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ORIGINAL ARTICLE

Enhancement of resistance to PVY in intragenic marker-free potato plants by RNAi-mediated silencing of eIF4E translation initiation factors

Dmitry Miroshnichenko[1,2] · Vadim Timerbaev[1,2] · Anna Okuneva[3] · Anna Klementyeva[1] · Tatiana Sidorova[1] · Alexander Pushin[1,2] · Sergey Dolgov[1,2]

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Abstract

Among various defence strategies used to protect potato plants from a viral infection, a deficiency of the eukaryotic translation initiation factors (eIF) provided by the host-mediated translational suppression has been characterized as a promising approach in modern crop breeding. The strategy used in the present study relies on knocking down the genes of the eIF4E-encoding family in a susceptible potato cultivar by the RNAi silencing mechanism. Taking into account the high concern regarding the potential effects of biotech crops on product safety and public acceptance, no bacterial/viral sequences and selectable marker genes were used. A hairpin construct carrying the fragment of potato *eIF4E1* gene was created under control of potato *Lhca3* (photosystem I 24 kDa light-harvesting protein) gene promoter and terminator. As a result of marker-free *Agrobacterium*-mediated transformation, two independent events were identified; one was confirmed to be clean from vector-backbone DNA sequences. Due to high homology between the genes encoding eIF4E1 and eIF4E2 potato factors, the silencing of both genes was achieved. During the two seasonable experiments, the intragenic line with higher production of small RNAs due to the expression of hpRNAi cassette showed a strong level of resistance after the mechanical inoculation with agriculturally significant PVY^{NTN} strain and produced tubers phenotypically similar to non-infected control. Overall, the results obtained here indicate that RNAi-mediated transcriptional regulation of targeted *eIF4E* gene family using plant tissue-specific promoters in marker-free intragenic potato plants is a promising strategy for improving the viral resistance in clonally propagated crops without affecting plant phenotype and productivity.

Key message

This study is a proof-of-concept for successful generation of viral resistance in potato by the RNAi-mediated transcriptional regulation of targeted *eIF4E* gene family using intragenic approach.

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✉ Sergey Dolgov
Dolgov@bibch.ru

[1] Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry RAS, Science Ave 6, Pushchino, Moscow Region, Russia

[2] All-Russia Research Institute of Agricultural Biotechnology, Moscow, Russia

[3] Doka-Gene Technology Ltd, Rogachevo, Moscow Region, Russia

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Introduction

The reduction of virus infection is still a major problem for potato growers. With the globalization of agriculture, various viral diseases have become widely distributed and significantly affected the potato production. Among more than forty viruses infecting potato, the *Potato virus Y* (PVY, *Potyvirus* genus, family *Potyviridae*) is considered as the most harmful viral disease. The potato yield losses in various geographical areas can reach 50–100% due to severe PVY infection depending on the cultivar, virus strain, developmental stage of plant and temperature during the plant growth (Visser et al. 2012). The control of PVY in fields is difficult. PVY has a wide natural host range (Brunt 2001) and easily transmitted in a non-persistent manner mechanically and by a range of aphid species and others vectors, capable to colonize potatoes (Loebenstein and Gaba 2012). A combination of classical agricultural activities with the vector control and the use of the certified high-grade potato seeds generally provide the relative effect in limitation of the virus spread (Jeffries et al. 2006). The best reliable way to withstand the PVY infection is the breeding of cultivars displaying viral resistance at the genetic level.

One of the antiviral genetic concepts used in the modern plant breeding is the disruptions of virus establishment in host plants using the RNA interference (RNAi) mechanism. In the last 15 years, many efforts have been made to inhibit virus infection in plants through the introduction and expression of constructs encoding virus-specific small RNAs (sRNAs) derived from various functional parts of the viral genome (Pooggin 2017). To date, most of PVY genome sequences were successfully used as targets for RNAi silencing. PVY represents a 9.7-kb positive-sense single-stranded RNA translating in host cells into a large polyprotein, that is processed into 10 mature proteins with different functions (Loebenstein and Gaba 2012). The expression of constructs contained cDNA of the inverted repeat sequences of the PVY genome, including the eight functional genes *P1*, *HC-Pro*, *P3*, *CI*, *NIa-Vpg*, *NIa-Pro*, *NIb* and *CP*, allowed researchers to inhibit PVY spreading in tobacco and potato plants (Gargouri-Bouzid et al. 2006; Bai et al. 2009; Chen et al. 2010; Kent et al. 2012). Despite the fact that the efficiency of viral gene silencing significantly varied when the different regions of the PVY genome were targeted, the abundant production of self-complementary virus-specific hairpin RNAs in transgenic plants conferred an effective resistance to a single or multiple PVY strain.

Although the introduction of viral-derived RNAi constructs has been shown as an effective technique, the transgenic resistance concept has several constraints. In many counties, such novel crops met various types of public opposition because of 'uncertain' biosafety of transgenic plants due to the introduction of foreign (viral, bacterial) and selective sequences. A survey of growers from the North American potato industry revealed that near 90% of respondents are agreed to support the biotech varieties if the new traits will be derived from the potato genome (Toevs et al. 2011). From this point, the cisgenic or intragenic concept based on the overexpression or suppression of the host genes, which are involved into the plant-virus interactions, could be considered as a more promising approach in terms of public acceptance of GM crops.

Recent studies have demonstrated that plant genes encoding the eukaryotic translation initiation factors (eIF) represent promising targets for engineering viral resistance using New Plant Breeding Techniques (Bastet et al. 2017). Plant *eIF4Es* are organized in small gene families, coding three main family members eIF4E, eIF(iso)4E (plant-specific), and nCBP (novel cap binding protein). The genes of the *eIF4E*-encoding family are presented in plant genomes by different paralogs, especially in polyploid species. In *Solanacea*, including potato, pepper, tomato and tobacco two variants of *eIF4E* gene, *eIF4E1* and *eIF4E2*, with a high homology (70–80%) have been identified, while protein encoded by the *eIF(iso)4E* share only approximately 50% amino acid identity with *eIF4E1*/*eIF4E2* genes (Jung and Yeam 2013). A functional deficiency of eIF4 factors have been reported to increase the immunity of the host plants against a set of viral species, belonging mostly to potyviruses, but also to carmoviruses, bymoviruses and cucumoviruses (Schmitt-Keichinger 2019). The loss of susceptibility to various plant viruses with single-stranded RNA genomes was initially found in various naturally resistant individuals bearing the mutated *eIF4E* alleles with point mutations or deletions (Sanfaçon 2015; Schmitt-Keichinger 2019). Such recessive resistance alleles encode the modified eIF4E proteins, thereby preventing the interaction between initiation factors and the multifunctional viral genome-linked protein (VPg), which is largely involved in the replication, translation, cell-to-cell and long-distance movement of viruses belonging to family *Potyviridae* (Wang and Krishnaswamy 2012; Sanfaçon 2015). Recessive natural resistance genes mapped to mutations in *eIF4E* locus have been found in more than 15 plant species (Sanfaçon 2015; Schmitt-Keichinger 2019), including tobacco, tomato, pepper and potato displaying resistance to PVY (Ruffel et al. 2002, 2005; Duan et al. 2012). Moreover, some of the natural evolved *eIF4E* variants have been successfully introgressed in some

cultivars of pepper (Ruffel et al. 2002), lettuce (Nicaise et al. 2003), pea (Gao et al. 2004) and barley (Stein et al. 2005).

In most of the crops, however, it's difficult or still impossible to incorporate recessive resistance into the best commercial cultivars in a few steps using conventional crossbreeding, even if the modified alleles of eIF4E factors are present in related species. In potato, due to high heterozygosity and tetrasomic inheritance in the offspring of modern cultivars (Muthoni et al. 2015), the extensive screening of large populations is required to eliminate unfavourable alleles and to breed new marketable cultivars. For these reasons, the introgression of recessive PVY resistance in potato is a complicated, laborious and time-consuming (12–15 years) process with an uncertain outcome.

Unlike traditional breeding, the time required for the introduction of a specific gene into the genome of commercial potato variety through genetic engineering is generally varied between 10 and 12 months. This time includes *Agrobacterium*-mediated delivery of construct, generation of plants in vitro, transfer of genetically modified plants into the greenhouse and preliminary analysis of morphological, molecular and physiological characteristics. As a result, the transgenic technique has been applied to confer the differential resistance against PVY by the overexpression of a natural allelic variant of the *eIF4E* genes (Bastet et al. 2017; Schmitt-Keichinger 2019). The introduction of constructs encoding the genes of the *eIF4E*-encoding family, such as *pvr1*^{2} and *Eva1*, isolated from pepper and the wild potato, increased the immunity of virus-susceptible potato variety to the three main PVY strains (Duan et al. 2012). Similarly, the transgene expression of a *pvr1* allele in tomato increased the resistance to the *Tobacco etch virus* and *Pepper veinal mottle virus*, representing the *Potyviridae* family (Kang et al. 2007). In addition, the functional *eIF4E* genes of potato and *Arabidopsis* were modified via chemical or site-directed mutagenesis and then introduced back into the plant genome to generate complemented plants displaying various degrees of the resistance to potyviruses (Cavatorta et al. 2011; Arcibal et al. 2016; Bastet et al. 2018).

