF(iso)4E* and *eIF(iso)4G*) (Diaz-Pendon *et al.,* 2004), other components from the translational machinery have been found to be involved in virus infection in some plants, including the translation initiation factor 4B (*eIF4B*), the translation elongation factors 1A and 1B (*eEF1A* and *eEF1B*) and poly(A)-binding proteins (PABPs) (Hwang *et al.,* 2013; Li *et al.,* 2014; Sasvari *et al.,* 2011). These proteins, along with eIF2Bϐ represent new targets and

mechanisms for antiviral strategies including via genome editing techniques and targeting induced local lesions in genomes (TILLING).

Eukaryote translation initiation is a highly regulated and complex stage of gene translation, which requires the action of at least 13 core initiation factors and 5 auxiliary factors (Hinnebusch and Lorsch, 2012; Jackson *et al.,* 2010). eIF2B is a multi-subunit protein critical for protein synthesis initiation and its control, it comprises 5 distinct subunits (α-ε) (Bogorad *et al.,* 2014; Jennings and Pavitt, 2014). The eIF2Bα subunit has been shown to be required for translation suppression of vesicular stomatitis virus, suggesting its regulatory role in virus infection in mammals (Elsby *et al.,* 2011). The association of *eIF2Bβ* with TuMV resistance is the new example of this gene acting as a resistance gene to a plant virus. eIF2B acts as a guanine nucleotide exchange factor (GEF) for its GTP-binding protein partner eIF2 via interaction with eIF2·GTP at an early step in translation initiation (Jennings and Pavitt, 2014). Mutations in eIF2B subunits impact on the stability of the eIF2B complex, or appear to alter the interaction with eIF2 in some cases (Jennings and Pavitt, 2014). It is possible that the mutation A120G affects the eIF2B-mediated interaction with eIF2, possibly the guanine nucleotide exchange for its GTP-binding protein partner eIF2. The SNP genotyping analysis demonstrated that the amino acid alanine at position 120 is required for TuMV susceptibility. The presence of the amino acids glycine, or tryptophan at this position in the resistant plant lines appears to be the determinant of resistance. The *B. juncea* lines investigated in this study were all landraces and of various types including oilseed, leafy vegetable and root; the resistant plant lines were root, or oilseed types originating from Tibet. It will be interesting to compare the phylogeny and evolutionary relationship between

resistant and susceptible types in order to look at the origin and evolution of the resistance. The presence of multiple copies of *eIF2Bβ* in brassicas allows redundancy, this along with the fact TuMV only seems to be able to use, or access one eukaryotic translation initiation complex (Nellist *et al*., 2014) facilitates recessive TuMV resistance in brassicas without the mutations resulting in plant death, or affecting the general growth of the plant. The genome multiplication event in brassicas resulted in high gene content and facilitated the gene redundancy, although substantial genome reshuffling and gene loss also occurred (Chalhoub, 2014; Lysak *et al.,* 2005; Wang *et al.,* 2011).

Recessive virus resistance in the allopolyploid brassicas is very rare. Despite a lot of research on one of the other allopolyploid species, *B*. *napus*, only dominant resistance has been found (Walsh and Jenner, 2002). The presence of recessive resistance in so many *B. juncea* accessions might suggest some big difference(s) in the evolution of these two allopolyploid species, perhaps related to selection pressure from different levels of exposure to TuMV and / or how long ago the two species originated from hybridisation between their respective diploid progenitors.

TuMV causes serious losses in *Brassica* crops and was considered to be the second most important virus infecting field vegetables (Tomlinson, 1987); most varieties of *B. juncea* are particularly susceptible to TuMV. The discovery of *eIF2Bβ*-mediated resistance to TuMV provides a new opportunity for the genetic control of this virus. RNA viruses have a high mutation frequency due to the low efficiency of proofreading-repair associated with RNA replicases and transcriptases (Steinhauer *et al.,* 1992). The development of efficient and

durable plant resistance able to withstand the genetic plasticity of viruses therefore represents a challenge for the coming years. The discovery of *retr03* (*eIF2Bß*) provides an extra target for the identification of resistance genes to a range of viruses, particularly the largest group of plant viruses the *Potyviridae*, many of which are known to be dependent on the eukaryotic translation complex for completing their life cycles (Robaglia and Caranta, 2006). More specifically, as *retr03* exists in the brassica A genome, there is the potential to deploy it in the other brassica species possessing the A genome, *B. rapa* and *B. napus*. It will also be possible to combine it with *retr01* which has been shown to confer broad-spectrum resistance to TuMV (Rusholme *et al.,* 2007; Walsh *et al.,* 2002) to potentially increase the durability of both resistance genes.

EXPERIMENTAL PROCEDURES

Plant material and virus isolate

A collection of 35 lines of *B. juncea* were evaluated for TuMV resistance. The resistant accession VC029 was crossed with the susceptible accessions STZ and WZD to generate F_1, F_2 and BC_1 populations for phenotypic and genetic analysis. The F_2 population from the cross between accessions VC029 and STZ was used for bulked segregant analysis by resequencing. The susceptible accession STZ was used for functional analysis. The TuMV isolate ZJ was collected from a field of *B. juncea* in Zhejiang Province of China. The coat protein (*CP*) gene was amplified by RT-PCR using primers GCAGATGAAACGCTTGACGCAG forward and

TACAACTTCATAACCCCTGAACGC reverse (Supplementary Table S7), the product sequenced and compared with those of other (Ohshima *et al*., 2002) TuMV isolates.

TuMV resistant germplasm identification and genetic analysis

To identify the TuMV-resistant *B. juncea* germplasm, plants at the 2-3 true-leaf stage were mechanically inoculated with the TuMV ZJ isolate (Jenner and Walsh, 1996). In order to reduce the chance of any escapes, a second inoculation was performed 3-5 days after the first inoculation. The mosaic, chlorotic, or necrotic symptoms of TuMV infection in inoculated plants were visually assessed 4 weeks after the second inoculation. Plants not showing symptoms were tested using a double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA) (Agdia, USA) to confirm the absence of TuMV. RT-qPCR was also used to check *TuMV-CP* gene expression following inoculation with TuMV. Plants with no obvious symptoms and that were negative in ELISA and RT-qPCR tests were classified as resistant and the others as susceptible (Walsh *et al.,* 1999). For genetic analysis, segregation ratios of resistant and susceptible phenotypes in F_2 and BC_1 families were analysed using the chi-square test for goodness of fit to the expected segregation ratio for the predicted genetic model. Primers used for checking *TuMV-CP* gene expression are listed in supplementary Table S7.

Bulked segregant analysis by resequencing

Resistant (49) and susceptible (49) plants from the F_2 population of the cross between accessions VC029 and STZ were selected. Total DNA was extracted from each plant and mixed equivalently to build the resistant (R) and susceptible (S) pools. The DNA from the R and S pools and accessions VC029 and STZ were submitted for pair-end (PE125) sequencing using Illunima Hiseq2500 according to the Illumina protocol. Filtered clean reads were mapped to the *B. juncea* genome to estimate insert size of the sequencing library and calculate read depth and distribution in the genome (Yang *et al.,* 2016) using the BWA program (Li and Durbin, 2009). Duplicated reads were removed using SAMtools (Li *et al.,* 2009) and single nucleotide polymorphisms (SNPs) were detected using the GATK toolkit in each pool and between pool referred to genome (McKenna *et al.,* 2010). SNPs linked to TuMV resistance were then identified through an association study using the Δ (SNP_index) (Fekih *et al.,* 2013; Takagi *et al.,* 2013). Regression and fitting analysis of the Δ (SNP_index) for SNPs markers in the same chromosome was used to decide the threshold of the Δ (SNP_index) (Hill *et al.,* 2013). The threshold (0.151181810500557) of Δ (SNP_index) was determined for the 99% quantile in this analysis. We annotated all genes in associated regions using nr, Swissprot, GO (Gene Ontology), COG (Cluster of orthologous groups) and KEGG (Kyoto Encyclopedia of Genes and Genomes) databases.

Functional analysis of *eIF2Bβ*

The *eIF2Bβ* gene (*BjuA006209*) was silenced in 30 day old STZ seedlings by using the one-step TYMV-based VIGS system (Pflieger *et al.,* 2008). Palindromic 40nt oligonucleotides including inverted 40nt repeats (total 80nt) were designed from the target gene (BjuA006209), 5'-TTAATGGGAAAGGGATTGGGTATCCTGCGATGTCTGCGGCGCCGCAGACATCGCAGGATACCCA ATCCCTTTCCCATTAA-3'. The pTY-S VIGS vector plasmid was digested with the SnaBI restriction enzyme. The self-hybridised custom oligonucleotide was ligated with the linearised vector. Amplification of a *TYMV-CP* gene of the expected size (560nt) was used to identify positive clones. For virus infiltration, 3 µg of purified pTY-S carrying the target gene plasmid DNA in 8 µL was diluted in 25 µL ddH_2O and infiltrated into 2-4 fully expanded plant line STZ leaves. The plants infiltrated with the empty pTY-S vector were used as a control. The pTY-PDS-IR vector used as a positive control causes photo-bleaching because of silencing of the phytoene desaturase gene. Infiltrated plants were maintained at 20/22 ºC with a 16h/8h light/dark cycle in a growth chamber. Three weeks post infiltration, the plants were inoculated with TuMV as described above and phenotypes were assessed 3 weeks post inoculation. Primers used for detecting the clone used to silence the *eIF2Bβ* gene (*BjuA006209*) are listed in supplementary Table S7.