As an alternative genetic defence mechanism, the RNAi induced silencing of *eIF4E* genes could be applied to increase the viral immunity in plants. To date, RNAi mediated silencing of *eIF4E* genes have been successfully used to confer resistance to potyviruses in tomato (Mazier et al. 2011), melon (Rodríguez-Hernández et al. 2012), plum (Wang et al. 2013), tobacco (Takakura et al. 2018) and to one of tombusviruses in melon (Rodríguez-Hernández et al. 2012). In above-mentioned reports, the synthesis of double-stranded RNA due to an expression of intron-spliced hairpin constructs with homology to the sequences of *eIF4E1*/*eIF4E2* or *eIF(iso)4E* leads to a degradation of RNA transcripts produced by targeted genes and deprives viruses of their ability to use the host translational machinery for the multiplication. At the same time, the silencing of the natural *eIF4E-1* gene in potato cv. 'Russet Burbank' by expression of the hairpin construct containing the strictly specific inverted repeats was not successful in conferring resistance to PVY (Duan et al. 2012). In contrast, the significant decrease in the accumulation of a resistance-breaking strain of PVY has been recently reported for tobacco (*Nicotiana tabacum* L.) silenced for two homeologous isoforms of eIF(iso)4E (Takakura et al. 2018).

Evidence from previous reports indicates that the success of RNAi defense strategy largely depended from selectivity toward different eIF4 isoforms targeted for the specific viral resistance. In melon and tomato, a broad spectrum resistance to potyviruses have been achieved by the silencing of the *eIF4E* genes, while the RNAi lines silenced for its isoform eIF(iso)4E remained susceptible to potyviral infection (Mazier et al. 2011; Rodríguez-Hernández et al. 2012). The opposite situation was observed in plum, where RNAi-mediated reduction of the *eIF(iso)4E* transcripts provided effective resistance to plum pox virus (PPV), while *eIF4E* silenced transgenic lines were still susceptible for the viral infection (Wang et al. 2013). In accordance to this, the silencing of two *eIF(iso)4E* genes in allotetraploid tobacco (respectively, derived from the *N. sylvestris* or *N. tomentosiformis* parent) successfully triggered the resistance to PVY (Takakura et al. 2018).

To date, the most of described transgenic plants have been produced using various non-plant sequences such as bacterial selection markers or viral-derived promoters and genes. To alleviate the public concern about health safety of virus-resistant crops, the intragenic strategy can be used as one of the alternative approaches. Intragenesis refers to new plant breeding techniques, through which the new functional sequence is introduced by the ordinary methods of genetic transformation, but consists of various genetic elements originating from the gene(s) or loci of the same or a crossable species (Holme et al. 2013). An integral part of the intragenesis is the generating of antibiotic- and marker-free plants. Last decades, several strategies have been proposed to generate plants free of selectable markers (Holme et al. 2013). The elimination of selectable markers using the co-transformation strategy is hardly applicable for vegetatively propagated crops like potato, as this strategy is based on the separate inheritance of the selectable gene and gene(s) of interest in the progeny. The site-specific recombinase-mediated self-excision is a more promising method for potato, as it allowed removing undesirable genes from genome without loss of the original properties of cultivars. For example, the application of heat shock inducible Cre/lox systems resulted in a 71% excision efficiency of the *nptII* antibiotic resistance marker gene in potato (Orbegozo et al. 2016). This method, however, requires the creation of a complicated construct, while certain additional manipulation should be

done to re-regenerate marker-free plant after the induced excision. For potato, however, a less simple approach could be applied. A number of potato varieties demonstrate a high regeneration and transformation activities, so an *Agrobacterium*-mediated introduction of desirable sequences could be carried out without selective pressure. In the past years, constructs lacking the selectable marker genes have been successfully used for the generation of intragenic low acrylamide potatoes (Richael et al. 2008), cisgenic potatoes resistant to late blight (de Vetten et al. 2003; Jo et al. 2014) and transgenic double-virus-resistant potatoes (Bai et al. 2009).

In the present study the hpRNAi construct directed to target homological *eIF4E*s family genes of potato have been successfully introduced into the genome of commercial variety to produce PVY-resistant plants. To fit the intragenic strategy, the designed construct was consisted of the regulatory sequences (promoter, intron and terminator) belonging to the same crop species and was free of any selectable and marker genes.

Materials and methods

Construction of the intron-spliced-hairpin construct for RNAi–mediated silencing

The identification of orthologous potato *eIF4E* genes was carried out using a comparative analysis of published tomato and potato genes with a help of NCBI basic local alignment search tool (BLAST, https://blast.ncbi.nlm.nih.gov). The localization of the sequences of the studied genes in the potato genome was identified using Ensembl Genomes (https://plants.ensembl.org/index.html). Sequence alignment was performed using SnapGene software version 4.1.9 (GSL Biotech, https://snapgene.com).

To achieve the post-transcriptional silencing of the *eIF4E1* and *eIF4E2* genes expression, an RNA interference vector was created. The expression cassette was assembled in the pUCXB1 vector (plasmid pUC18 modified by the addition of *Xba*I and *Bam*HI restriction sites). A 474 bp fragment of the *eIF4E1* gene (Fig. S1) was amplified from cDNA of potato 'Pirol NN' variety. For this, RNA was isolated from young leaves of in vitro plants using a GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, Waltham, USA). cDNA was synthesized using primers (Tables S1) and RevertAid Premium Reverse Transcriptase (Thermo Scientific) as recommended by the manufacturer. For the amplification of *eIF4E1* gene repeats in the reverse and forward orientations primers E1(as)-up–E1(as)-low and E1(sa)-up–E1(sa)low, respectively, were designed (Table S1). All remaining intragenic sequences were amplified from genomic DNA of potato cv. 'Manhattan'. A strong promoter of the potato *Lhca3* gene (chlorophyll a/b binding protein; apoprotein II of the light-harvesting complex of photosystem I, GenBank no. S66876) was chosen as a regulatory element and its 969 bp fragment was amplified using prL3-up and prL3-low primers (Table S1). The 299 bp fragment of terminator from the same *Lhca3* gene (GenBank no. EU293853) was amplified with the use of trL3-up and trL3-low primer pairs (Table S1). The inverted repeats were separated by a functional intron from ribulose-(1.5)-bisphosphate carboxylase/oxygenase small subunit gene (*rbsc1*, GenBank no. X69759) 250 bp in size, which was amplified using inRB-up and inRB-low primers (Table S1). All PCR reactions were performed using Phusion Hot Start II DNA Polymerase (Thermo) on a SimpliAmp Thermal Cycler (Thermo) instrument. The PCR products were excised from the agarose gel and cloned into the appropriate vectors. The correctness of the cloning at each stage was controlled using restriction analysis, while the final vector was sequenced. The length of foreign sequences in the joints between the elements of the hairpin structure was no more than 6 nt. These minimal DNAs remained from the restriction sites used for cloning and cassette assembly. In our work, pMF1 (Schaart et al. 2004) was taken as the basis to create a vector that does not contain selective genes. With this purpose a complete T-DNA region of pMF1 was removed by *Vsp*I sites with the exception of both left and right borders. The resulted expression cassette was cut out at the *Asc*I and *Sbf*I sites, the ends were blunt and the previously assembled hairpin sequence was introduced. The resulting marker-free RNAi expression vector with a size of 9381 bp was named pWS-E1-Lhca3 (WS—without selection) (Fig. 1) and transferred into *Agrobacterium tumefaciens* strain AGL0.

Plant transformation and marker-free plant detection

Stem internodal segments of in vitro grown potato plants (*Solanum tuberosum* L. cv. 'Pirol NN', kindly provided by the Doka-Gene Technology Ltd, Russia) were used as explants for *Agrobacterium*-mediated transformation. Potato plants were grown in vitro in plastic containers inside a growth chamber under controlled conditions (16 h light 40 lE m^{-2} s^{-1} and 8 h dark at 22–25 °C) on phytohormone–free MS medium (Murashige and Skoog 1962) supplemented with 30 g l^{-1} sucrose and solidified with 0.8% (w/v) agar, pH 5.8. For transformation, the *A. tumefaciens* colonies were cultured overnight in a shaker (150 cycles min^{-1}) at 28 °C in liquid LB medium containing 100 mg l^{-1} kanamycin. Immediately before the explants cuttings, the agrobacterial suspension was diluted (1:10) with hormone-free MS medium to a final density of $OD_{600} = 0.4$. Fresh cut explants were incubated in the diluted agrobacterial suspension for 15–20 min, then blot-dried on sterilized paper and transferred to the regeneration medium supplemented

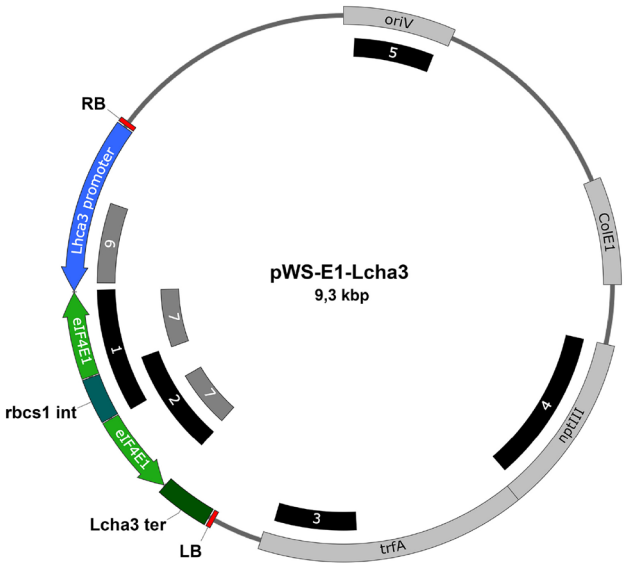

Fig. 1 Schematic presentation of the pWS-E1-Lhca3 vector, plasmid used for marker-free transformation of potato. *Lhca3* gene of Photosystem I chlorophyll a/b-binding protein 3-1, eIF4E1 eukaryotic initiation translation factor, *rbcs* ribulose-(1.5)-bisphosphate carboxylase/oxygenase small subunit gene, *RB* right border, *LB* left border; *trfA* replication protein, *nptIII* neomycin phosphotransferase III, *ColE1 E. coli* origin of replication, *oriV A. tumefaciens* origin of replication. Bars represent the elements that were detected by PCR (black bars) and Southern blot (gray bars). The number of primer pair is described in Supplementary Table 1