To generate a construct for expression of *eIF2Bβ* from the susceptible line (STZ) in the resistant line (VC029) using the TYMV derived pTY-S vector, full length copies of *eIF2Bβ* from STZ were amplified using ATCCCGGGATGCCGGACGTGCAATCGA as the forward primer with

the *Sma*I restriction enzyme site and CTACCGGTCATCACCAAATCATCAGCGGA as the reverse primer with the *Age*I restriction enzyme site. Products were cloned into the pTY-S virus-derived vector containing the CaMV 35S promoters to generate pTY-S- eIF2Bβ using the T_4-DNA ligase system (Clontech TaKaRa, USA). The pTYCP-F: TCCACCCTCACCACCTTC and pTYCP-R: CCCTAATTCCCTTATCTGGG primer pair was used to check for the presence of *eIF2Bϐ* in the clone. The TuMV isolate and inoculation procedure were as described above. *eIF2Bϐ* was cloned from VC029 plants inoculated with the TYMV vector pTY-S/eC expressing *eIF2Bϐ* from the susceptible plant line STZ.

Genotyping analysis of *eIF2Bϐ*

We identified *eIF2Bϐ* orthlogs in *B. rapa*, *B. nigra*, *B. juncea* and *Arabidopsis* using Blastn. After alignment by ClustalW, a Maximum Likelihood tree was constructed by using MEGA 5.0, with 1000 as the number of bootstrap replications. Because of the genome triplication event in the diploid brassica species and the subsequent duplication during the evolution of *B. juncea*, the alleles of *eIF2Bϐ* (*BjuA006209*) from TuMV-resistant and TuMV-susceptible *B. juncea* lines were analysed. A specific fragment including the non-synonymous *eIF2Bϐ* SNP was amplified by PCR from plants of 7 resistant lines (VC029, VC028, VC024, WT-28, 03B0103, 03D0102, 4D101) and 7 susceptible lines (STZ, WZD, MF, 03C0804, 03C0909, VB109, VA003) to analyze the SNP further. The primers are listed in supplementary Table S7.

Molecular marker development based on *eIF2Bβ*

To identify the genomic variation between resistant (R) and susceptible (S) *B. juncea* plant lines at the *BjueIF2Bβ* loci, genomic DNA was extracted from 100-200 mg of leaves using the DNeasy kit (Qiagen) and genes were amplified and genomic sequences obtained. Full genomic sequences were aligned and variation analysed using Clustalx. Based on indel variation between genomic sequences of resistant (R) and susceptible (S), degenerate primers (*BjTuR*, forward: GTTAATGGGAAAGGGATTGGGTATCCTTG and Reverse: ATAGCTTGCTCGGCGATCTGCTCAT) were used to amplify specific fragments with different lengths for resistant and susceptible plants (Supplementary Table S7).

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The authors declare no conflict of interest.

SUPPORTING INFORMATION

Figure S1. A phylogenetic tree of the coat protein nucleotide sequences of Turnip mosaic virus (TuMV) isolates including the isolate used in this study (TuMV_ZJ).

Figure S2. Plant (Bar=5 cm) and leaf (Bar=4 cm) symptoms in 35 *Brassica juncea* accessions after inoculation with Turnip mosaic virus isolate ZJ.

Figure S3. Turnip mosaic virus (TuMV) *Coat Protein* (*CP*) gene expression in 35 *Brassica juncea* accessions following virus inoculation, determined using qRT-PCR.

Figure S4. Measurement of CP accumulation by ELISA as a proxy for virus accumulation of 35 *Brassica juncea* accessions after Turnip mosaic virus inoculation, determined by enzyme-linked immunosorbent assay (ELISA).

Figure S5. Distribution of coverage depth of resequencing data on *Brassica juncea* chromosomes. A, susceptible F_2 bulk. B, resistant F_2 bulk.

Figure S6. GO (Gene Ontology) analysis of genes linked to Turnip mosaic virus resistance in *Brassica juncea* from the association study.

Figure S7. COG (Cluster of orthologous groups) analysis of genes linked to Turnip mosaic virus resistance in *Brassica juncea* from the association study.

Figure S8. Alignment of *eIF2Bβ* (*BjuA006209*) cDNA sequences from a Turnip mosaic virus resistant (R) and a susceptible (S) line of *Brassica juncea*.

Figure S9. Alignment of *eIF2Bβ* (*BjuA006209*) putative amino acid sequences from a Turnip mosaic virus resistant (R) and a susceptible (S) line of *Brassica juncea*.

Figure S10. Alignment of *eIF2Bβ* (*BjuA006209*) genomic sequences between Turnip mosaic virus resistant (R) and susceptible (S) *Brassica juncea* lines.

Figure S11. Turnip mosaic virus (TuMV) infection and *eIF2Bβ* (*BjuA006209*) expression levels in *Brassica juncea* STZ plants following silencing with pTY-S/eS and challenge with Turnip mosaic virus isolate ZJ. **(a)** Expression levels of *eIF2Bβ* (*BjuA006209*) in plants showing different susceptibilities to TuMV. **(b)** Leaf symptoms of plants showing different susceptibilities to TuMV. Mock, buffer only inoculation of STZ plants; R, Resistant; MS, intermediate level of susceptibility; S, Susceptible. Bar=4cm.

Figure S12. Indel variation between Turnip mosaic virus resistant and susceptible lines of *Brassica juncea*.

Table S1. Resequencing of Turnip mosaic virus resistant and susceptible *Brassica juncea* F_2 bulks.

Table S2. Statistics of single nucleotide polymorphisms (SNPs) in Turnip mosaic virus resistant and susceptible *Brassica juncea* F_2 bulks.

Table S3. Statistics of single nucleotide polymorphisms (SNPs) between Turnip mosaic virus resistant and susceptible *Brassica juncea* gene pools.

Table S4. Chromosome regions associated with Turnip mosaic virus resistance in *Brassica juncea*.

Table S5. The candidate genes associated with Turnip mosaic virus resistance in *Brassica juncea*.

Table S6. Turnip mosaic virus resistance and susceptibility in *Brassica juncea* plant line STZ inoculated with the VIGS vector.

Table S7. Primers used in this study.

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Table 1. Identification of Turnip mosaic virus-resistant *Brassica juncea* germplasm

Accessions	Phenotypic symptom	RT-qPCR of *TuMV-CP*	ELISA	Category
03A0106	+	+	+	S
VC021	+	+	+	S
VC024	-	-	-	R
VC026	-	+	+	S
VC028	-	-	-	R
VC029	-	-	-	R
VC032	-	-	+	S
WZD	+	+	+	S
STZ	+	+	+	S
VB005	+	+	+	S
VB017	+	+	+	S
VB109	+	+	+	S
03B0103	-	-	-	R
03B0304	+	+	+	S
VA003	+	+	+	S
VA202	+	+	-	S
VA274	+	+	+	S
VA365	+	+	+	S
VA377	+	+	+	S
03C0804	+	+	+	S

03C0909	+	+	+	S
03C1112	+	+	+	S
4D101	-	-	-	R
4D105	-	+	-	S
4D106	+	+	+	S
03D0102	-	-	-	R
03D0110	+	+	+	S
VE001	+	+	+	S
VE002	+	+	+	S
JCX	+	+	+	S
WT-6	+	+	+	S
WT-8	+	+	+	S
WT-12	+	+	+	S
WT-14	+	+	+	S
WT-28	-	-	-	R

+, infected; - not infected; S, susceptible; R, resistant.

Table 2. Inheritance of Turnip mosaic virus resistance in *Brassica juncea*

Crosses	Generation	Resistant	Susceptible	Expected ratio (R:S)	Chi-square	*P*[a]
VC029 ×STZ	F_1	0	20			
	F_2	55	171	1:3	0.05	0.82
VC029 × WZD	F_1	0	20			
	F_2	30	94	1:3	0.04	0.84
(VC029 × WZD) ×VC029	BC_1	44	36	1:1	0.8	0.37

Phenotypic observation was used to determine resistance/susceptiblity to TuMV infection in plants 19 days after inoculation.

[a]Observed segregation ratios are statistically consistent with expected ratios (Chi-square test, P > 0.05).

FIGURE LEGENDS

Figure 1. Phenotypes of *Brassica juncea* lines VC029 and STZ and F_1 plants following Turnip mosaic virus (TuMV) ZJ inoculation. **(a)** Leaf symptoms (Bar=1 cm). **(b)** *TuMV-coat protein* (*CP*) expression in leaves 2 weeks after TuMV inoculation. **(c)** TuMV isolate concentration in leaves 2 weeks after TuMV inoculation determined by enzyme-linked immunosorbent assay (ELISA).

Figure 2. Mapping of Turnip mosaic virus (TuMV) resistance candidate genes using bulked segregant analysis by resequencing. **(a)** SNP-index statistics of resistant (R) and susceptible (S) gene pools. **(b)** SNP distribution of candidate genes in R and S gene pools.

Figure 3. Genotyping analysis of the Turnip mosaic virus resistance candidate gene (*BjuA006209*). **(a)** Alignment of *BjuA006209* with its orthologues from *Arabidopsis thaliana*, *Brassica rapa*, *Brassica nigra* and *Brassica juncea*. **(b)** Schematic diagram of *eIF2Bϐ* during the *Brassicaceae* triplication event (WGT). Only two copies of *eIF2Bϐ* were found in *B. rapa* and *B. nigra* indicating that both species had lost one copy. The blue copies in *B. rapa* (r) and in *B. nigra* (n) have the highest amino acid homology with the single copy in Arabidopsis, whereas the red copies are thought to have resulted from genome duplication. Of the three copies of *eIF2Bϐ* in *B. juncea*, homologies indicate that the two copies with highest homology to Arabidopsis originated from *B. rapa* and *B. nigra* and the copy originating from the duplication event came from *B. rapa*. **(c)** Genotyping of the non-synonymous substitution (A120G) of *BjuA006209* in resistant (R) and susceptible (S) lines of *Brassica juncea*. **(d)** Genotyping of the non-synonymous substitution (A120G) of *BjuA006209* and its homologue gene (*BjuB006522*) in resistant (R) and susceptible (S) *B. juncea* plants. WGD, whole genome duplication; WGT, whole genome triplication.