with MS macro- and micro-elements, MS vitamins, 30 g l^{-1} sucrose, 100 mg l^{-1} myo-inositol, 0.5 mg l^{-1} indoleacetic acid, 3 mg l^{-1} zeatin-riboside and solidified with 7 g l^{-1} agar, pH 5.8. After the co-cultivation for 3 days at 25 °C in the dark, nodal segments were transferred in the growth chamber under the light (16 h light 40 lE m^{-2} s^{-1} and 8 h dark at 22–25 °C) onto the fresh regeneration medium supplemented with 500 mg l^{-1} cefotaxime to eliminate *Agrobacterium.* The explants were subcultured every 10 days on fresh regeneration medium. To reduce the number of analyzed plants, all the shoots 1.5–3.0 cm long developed on inoculated explants under-non selective conditions were discarded during the early regeneration phase (20–45 days post inoculation), while smaller plantlets remained to grow further. When the remaining plantlets reached 1.5–2.5 cm long, they were detached from explants at 55, 65 and 70 days post inoculation. The independent shoots were cultured in culture vessels (eight plants per vessel) on phytohormone-free MS medium supplemented with 150 mg l^{-1} cefotaxime to develop plants of 5–8 cm long. The upper leaves of the developed plantlets were harvested for isolating genomic DNA for screening the presence of intragenic constructs by PCR. To reduce the number of manipulation, the leaf material of eight independent shoots growing in the same culture vessel was harvested and pooled prior extraction. If the polled DNA was positive for the presence of the hairpin construct, DNA from each plant of the pool (vessel) was isolated and analyzed by PCR to detect the single transformation event. The positive lines were further multiplicated and rooted by the nodal and apical segments using the same phytohormone-free MS medium supplemented with 150 mg l^{-1} cefotaxime. Well rooted in vitro plants were transferred to the greenhouse into the 5-cm pots containing a mixture of disinfected moss. Plants were covered with a semi-transparent film to maintain humidity over a period of 5 days when plants became strong enough to grow autonomously. The hardened plantlets were transplanted into the new pots (14 mm in diameter) to analyze viral resistance and morphological abnormalities.

Molecular characterization of intragenic plants

PCR analysis

Genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method as described (Rogers and Bendich 1994). The transfer of the hairpin sequence into the plants regenerated through the marker-free transformation was analysed by PCR. To provide amplification of a 756 bp fragment comprising the sequence of a part of the *eIF4E1* gene and a part of the intron of *rbcs* gene (Fig. 1, position 1), the E1(as)low and the inRBlow primers were used. For the amplification of an 750 bp fragment comprising the sequence of a part *eIF4E1* gene and a part of the intron of *rbcs* gene (Fig. 1, position 2) the forward primer inRBup and the primer E1(as)low were used. Primer pairs specific for the *trfA*, *nptIII*, and *oriV* genes, corresponding to a 501 bp, 943 bp, and 489 bp fragments, were also designed to study the presence of backbone-vector DNA (Fig. 1, positions 3, 4 and 5, respectively) in the lines carrying hairpin construct. All the primers used for the analysis of the integration of hairpin sequence and vector backbone are listed in Table S1. The amplified DNA fragments were visualized under ultraviolet light after electrophoresis on 2.0% agarose gel containing a TAE running buffer and ethidium bromide.

Southern blot analysis

Potato genomic DNA (30 µg) was digested overnight at 37 °C with 60 U *Eco*RV. The fragments were separated on 0.9% agarose gel and transferred to a positive-charged nylon membrane Hybond N + (GE Healthcare, Little Chalfont, United Kingdom) by capillary blotting following the manufacturer's instructions. The DNA probes were constructed by PCR using plasmid pWS-E1-Lhca3 as the template and pairs of primers for the *Lhca3* gene promoter and *eIF4E1* gene fragment (Fig. 1, Table S1) were used for amplification. DNA probe of 0.5 kb (PCR fragment of promoter of *Lhca3*

gene) were labeled with alkaline phosphatase using Amersham Gene Images AlkPhos Direct Labelling and Detection System (GE Healthcare). Prehybridization, hybridization (overnight at 60 °C) with alkaline phosphatase-labeled probes, and subsequent washings of the membrane were carried out according to the AlkPhos Direct Labeling System protocol. Detection was performed using CDP-Star detection reagent following the manufacturer's directions (Amersham CDP-Star Detection reagent, GE Healthcare). The signal from the blot was accumulated on X-ray film (Retina XBE blue sensitive, Carestream Health INC., NY, United States) in film cassette at room temperature for 24 h. X-ray films were scanned on Amersham imager 600 (GE Healthcare Life Sciences, Japan) after development.

RT-PCR and qRT-PCR analysis

Total plant RNA was isolated from inoculated and non inoculated plants using the Aurum Total RNA Fatty and Fibrous Tissue kit (Bio-Rad, USA). RNase-free DNase I polymerase was used to degrade plant genomic DNA before the RT reaction. To examine virus, cDNAs were generated from 2 µg of the total RNAs using RevertAid Reverse Transcriptase (RT) (Thermo Scientific, Lithuania) and were subjected to PCR. Viral genomic components including *Nib, CP, HC-Pro* and *P3* genes were detected using various sets of primers (Table S1) as described (Lorenzen et al. 2006; Chikh Ali et al. 2010). PCR products were separated by electrophoresis on 2% (w/v) agarose-ethidium bromide gels.

To quantify the RNA levels of the *eIF4E1* and *eIF4E2* genes by qRT-PCR, the cDNA of the wild-type (WT) plants and the lines carrying the hairpin construct were used. *ef1*α, a housekeeping gene, was used as endogenous control (Table S1). The experiments were performed on a LightCycler 96 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the PowerUp SYBR Green Master Mix reagent (Thermo) in strips in a volume of 20 µl. The PCR conditions were adjusted according to the manufacturer's recommendations with the use of primers listed in Table S1. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) was used to normalize the *eIF4E1* and *eIF4E2* values compared to the endogenous controls. Transcript levels were quantified with three biological repetitions (samples). Every sample was analyzed in triplicate. Data calculation and statistical analysis were performed using the software supplied with the Roche device.

Northern blot hybridization

An extracted potato total RNA (20 µg) was loaded onto 15% polyacrylamide gel containing 7 M urea, and then electrotransferred onto a membrane (Hybond-N+, GE Healthcare, United Kingdom). siRNA bands were probed within the 491 bp PCR fragment of *eIF4E1* gene (primers E1(as)up and E1(as)low) that was labeled with alkaline phosphatase using the Amersham Gene Image AlkPhos Direct Labeling and Detection System (GE Healthcare, United Kingdom). Detection was performed using CDP-Star detection reagent following manufacturer's directions (Amersham CDP-Star Detection reagent, GE Healthcare, United Kingdom).

Virus inoculation and virus-resistance analysis

In vitro-derived WT and *eIF4E*-silenced plants were manually inoculated with the *Potato virus Y*, stain PVY^{NTN} isolated from plants of commercially grown field (Doka-Gene Technology, Ltd) in Moscow region, Russia. For viral resistance analysis, in vitro rooted plants were hardened and raised in the controlled, insect-proof greenhouse with a 16 h natural daylight photoperiod and 18–20/22–25 °C (night/day) temperature regime. For artificial inoculation the fresh infected sap was prepared from previously infected potato leaves at a rate of 2.5 g tissue/10 ml phosphate buffer (pH 7.4) and the filtered inoculum was applied to plants. Plants were inoculated twice after a 2-day interval at the same time. The first inoculations were conducted at a stage of 6–8 leaves by rubbing leaf extracts onto the two leaves in the middle part of the plant. All plants were checked daily for symptoms for a month.

Two independent experiments were carried out to evaluate resistance to PVY. The first experiment was conducted in the summer when potato plants were grown in June–August. The second experiment was carried out during the winter season, and the plants were grown in November–February. Eight plants (replicates) of each line as well as WT plants were inoculated in each experiment. The PVY infection was analyzed by the RT-PCR and serologically through the enzyme-linked immunosorbent assay (ELISA). For this, the foliage (upper leaves) of plants was sampled two times after 15 and 25 days of the first inoculation. The protein extracts for ELISA test were prepared as described (Sidorova et al. 2019). PVY infection was evaluated using Double Antibody Sandwich ELISA (DAS-ELISA) by the kit purchased from LOEWE Biochemica GmbH (Sauerlach, Germany). DAS-ELISA assay was performed according to the manufacturers' instructions using rabbit polyclonal antibodies against the coat protein of PVY (Anti-Virus-IgG, Anti-Virus-IgG-AP-conjugate). Absorbance was measured with an iMark Microplate reader (Bio-Rad, USA).