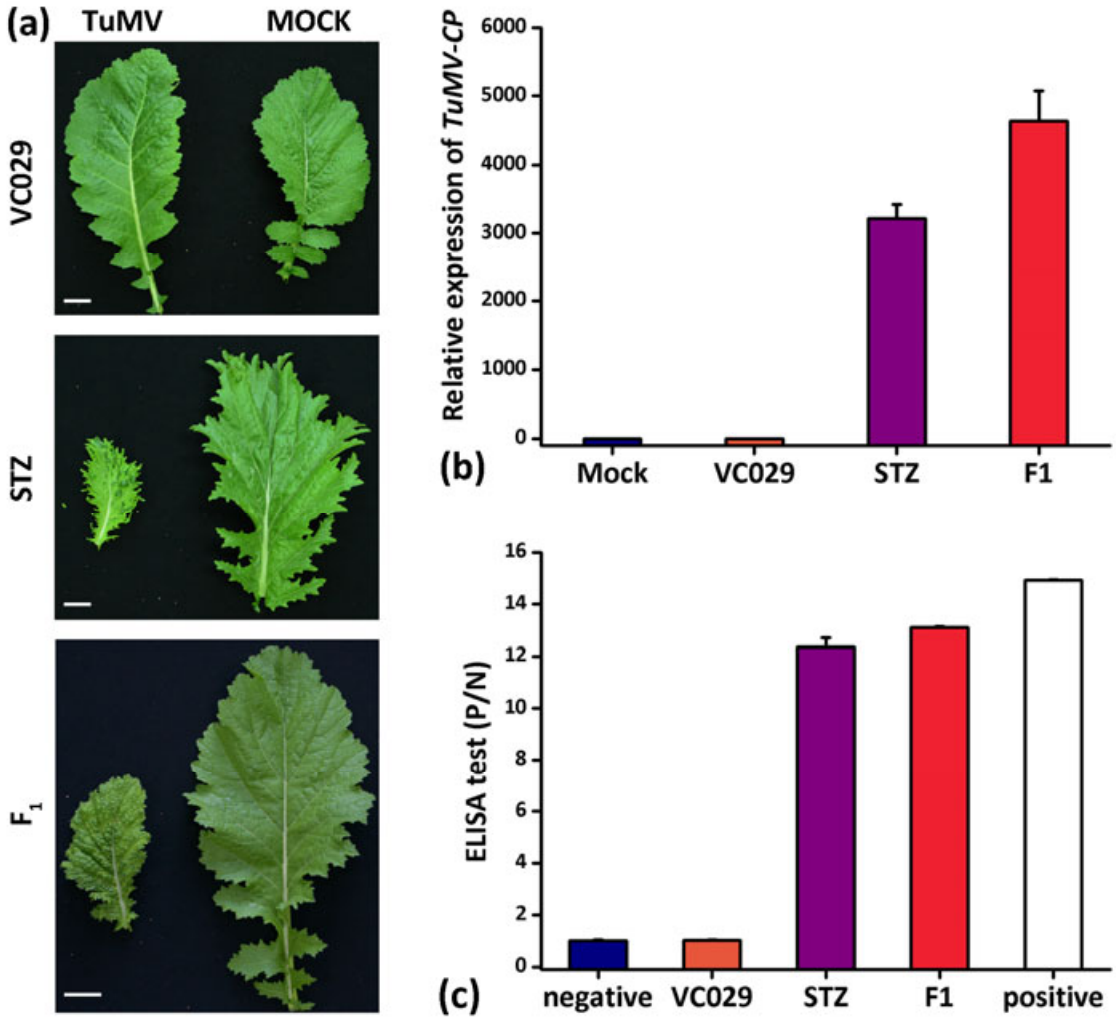
Figure 4. Phenotypes of *Brassica juncea* plant lines inoculated with the *eIF2Bϐ* (*BjuA006209*) VIGS vector following Turnip mosaic virus (TuMV) ZJ inoculation. **(a)** Plant growth (Bar=5 cm). **(b)** TuMV symptoms in leaves (Bar=4 cm). **(c)** *eIF2Bϐ* expression before TuMV

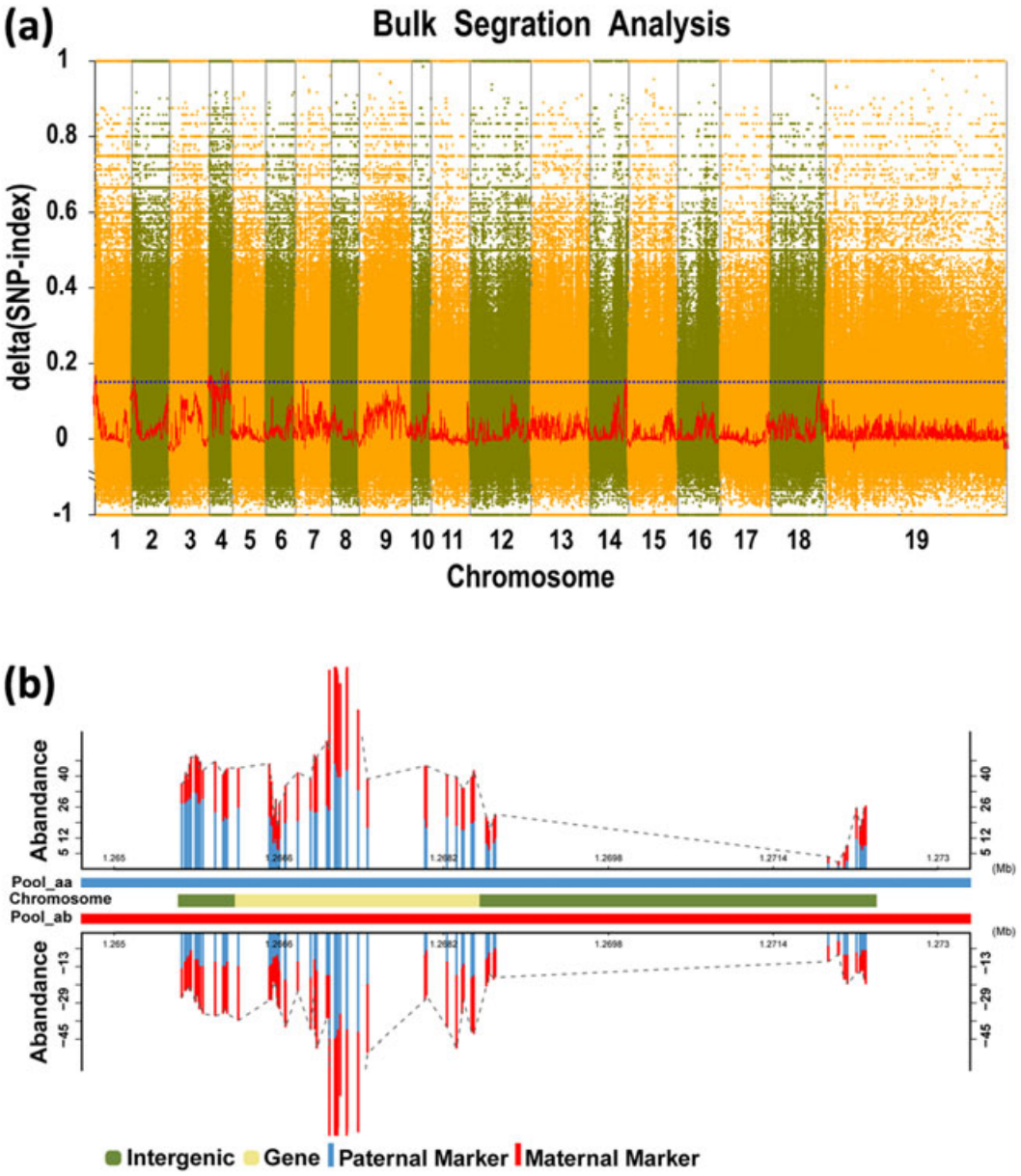
inoculation. **(d)** *eIF2Bβ* (*Bju020776* and *BjuB006522*) expression 2 weeks after TuMV ZJ inoculation. **(e)** *eIF2Bβ* (*BjuA006209*) expression 2 weeks after TuMV ZJ inoculation. **(f)** *TuMV-CP* expression 2 weeks after TuMV ZJ inoculation. **(g)** TuMV isolate ZJ content 2 weeks after TuMV inoculation. MOCK, inoculation buffer only; TuMV, Turnip mosaic virus (TuMV) ZJ isolate inoculation; pTY-S, TYMV-derived vector inoculation; pTY-S/TuMV, TYMV-derived vector inoculation followed by TuMV ZJ isolate inoculation; pTY-S/eS, TYMV-derived vector with *eIF2Bβ* silencing construction inoculation; pTY-S/eS/TuMV, TYMV-derived vector with *eIF2Bβ* silencing construction inoculation followed by TuMV ZJ inoculation.