Results

eIF4Es gene analysis for vector construction

In this study, we targeted the *eIF4E1* gene for post-transcriptional gene silencing based on the alignment and comparison of the published sequences of potato and tomato *eIF4E* genes. The *eIF4E1* gene includes 5 exons and 4 introns and the coding region consists of 696 bp. *eIF4E1* gene of potato demonstrates 97–99% identity with the sequences available in the GenBank, including homologs isolated from 'Russet Burbank' cultivar (JF927213), two alleles from unknown potato Finish cultivar (FN666435, FN666436), the ortholog from *Solanum etuberosum* (JF927214), known as *Eva1* (Duan et al. 2012), and the modified allele from 'Russet Burbank' strain 'Ida' (JN831440), which contains a few point mutations homologous to those of $pvr1^2$ (the recessive potyvirus resistance allele in pepper) (Cavatorta et al. 2011). The sequences of potato *eIF4E1* homologs share more than 96% nucleotide identity with the tomato *eIF4E1* gene (AY723733). The silencing of tomato *eIF4E1* gene, as well as of its closest homolog, *eIF4E2* (GQ451831), was previously found to confer the broad-spectrum resistance against potyviruses in transgenic tomato (Mazier et al. 2011). In BLAST searches, the closest match to the tomato *eIF4E2* gene in the potato genome was the sequence of the 4E-1-like protein mRNA (JN564590, 97% homology). In both potato and tomato, the locus for *eIF4E2* gene maps to chromosome 2 and the coding region consists of 663 bp. Similarly, the potato and tomato *eIF4E1* homologs are located on chromosome 3 of their genomes. The BLAST run also revealed nucleotide matches of the studied genes with parts of the potato genome located on other chromosomes. The discovered regions of the genome, however, are not extended and do not coincide exactly with the studied genes, suggesting that they are not paralogs of *eIF4E* genes. Since the nucleotide sequences of potato *eIF4E1* and *eIF4E2* genes show 72% identity, we designed an hpRNAi construct, which is directed to target both *eIF4Es* family genes using the homological 474 bp fragment. This fragment includes the complete sequence of the second exon and large parts of exons 1 and 3 (Fig. S1). By cloning self-complementary hairpin structures of this fragment we assembled the intragenic cassette (Fig. 1) which was further used for marker-free genetic transformation of potato.

Generation of marker-free potato lines and the analyses of events carrying the hairpin sequence

PVY susceptible potato 'Pirol NN' (Norika GmbH), an important cultivar for chip production and for table usage, was used for the generation of intragenic plants. Preliminary experiments indicated that 'Pirol NN' has a high ability for in vitro plant regeneration from nodal segments displaying 100% shoot formation rate on the MS medium supplemented with 3 mg l^{-1} zeatin-riboside and 0.5 mg l^{-1} indoleacetic acid. In the present study, 100 nodal explants of 'Pirol NN' were transformed by *A. tumefaciens* carrying the marker-free pWS-E1-Lhca3 vector. Overall 224 plantlets were collected under non-selective conditions and transferred to phytohormone-free medium for elongation. To simplify the search for the intragenic individuals, every 8 plantlets were pooled by planting into the one culture vessel, and after the plants have reached a height of 5–8 cm, leaves from every plant (one leaf from each plant) were collected and mixed together into one sample for DNA extraction. The analysis of samples through amplification of the chimeric union of the left arm of the hairpin construct with the *rbsc1* intron revealed the presence of intragenic plants in samples taken from the 4th and 10th vessels (Fig. S2). To identify individual events, the new DNA samples of the all plants cultured in these vessels were extracted, and two independent events (pws4.6 and pws10.1) were found to be positive in the PCR analysis (Fig. S2). Upon further analysis, by the amplification of the chimeric union of *rbsc1* intron and the right arm of the hairpin construct (Fig. S2), the integrity of the hairpin construct was confirmed in both identified lines. Thus, marker-free transformation efficiency defined as the percentage of shoots that is PCR positive for hairpin sequences was 0.9%; while defined as a number of PCR positive plants over the number of *Agrobacterium*-infected nodal explants it was achieved 2%.

PCR positive plants were further analyzed by Southern blot hybridization (Fig. 2). Hybridization was carried out using probes specific for the promoter sequence of *Lhca3*

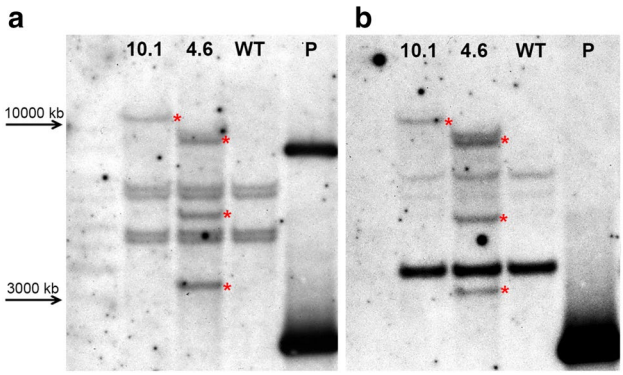

Fig. 2 Southern blot analysis of *Eco*RV-digested DNA isolated from PCR-positive potato lines using the probes specific for the sequence of *Lhca3* gene promoter (**a**) and the sequence of eIF4E1 translation initiation factor (**b**). *Lane* P: positive control, plasmid pWS-E1-Lhca3 digested with *Bgl*II; *lane* WT: control plant cv. 'Pirol NN'; *lanes* 4.6, 10.1: PCR-positive lines

gene and the sequence of eIF4E1 translation initiation factor. As expected, partially identical patterns were detected after Southern blot hybridisation between WT and lines generated after genetic transformation. All the plants contained four identical copies of *Lhca3* gene promoter (Fig. 2a), presumably corresponding to the number of alleles in tetraploid potato cv. 'Pirol NN', while three visible bands respecting to the *eIF4E* family genes were hybridized with the eIF4E1 probe (Fig. 2b). At the same time, evident additional copies of *Lhca3* promoter and *eIF4E1* fragment were detected in both events due to T-DNA insertion. Pws10.1 line contained one additional insertion, while at least three extra copies were detected in the genome of pws4.6 line (Fig. 2).

Both lines were also analysed by PCR for the presence of vector-backbone DNA sequences using primers specific for three genes outside the T-DNA of the pWS-E1-Lcha3 vector, such as *nptIII*, *trfA*, and *oriV* (Fig. S2). We found that line pws4.6 was positive for two of the PCR reactions, whereas line pws10.1 was negative for the presence of all analysed vector-backbone fragments. Despite the fact that the pws4.6 line was not completely free from the unwanted DNA insertion, along with pws10.1 it was further analysed for the RNAi mediated silencing of the *eIF4E1* and *eIF4E2* genes and for resistance to PVY.

Specific silencing of the *eIF4E1* and *eIF4E2* genes in potato plants carrying the hairpin construct

The expression levels of the targeted potato *eIF4E1* and *eIF4E2* genes in pws4.6 and pws10.1 lines were measured by reverse transcription-quantitative polymerase chain reaction (qRT-PCR). The analysis with primers specific for the fragment of potato *eIF4E1* gene showed approximately tenfold reduction of the accumulation level of *eIF4E1* mRNAs in both lines compared with the control 'Pirol NN' plants (Fig. 3a). Both potato lines with introduced RNAi constructs also showed a clear decrease of transcript levels of the *eIF4E2* isoform; mRNAs level of the endogenous *eIF4E2* gene in WT potato plants was 4.2 and 6.7 times higher than that in the samples of pws4.6 and pws10.1, respectively (Fig. 3a).

To assess whether the presence of reduced mRNA levels of the *eIF4E1* and *eIF4E2* genes in both lines are due to the RNA silencing, the accumulation of hairpin-derived small interfering RNAs (siRNAs) was analyzed by Northern blot. Blotting of two independent samples of low molecular-weight RNAs extracted from the greenhouse grown WT, pws4.6 and pws10.1 plants revealed a higher level of siRNA accumulation in pws10.1 and weaker, but equal intensities of siRNAs in two samples of the pws4.6 line (Fig. 3b). Since no visible bands were found in extracts of WT plants, this observation prompts us to conclude that the presence of small RNAs of 25 nt in both analyzed potato lines is the consequence of the degradation of hairpin RNAs due to the RNAi silencing.

Reducing *eIF4E* transcript accumulation enhances resistance to PVY

Plant of pws4.6 and pws10.1 lines together with WT 'Pirol NN' were mechanically inoculated at the 6–8 leaf stage (3 weeks of ex-vitro growth) by the same PVY^{NTN} isolate. To

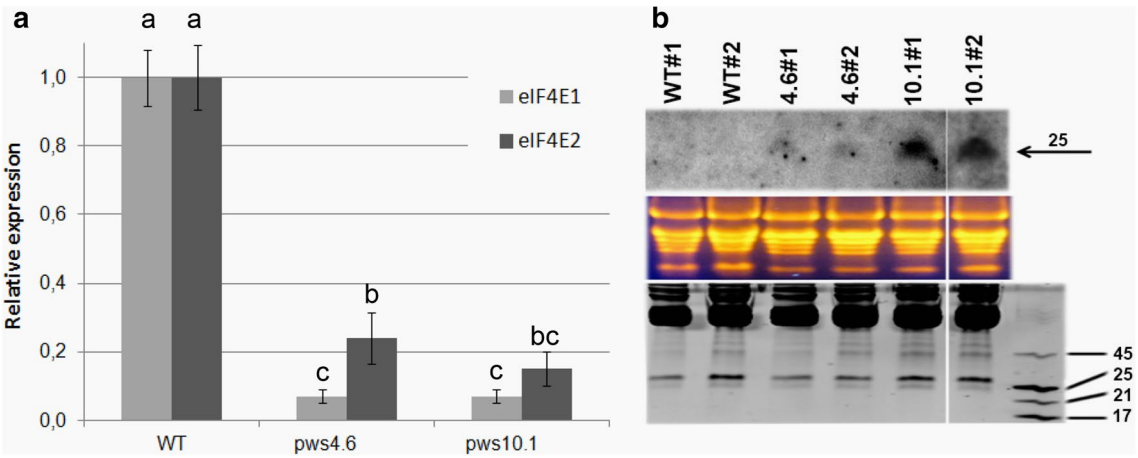

Fig. 3 Relative expression of *eIF4E1* and *eIF4E2* genes and siRNAs analysis of potato plants carrying the hairpin construct. **a** Reduction in the levels of *eIF4E* paralogs transcripts quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The presented values are means of three replicates and the bars are standard deviations. Means having the same letter in the column has no significant differences according to Duncan's multiple range test ($p < 0.05$). **b** RNAi-derived siRNAs detected by Northern blot. *Central panel* shows total RNAs as a loading control (ethidium bromide stained gel); *lower panel* shows the distribution of small RNAs of the same sample (acrylamide gel). WT#1 and WT#2—samples of two wild-type 'Pirol NN' plants, 4.6#1, 4.6#2, 10.1#1, 10.1#2—samples of low molecular-weight RNAs extracted from the greenhouse grown potato lines carrying the hpRNAi construct

Table 1 Resistance analysis of potato plants challenged with PVY^{NTN} in two independent experiments

	Plants with symptoms/plants inoculated					Type of symptoms
	8 dpi[a]	10 dpi	13 dpi	15 dpi	21 dpi	
Experiment 1[b]						
WT (Pirol NN)	0/14	11/14	13/14	14/14	14/14	Severe
Pws4.6	0/8	0/8	0/8	4/8	6/8	Mild
Pws10.1	0/8	0/8	0/8	0/8	0/8	No symptoms
Experiment 2[c]						
WT (Pirol NN)	0/12	9/12	12/12	12/12	12/12	Severe
Pws4.6	0/8	0/8	0/8	3/8	7/8	Mild
Pws10.1	0/8	0/8	0/8	0/8	0/8	No symptoms

[a]Days post inoculation

[b]Plants were grown in greenhouse from June to August

[c]Plants were grown in greenhouse from November to February

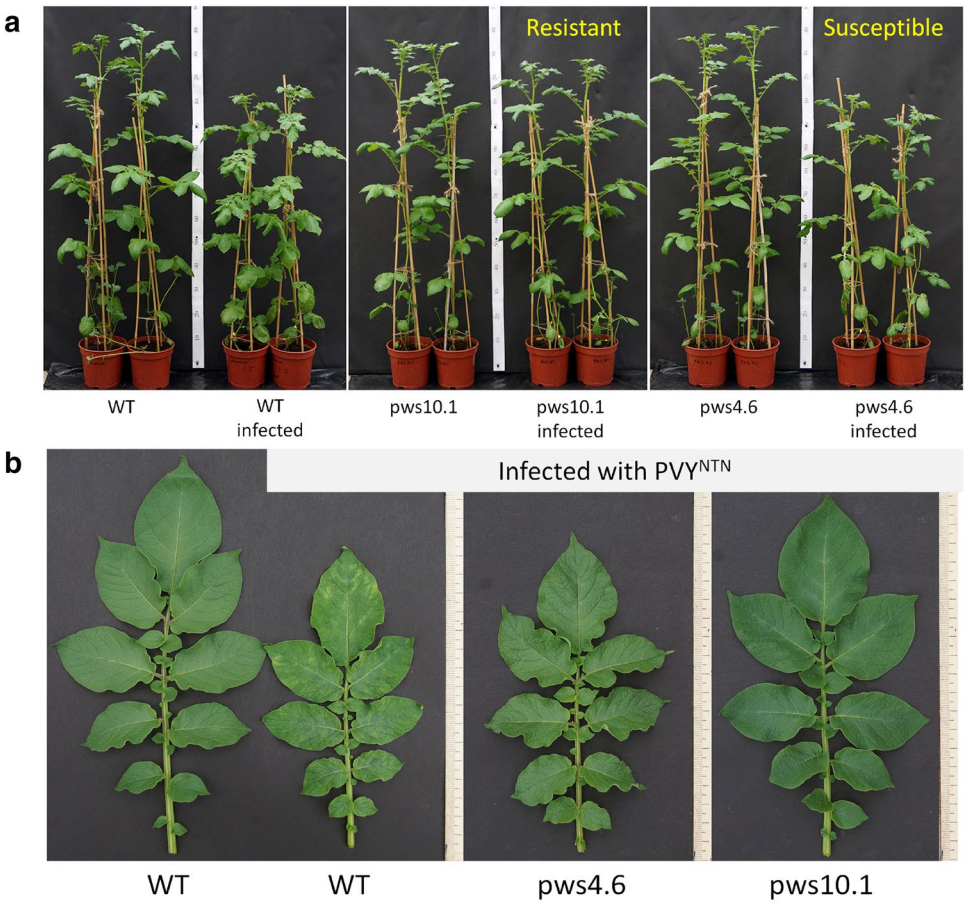

Fig. 4 Testing of potato lines carrying the eIF4E1 hairpin construct for virus resistance against PVY^{NTN}. Symptoms of viral infection at 25 days post inoculation on plants (**a**) and leaves (**b**) of in vitro-derived plants of pws4.6, pws10.1 and wild-type 'Pirol NN' (WT)

determine the infection, the development of symptoms was monitored visually (Table 1, Fig. 4), the presence of the viral proteins was evaluated by ELISA test (Fig. 5a) and accumulation of viral RNAs was detected by RT-PCR analysis (Fig. 5b). Two independent seasonal experiments, carried out in summer and winter, showed that all inoculated potato plants were symptomless after inoculation with PVY during the first 8 days. Starting from the 9th day of post-inoculation (dpi) clear symptoms appeared on the WT plants. By the 2 weeks, all the control plants were infected with PVY and exhibited severe mosaic symptoms, especially on younger leaves (Fig. 4b). By contrast, the RNAi plants with reduced transcripts of both *eIF4E1* and *eIF4E2* did not show any visual symptoms for the 2 weeks. Starting from 14th dpi, mild viral symptoms, such as the appearance of yellowish and light green spots on leaves were found in some pws4.6

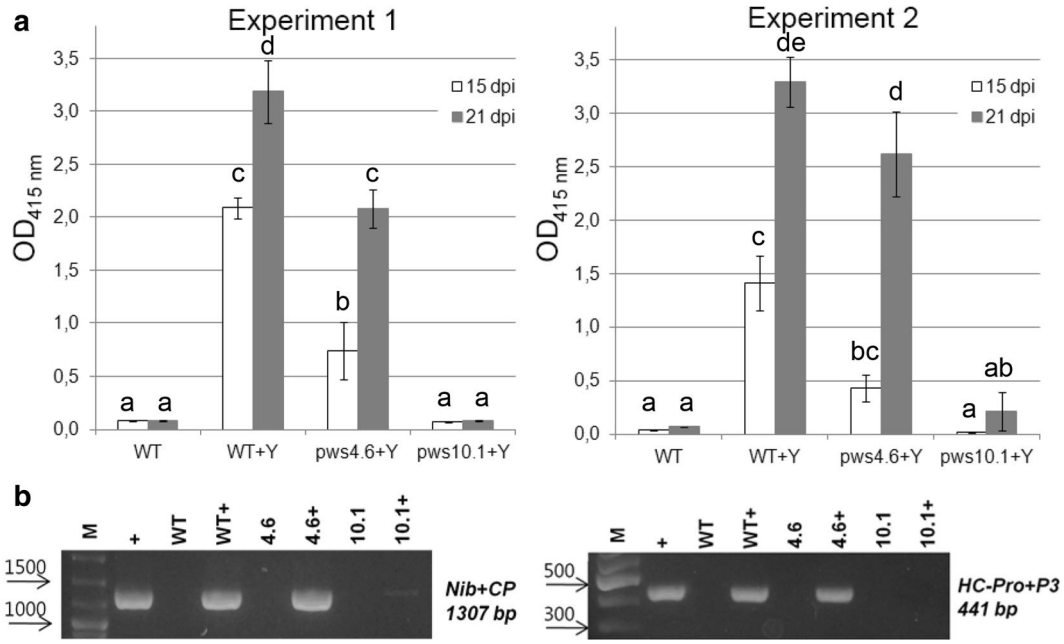

Fig. 5 Analysis of potato lines carrying the *eIF4E1* hairpin construct infected with PVY^{NTN}. **a** ELISA plate readings 15 and 21 days post inoculation (optical density at 415 nm). Two seasonal experiments were conducted. Potato plants were grown in June–August (Experiment 1) and in November-February (Experiment 2). The bars represent means of 6 leaf samples. The error bars represent standard deviations of the readings. Means having the same letter in the column has no significant differences according to Duncan's multiple range test ($p < 0.05$). **b** RT-PCR assay of infected and uninfected potato plants (data from the Experiment 2) for the presence of viral RNAs sequences corresponding to 3′ (left panel) and 5′ (right panel) parts of the PVY genome. WT, pws4.6, and pws10.1—samples of uninfected wild-type 'Pirol NN' plants, 4.6 and 10.1—samples from the lines carrying the hairpin construct, WT+, 4.6+ and 10.1+—samples of infected plants, +—sample of infected sap used to inoculation

individuals (Table 1). At 21 dpi, the most of the inoculated pws4.6 plants (75% in summer experiment and 89% in winter experiment) displayed mild symptoms: the mosaic pattern was muted but distinguished and the certain modification of leaflet shape was observed (Fig. 4b). Despite the fact that symptoms to appear 1 week later and changes in leaf colour and size were sometimes unobvious, the ELISA assay, as well as RT-PCR analysis for the PVY RNAs, confirmed the presence of the virus in leaf extracts of all analysed pws4.6 plants (Fig. 5). ELISA test performed during both seasonal experiments generally correlated with the visual observations and confirmed that PVY spread in pws4.6 plants was less pronounced than in control plants. Nonetheless, both pws4.6 and control plants displayed a growth delay due to PVY infection compared with uninfected individuals during the first 3 to 4 weeks after mechanical inoculation (Fig. 4a). Analysis of the plant height revealed that the PVY infection causes relatively higher reduction of height in pws4.6 and WT compared to pws10.1 (Fig. S3a). At the same time, without viral infection, the extent of the plant height alterations between WT and lines expressing hpRNAi construct was not statistically proven (Fig. S3a).