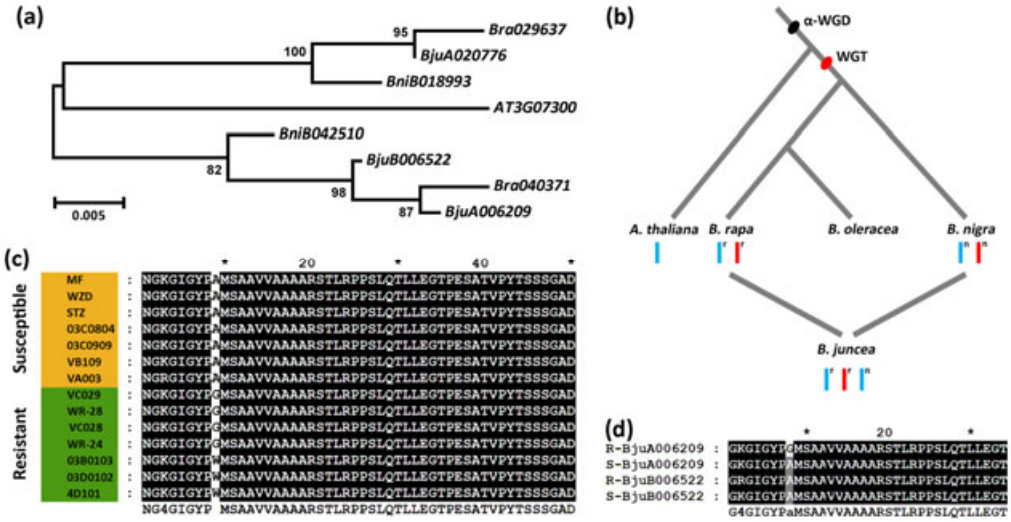
Figure 5. Phenotypes of the resistant plant line (VC029) following expression of *eIF2Bβ* (*BjuA006209*) from the susceptible plant line (STZ) and challenge with Turnip mosaic virus (TuMV) ZJ isolate. **(a)** Plant growth (Bar=1 cm). **(b)** TuMV symptoms in leaves (Bar=1 cm). **(c)** Confirmation of the presence of *eIF2Bβ* from STZ in VC029 following pTY-S/eC inoculation by sequencing *eIF2Bβ* clones from VC029 plants. Seven clones with C and three clones with G were observed. **(d)** *TuMV-CP* expression 2 weeks after TuMV ZJ inoculation. **(e)** TuMV isolate ZJ content 2 weeks after TuMV inoculation determined by ELISA. MOCK, buffer inoculation of VC029 plants; TuMV, Turnip mosaic virus (TuMV) ZJ isolate inoculation of VC029 plants; pTY-S, TYMV-derived vector inoculation of VC029 plants; pTY-S/TuMV, TYMV-derived vector inoculation of VC029 plants followed by TuMV ZJ isolate inoculation; pTY-S/eC, TYMV-derived vector expressing *eIF2Bβ* from STZ plants inoculation of VC029

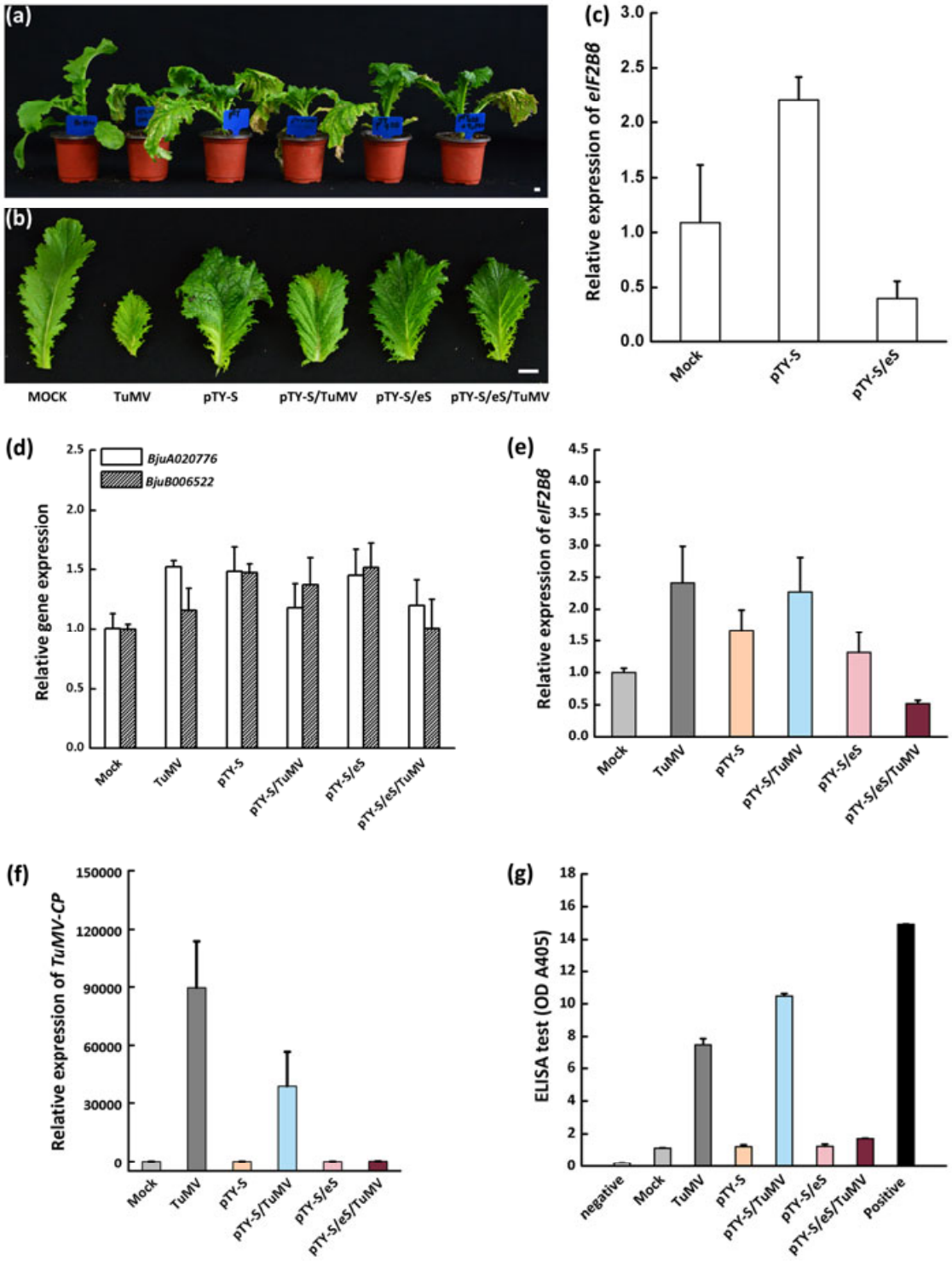
plants; pTY-S/eC/TuMV, TYMV-derived vector expressing *eIF2Bß* from STZ plants inoculation of VC029 plants followed by TuMV ZJ isolate inoculation.

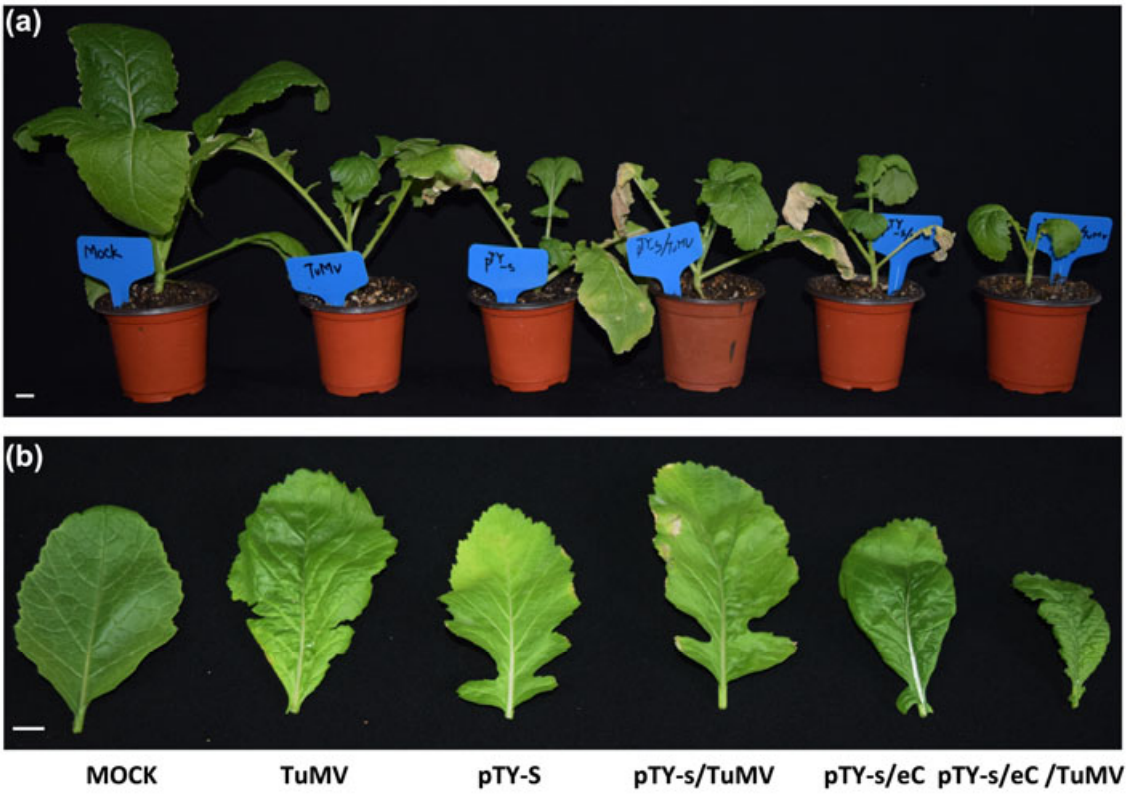
Figure 6. Molecular marker-assisted selection of Turnip mosaic virus (TuMV) resistance in *Brassica juncea*. **(a)** Genomic sequence comparison of *eIF2Bß* (*BjuA006209*) alleles from resistant (R) and susceptible (S) lines showing the non-synonymous SNP and Indel variation. **(b)** Molecular marker (*BjTuR*) development based on the Indel variation between R and S lines. **(c)** Correlation of the *BjTuR* marker with TuMV resistance in the F_2 population (Bar=1 cm). **(d)** Correlation of the *BjTuR* marker with TuMV resistance in the BC_1 population. **(e)** *BjTuR*-assisted selection of TuMV resistance in *B. juncea* (Bar=1 cm).











(c)

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Mock            : GATGTTAATGGGAAAGGGATTGGGTATCCTGCGATGTCTGCGGC
pTY-S/eC/TuMV-1 : GATGTTAATGGGAAAGGGATTGGGTATCCTGCGATGTCTGCGGC
pTY-S/eC/TuMV-2 : GATGTTAATGGGAAAGGGATTGGGTATCCTGCGATGTCTGCGGC
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