Unlike pws4.6, infected intragenic pws10.1 plants displayed apparent limitation of the viral multiplication to almost undetectable levels. During the entire period of visual monitoring until the harvesting of tubers, there were no visible viral symptoms on leaves of inoculated pws10.1 plants (Fig. 4b). The absence of symptoms was consistent with the ELISA results since both the uninfected control and infected pws10.1 line showed almost the same low DAS-ELISA values (Fig. 4a). The only exception was found in winter experiment when some increase of the OD value was detected in one of pws10.1 individual at 21 dpi. When this plant was analyzed through the end-point RT-PCR using the primers specific to the *Nib + CP* viral sequences, an amplification of a very faint band was observed, that fit to the minor increase of the OD values; while the control and pws4.6 plants showed a much higher accumulation of PVY CP (Fig. 5b). At the same time, the presence of the viral transcripts of such genes as *HC-Pro* and *P3* was not detected by RT-PCR, however, they were easily found in leaves of control and pws4.6 plants (Fig. 5b).

The knock-down of host *eIF4E1* and *eIF4E2* genes using a green-tissue-specific *Lhca3* promoter did not affect the morphology of harvested tubers (Fig. 6a). Clear changes in the shape of tubers associated with susceptibility to PVY were observed in infected control and pws4.6 plants (Fig. 6a). Tubers harvested from infected plants of pws10.1

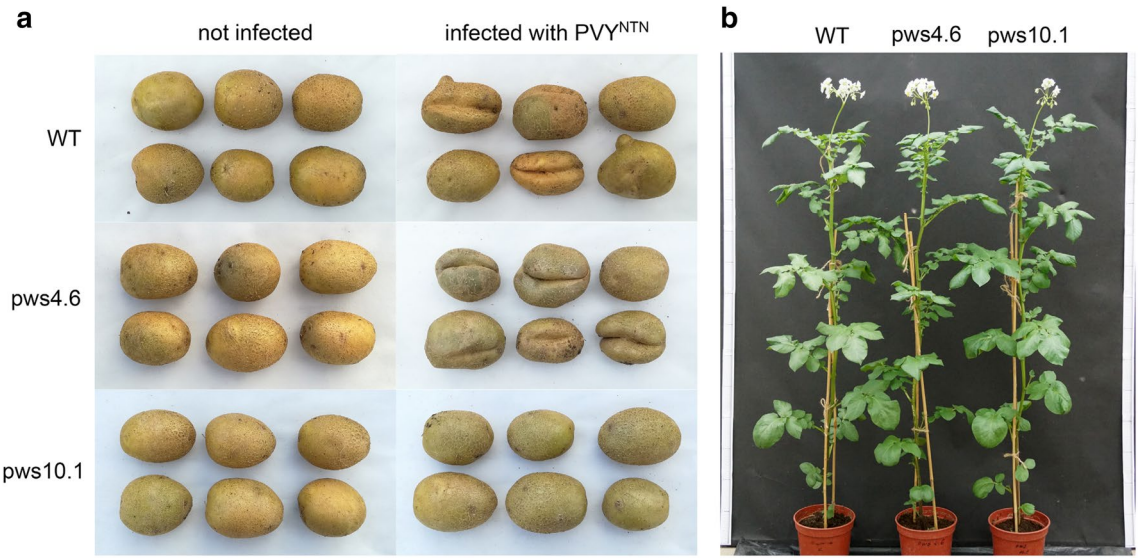

Fig. 6 Effect of RNAi-mediated silencing of *eIF4E1* and *eIF4E2* genes on the tuber shape (**a**) and terminal height of potato plants (**b**). **a** Tubers from infected and non-infected in vitro-derived plants; 1 month after the harvesting. **b** Representative plants at the flowering stage, which are grown from tubers harvested from non-infected plants (first vegetative generation). WT—wild-type 'Pirol NN' plants, pws4.6 and pws10.1—lines carrying the *eIF4E1* hairpin construct

line were asymptomatic for infection and similar to the healthy tubers, indicating an effective suppression of PVY by expression of ihpRNAi cassette. Since the morphological and developmental effects related to the suppression of plant eIF4E initiation factors are of great concern, tubers produced by uninfected WT, pws4.6 and pws10.1 lines were planted after breaking of dormancy to evaluate plant growth under greenhouse conditions. Plants grown from tubers produced by both two lines expressing the ihpRNAi cassette showed no clear morphological abnormalities during the active growth (Fig. 6b). Although a tendency to a slight plant height reduction was noted, the analysis of variance showed no significant difference in terminal plant height between the uninfected WT 'Pirol NN' plants and the plants carrying hairpin construct (Fig. S3b).

Discussion

Nowadays, there is an increasing interest in using new plant breeding techniques, such as genome editing, cisgenesis and intragenesis as alternatives to transgenic crop development. Towards developing an intragenic approach to imparting viral resistance in potato, we isolated the potato promoter and terminator and engineered the vector, T-DNA of which consisted of entirely plant-derived sequences to achieve the post-transcriptional silencing of the plant initiation translation factors. The hairpin cassette was further introduced into the potato genome using the marker-free transformation protocol. There is general agreement that a successful generation of marker-free transgenic/cisgenic/intragenic plants without selection pressure is relayed on the ability of certain species to regenerate plants in vitro with a high frequency (de Vetten et al. 2003; Weeks et al. 2008; Bhatnagar et al. 2010; Li et al. 2009; Richardson et al. 2014). In the present study, marker-free plants were produced on the antibiotic-free medium by exploring the high plant regeneration and transformation capacity of potato nodal explants (de Vetten et al. 2003; Barrell et al. 2013). To minimize the laborious manipulations, a DNA pooling method was applied to facilitate the identification of transformation events by PCR. As a result, molecular analyses confirmed that 0.9% of the regenerated plantlets were positive for the hairpin cassette. The effectiveness of marker-free plants detection obtained here is generally consistent with the efficiency of 0.5–5% achieved in similar experiments using non-selected transformation approach for the production of transgenic, cisgenic or intragenic plants of potato (de Vetten et al. 2003; Richael et al. 2008; Bai et al. 2009; Jo et al. 2014).

Similarly to the other experiments using non-selected transformation methods (de Vetten et al. 2003; Jo et al. 2014; Li et al. 2009), the introduction of undesirable vector backbone sequences was found. The backbone DNA insertions were apparently linked with an integration pattern. The analysis for the integration sites revealed numerous insertions of the hairpin cassette in pws4.6 line, while the other event, pws10.1, carrying only one intragenic sequence, was free from unwanted fragments. The pattern of insertions was also found to correlate with a detectable level of specific siRNAs in intragenic potato lines. Pws10.1 plants

with simple integration of the hpRNA construct displayed a higher accumulation of siRNAs, whereas the pws4.6 plants with complex cassette insertions contained a lower amount of intragene-derived siRNA. This, in turn, influenced the efficiency of the RNAi mediated resistance. After the challenge with PVY, plants of pws10.1, producing a higher level of detectable sRNAs, remained unsusceptible to the viral infection. At our thought, the multiple (and probably truncated insertions) might cause some cross-silencing effect between hairpin fragments and endogenous *eIF4E* genes, thus resulting in lower production of siRNAs. At the same time, the observed differences could be a consequence of the insertion of hpRNAi structure into different chromosomal loci that can lead to either silencing or a higher expression of a construct due to position effects.

Managing cellular translation initiation factors to achieve eIF4-based resistance to various RNA viruses is a promising strategy, intensively explored last decade by plant biotechnology (Bastet et al. 2017; Schmitt-Keichinger 2019). In the present study, to combat PVY infection we used the hpRNA construct driven by *Lhca3* promoter to induce the accumulation of small interfering RNAs specific to *eIF4E* gene family. Since it is based on interfering with the plant translation machinery to prevent viral replication, certain problems arose when an artificial knockdown of functional *eIF4E* genes was previously achieved in some plant species. For example, the overexpression of antisense construct contained transcribed cDNAs of *NteIF4E1* gene led to a consistent lag in the growth of tobacco plants, while mature plants were still indistinguishable from wild-type (Combe et al. 2005). Similarly, the RNAi-mediated silencing for several *eIF4E* genes in transgenic tomato significantly impaired growth and fertility, as plants demonstrated a semi-dwarf phenotype, while the harvested fruits were smaller and lighter (Mazier et al. 2011). The specific silencing of *Cm-eIF4E* genes induced by the ihpRNA in melon also affected the growth and fertility; therefore, only one out of eight independent events produced an abundant amount of seeds (Rodríguez-Hernández et al. 2012). In all mentioned reports, the silencing of *eIF4E* genes was achieved through the overexpression of introduced constructs under the control of the strong viral 35S promoter.

In the present study, we used the green-tissue-specific *Lhca3* gene promoter to knock-down *eIF4E* genes to minimize the possible negative developmental effects in potato plants. This promoter is known to be a light-inducible foliage-specific promoter, which has been successfully used in potato, tobacco and chrysanthemum to regulate transcriptional control of foreign genes expression (Nap et al. 1993; Annadana et al. 2002; Meiyalaghan et al. 2005). Preliminary we have investigated the activity of the potato *Lhca*3 promoter in various tissues of greenhouse-grown transgenic potato lines and found a high accumulation of foreign protein (GUS) in leaves (data not shown).

In line with this, the promoter activity was rather strong to induce the specific silencing of *eIF4E1* gene in potato leaves up to 10 times compared to WT plants. Interesting, that the similar level of silencing was observed in transgenic plum and melon, when a CaMV 35S promoter was used to drive ihpRNA construct to reduce *eIF4E* or *eIF(iso)4E* transcripts accumulation (Rodríguez-Hernández et al. 2012; Wang et al. 2013). Since the hairpin construct transferred to potato genome consisted of the fragment with high homology to the sequence of *eIF4E2* gene its substantial silencing was also achieved. The decreasing in the level of *eIF4E2* transcripts in our study was not as high as in the recent study on RNA-mediated silencing of two homeologous tobacco isoforms of *eIF4E2* (Takakura et al. 2018), but in the mentioned research the hairpin construct was designed to specifically reduce the expression of *eIF4E2* genes.

In our study, the simultaneous RNAi-induced silencing of both eIF4E1 and eIF4E2 factors in intragenic potato plants significantly reduce susceptibility to harmful PVY strain. In contrast to tomato and melon (Mazier et al. 2011; Rodríguez-Hernández et al. 2012), such functional deficiency, however, did not affect the plant growth and morphology in both ex-vitro plants and plant raised from the harvested tubers. Moreover, no obvious defects were also found in tubers of *eIF4E*-silenced plants, since a green-specific promoter was used to induce gene silencing.

The application of RNAi technology to knockdown the expression of translation factors to trigger virus resistance, however, may not necessarily lead to developmental defects. As example, no significant phenotypic and generative alterations were observed, when the active knock down of *eIF4E* or *eIF(iso)4E* genes families was achieved in plum, peanut and tobacco by use of strong constitutive CaMV 35S promoter for antisense and RNAi-mediated silencing (Wang et al. 2013; Xu et al. 2017; Takakura et al. 2018). It is assumed that the prevention of side effects is associated with cross-redundancy among the various isoforms of eIF4Es, which allows plants to maintain the normal functioning of translation machinery, despite the knockdown of one of the genes (Gauffier et al. 2016). From this point of view, the silencing of *eIF4E1* and *eIF4E2* genes in generated potato plants could probably be balanced due to redundancy among eIF4E initiation factors, which made it possible to provide virus resistance without compromising the plant development. The problem is that a specific virus can use for translation the factor, the lack of which leads to impaired growth and development. Moreover, as was previously shown in pepper, some viruses can interact with both eIF4E and eIF(iso)4E isoforms to infect the plants (Ruffel et al. 2005). Nevertheless, as have been shown by numerous studies, viruses generally selectively recruit one member

of the eIF4E family for their infection, and such virus-host interaction appears to depend on the specific virus and species (Sanfaçon 2015; Schmitt-Keichinger 2019).

The resistance to PVY in various species, including potato, tomato, pepper and tobacco, is generally associated with the eIF4E1, rather than with eIF4E2 and eIF(iso)4E isoforms (Sanfaçon 2015; Bastet et al. 2017; Schmitt-Keichinger 2019). Moreover, the combination of *eIF4E1-pvr2* resistance allele with *eIF(iso)4E* loss-of-function allele resulted in the disappearance of PVY resistance in pepper (Quenouille et al. 2016). Duan et al. (2012) suggested that the second eIF4E isoform of potato may not be involved in translation initiation of viral mRNA since they did not observed diversity of *eIF4E2* sequences among 14 PVY-resistant accessions of wild species of potato. In line of this, most of the tobacco RNAi plants with reduced levels of transcripts of *eIF4E2* showed no resistance to PVY; by contrast, the RNAi plants with reduced transcripts of *eIF(iso)4E* orthologs exhibited decrease of susceptibility to a resistance-breaking strain of PVY^{NTN}, but were susceptible to other harmful PVY^{NTN} strain (Takakura et al. 2018).

Surprisingly, transgenic potato lines with strongly reduced *SteIF4E-1* transcript levels due to the expression of RNAi construct did not limit PVY replication, because all transgenic events developed disease symptoms consistent with untransformed controls (Duan et al. 2012). Unfortunately, in above-mentioned study, no data concerning the accumulation of siRNA was presented. Moreover, Duan et al. (2012) used a shorter (286-bp) specific fragment containing 251-bp of the 30-untranslated trailer and 35-bp of the 50-untranslated leader of *eIF4E*-1 gene for the assembly of RNAi construct. In the present research, the extended sequence (474 bp) was used for silencing, so our study cannot be directly compared. Due to high homology between the *eIF4E*s genes, the potato lines obtained here showed a clear decrease of the level of e*IF4E1* and *eIF4E2* mRNA transcripts in the leaves and the enhanced resistance to PVY correlated with a higher accumulation of *eIF4E*-specific siRNAs. Further investigations involving a greater number of independent events are required to elucidate the importance of the potentially active siRNAs from the certain parts of *eIF4E1/eIF4E2* genes or their specific proportion for the successful engineering of the PVY resistance in potato.

Conclusions

This study is a proof-of-concept for successful generation of viral resistance in potato by the RNAi-mediated transcriptional regulation of targeted *eIF4E* gene family using plant tissue-specific promoter without affecting plant phenotype. We have demonstrated that knocking down potato *eIF4E* paralogs can substantially reduce the susceptibility of greenhouse grown plants to PVY. Important to note, that the integrated hpRNAi cassette is not of viral origin and is not translated into a protein. Combined with intragenic strategy this approach should be further exploited for the intelligent breeding of potato and other clonally propagated crops to a broader spectrum of viral pathogens.

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Author contributions DM, VT, SD conceived and designed the experiments; SD contributed reagents/materials and coordinate the research; VT designed and engineered the vector for RNAi interference and performed qRT-PCR analysis; DM and AO carried out the *Agrobacterium*-mediated transformation and selected intragenic plants; DM and AK made the inoculation with virus; AK, TS and AP performed ELISA test; AK and AP carried out the PCR, RT-PCR and Southern blot; TS and AP extracted sRNAs and performed Northern blot; DM, VT, AP, SD analyzed the data; DM wrote the paper with assistance from all authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Annadana S, Mlynarova L, Udayakumar M, de Jong J, Nap JP (2002) The potato *Lhca3.St.1* promoter confers high and stable transgene expression in chrysanthemum, in contrast to CaMV-based promoters. Mol Breed 8:335–344. https://doi.org/10.1023/A:1015212312928

Arcibal E, Morey Gold K, Flaherty S, Jiang J, Jahn M, Rakotondrafara AM (2016) A mutant eIF4E confers resistance to Potato Virus Y strains and is inherited in a dominant manner in the potato varieties Atlantic and Russet Norkotah. Am J Potato Res 93(1):64–71. https://doi.org/10.1007/s12230-015-9489-x

Bai Y, Guo Z, Wang X, Bai D, Zhang W (2009) Generation of double-virus-resistant marker-free transgenic potato plants. Prog Nat Sci 19:543–548. https://doi.org/10.1016/j.pnsc.2008.08.005

Barrell PJ, Meiyalaghan S, Jacobs JM, Conner AJ (2013) Applications of biotechnology and genomics in potato improvement. Plant Biotechnol J 11(8):907–920. https://doi.org/10.1111/pbi.12099

Bastet A, Robaglia C, Gallois JL (2017) eIF4E resistance: natural variation should guide gene editing. Trends Plant Sci 22:411–419. https://doi.org/10.1016/j.tplants.2017.01.008

Bastet A, Lederer B, Giovinazzo N, Arnoux X, German-Retana S, Reinbold C, Brault V, Garcia D, Djennane S, Gersch S, Lemaire O, Robaglia C, Gallois JL (2018) Trans-species synthetic gene design allows resistance pyramiding and broad-spectrum engineering of virus resistance in plants. Plant Biotechnol J. https://doi.org/10.1111/pbi.12896

Bhatnagar M, Prasad K, Bhatnagar-Mathur P, Narasu ML, Waliyar F, Sharma KK (2010) An efficient method for the production of marker-free transgenic plants of peanut (*Arachis hypogaea*

L.). Plant Cell Rep 29:495–502. https://doi.org/10.1007/s00299-010-0838-4
Brunt AA (2001) Potyviruses. In: Loebenstein G, Berger PH, Brunt AA, Lawson RH (eds) Virus and virus-like diseases of potatoes and production of seed-potatoes. Kluwer Academic Publishers, Dordrecht, pp 77–86
Cavatorta J, Perez KW, Gray SM, Van Eck J, Yeam I, Jahn M (2011) Engineering virus resistance using a modified potato gene. Plant Biotechnol J 9:1014–1021. https://doi.org/10.1111/j.1467-7652.2011.00622.x
Chen X, Liu J, Xu L, Jiang F, Xie X, Zhu C, Wen F (2010) Inhibiting virus infection by RNA interference of the eight functional genes of the potato virus Y genome. J Phytopathol 158:776–784. https://doi.org/10.1111/j.1439-0434.2010.01701.x
Chikh Ali M, Natsuaki T, Maoka T, Natsuaki KT (2010) The simultaneous differentiation of *Potato virus Y* strains including the newly described strain PVY(NTN-NW) by multiplex PCR assay. J Virol Methods 165(1):15–20. https://doi.org/10.1016/j.jviromet.2009.12.010
Combe JP, Petracek ME, van Eldik G, Meulewaeter F (2005) Translation initiation factors eIF4E and eIFiso4E are required for polysome formation and regulate plant growth in tobacco. Plant Mol Biol 57:749–760. https://doi.org/10.1007/s11103-005-3098-x
de Vetten N, Wolters A, Raemakers K, van der Meer I, ter Stege R, Heeres E, Heeres P, Visser R (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. Nat Biotechnol 21:439–442. https://doi.org/10.1038/nbt801
Duan H, Richael C, Rommens CM (2012) Overexpression of the wild potato *eIF4E-1* variant *Eva1* elicits Potato virus Y resistance in plants silenced for native *eIF4E-1*. Transgenic Res 21:929–938. https://doi.org/10.1007/s11248-011-9576-9
Gao Z, Johansen E, Eyers S, Thomas CL, Ellis THN, Maule AJ (2004) The potyvirus recessive resistance gene, *sbm1*, identifies a novel role for translation initiation factor eIF4E in cell-to-cell trafficking. Plant J 40:376–385. https://doi.org/10.1111/j.1365-313X.2004.02215.x
Gargouri-Bouzid R, Jaoua L, Rouis S, Saïdi MN, Bouaziz D, Ellouz R (2006) PVY-resistant transgenic potato plants expressing an anti-NIa protein scFv antibody. Mol Biotechnol 33(2):133–140. https://doi.org/10.1385/mb:33:2:133
Gauffier C, Lebaron C, Moretti A, Constant C, Moquet F, Bonnet G, Caranta C, Gallois JL (2016) A TILLING approach to generate broad-spectrum resistance to potyviruses in tomato is hampered by *eIF4E* gene redundancy. Plant J 85(6):717–729. https://doi.org/10.1111/tpj.13136
Holme IB, Wendt T, Holm PB (2013) Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotechnol J 11(4):395–407. https://doi.org/10.1111/pbi.12055
Jeffries C, Barker H, Khurana SMP (2006) Potato viruses (and viroids) and their management. Potato production, improvement and post-harvest management. In: Gopal J, Khurana SMP (eds) Handbook of potato production, improvement and post-harvest management. The Haworth's Food Products Press, New York, pp 387–448
Jo KR, Kim CJ, Kim SJ, Kim TY, Bergervoet M, Jongsma MA, Visser RG, Jacobsen E, Vossen JH (2014) Development of late blight resistant potatoes by cisgene stacking. BMC Biotechnol 14:50. https://doi.org/10.1186/1472-6750-14-50
Jung J, Yeam I (2013) Exploring natural variations in *eIF4E* and screening for potyviral resistance in diverse *Nicotiana* species. Hortic Environ Biotechnol 54(5):430–440. https://doi.org/10.1007/s13580-013-0045-7
Kang BC, Yeam I, Li H, Perez KW, Jahn MM (2007) Ectopic expression of a recessive resistance gene generates dominant potyvirus resistance in plants. Plant Biotechnol J 4:526–536. https://doi.org/10.1111/j.1467-7652.2007.00262.x
Kent F, McCue Ponciano G, Rockhold DR, Whitworth JL, Gray SM, Fofanov Y, Belknap WR (2012) Generation of PVY coat protein siRNAs in transgenic potatoes resistant to PVY. Am J Pot Res 89:374–383. https://doi.org/10.1007/s12230-012-9257-0
Li B, Xie C, Qiu H (2009) Production of selectable marker-free transgenic tobacco plants using a non-selection approach: chimerism or escape, transgene inheritance, and efficiency. Plant Cell Rep 28(3):373–386. https://doi.org/10.1007/s00299-008-0640-8
Livak K, Schmittgen T (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta CT}$. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
Loebenstein G, Gaba V (2012) Viruses of potato. Adv Virus Res 84:209–246. https://doi.org/10.1016/B978-0-12-394314-9.00006-3
Lorenzen JH, Piche LM, Gudmestad NC, Meacham T, Shiel P (2006) A multiplex PCR assay to characterize *Potato virus Y* isolates and identify strain mixtures. Plant Dis 90:935–940. https://doi.org/10.1094/PD-90-0935
Mazier M, Flamain F, Nicolaï M, Sarnette V, Caranta C (2011) Knockdown of both *eIF4E1* and *eIF4E2* genes confers broad-spectrum resistance against potyviruses in tomato. PLoS ONE 6:e29595. https://doi.org/10.1371/journal.pone.0029595
Meiyalaghan S, Takla MF, Jaimess O, Yongjin S, Davidson MM, Cooper PA, Barrell PJ, Jacobs ME, Wratten SD, Conner AJ (2005) Evaluation of transgenic approaches for controlling tuber moth in potatoes. Commun Agric Appl Biol Sci 70(4):641–650
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497
Muthoni J, Kabira J, Shimelis H, Melis R (2015) Tetrasomic inheritance in cultivated potato and implications in conventional breeding. Aust J Crop Sci 9:185–190
Nap JP, Spanje M, Dirkse WG, Baarda G, Mlynarova L, Loonen A, Grondhuis P, Stiekema WJ (1993) Activity of the promoter of the *Lhca3*.St.1 gene, encoding the potato apoprotein 2 of the light-harvesting complex of photosystem I, in transgenic potato and tobacco plants. Plant Mol Biol 23:605–612
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. https://doi.org/10.1104/pp.102.017855
Orbegozo J, Solorzano D, Cuellar WJ, Bartolini I, Roman ML, Ghislain M, Kreuze J (2016) Marker-free PLRV resistant potato mediated by Cre-loxP excision and RNAi. Transgenic Res 25(6):813–828. https://doi.org/10.1007/s11248-016-9976-y
Pooggin MM (2017) RNAi-mediated resistance to viruses: a critical assessment of methodologies. Curr Opin Virol 26:28–35. https://doi.org/10.1016/j.coviro.2017.07.010
Quenouille J, Saint-Felix L, Moury B, Palloix A (2016) Diversity of genetic backgrounds modulating the durability of a major resistance gene. Analysis of a core collection of pepper landraces resistant to *Potato virus Y*. Mol Plant Pathol 17:296–302. https://doi.org/10.1111/mpp.12277
Richael CM, Kalyaeva M, Chretien RC, Yan H, Adimulam S, Stivison A, Weeks JT, Rommens CM (2008) Cytokinin vectors mediate marker-free and backbone-free plant transformation. Transgenic Res 17:905–917. https://doi.org/10.1007/s11248-008-9175-6
Richardson T, Thistleton J, Higgins TJ, Howitt C, Ayliffe M (2014) Efficient *Agrobacterium* transformation of elite wheat germplasm without selection. Plant Cell Tiss Organ Cult 119:647–659. https://doi.org/10.1007/s11240-014-0564-7

Rodríguez-Hernández AM, Gosalvez B, Sempere Raquel N, Burgos L, Aranda Miguel A, Truniger V (2012) Melon RNA interference (RNAi) lines silenced for *Cm-eIF4E* show broad virus resistance. Mol Plant Pathol 13:755–763. https://doi.org/10.1111/j.1364-3703.2012.00785.x

Rogers S, Bendich A (1994) Extraction of total cellular DNA from plants, algae and fungi. In: Gelvin S, Schiperoort R (eds) Plant molecular biology manual. Springer, Dordrecht, pp 183–190

Ruffel S, Dussault M-H, 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. https://doi.org/10.1046/j.1365-313X.2002.01499.x

Ruffel S, Gallois JL, Lesage ML, Caranta C (2005) The recessive potyvirus resistance gene *pot-1* is the tomato orthologue of the pepper *pvr2-eIF4E* gene. Mol Genet Genomics 274:346–353. https://doi.org/10.1007/s00438-005-0003-x

Sanfaçon H (2015) Plant translation factors and virus resistance. Viruses 7:3392–3419. https://doi.org/10.3390/v7072778

Schaart JG, Krens FA, Pelgrom KTB, Mendes O, Rouwendal GJA (2004) Effective production of marker-free transgenic strawberry plants using inducible site-specific recombination and a bifunctional selectable marker gene. Plant Biotechnol J 2:233–240. https://doi.org/10.1111/j.1467-7652.2004.00067.x

Schmitt-Keichinger C (2019) Manipulating cellular factors to combat viruses: a case study from the plant eukaryotic translation initiation factors eIF4. Front Microbiol 10:17. https://doi.org/10.3389/fmicb.2019.00017

Sidorova T, Mikhailov R, Pushin A, Miroshnichenko D, Dolgov S (2019) *Agrobacterium*-mediated transformation of Russian commercial Plum cv. "Startovaya" (*Prunus domestica* L.) with virus-derived hairpin RNA construct confers durable resistance to PPV infection in mature plants. Front Plant Sci 10:286. https://doi.org/10.3389/fpls.2019.00286

Stein N, Perovic D, Kumlehn J, Pellio B, Stracke S, Streng S, Ordon F, Graner A (2005) The eukaryotic translation initiation factor 4E confers multiallelic recessive *Bymovirus* resistance in *Hordeum vulgare* (L.). Plant J 42:912–922. https://doi.org/10.1111/j.1365-313X.2005.02424.x

Takakura Y, Udagawa H, Shinjo A, Koga K (2018) Mutation of a *Nicotiana tabacum* L. eukaryotic translation-initiation factor gene reduces susceptibility to a resistance-breaking strain of *Potato virus Y*. Mol Plant Pathol 19:2124–2133. https://doi.org/10.1111/mpp.12686

Toevs E, Guenthner J, Johnson A, McIntosh C, Thornton M (2011) Identity preservation systems for genetically modified potatoes. Am J Pot Res 88:303–308. https://doi.org/10.1007/s12230-011-9194-3

Visser JC, Bellstedt DU, Pirie MD (2012) The recent recombinant evolution of a major crop pathogen, *Potato virus Y*. PLoS ONE 7(11):e50631. https://doi.org/10.1371/journal.pone.0050631

Wang A, Krishnaswamy S (2012) Eukaryotic translation initiation factor 4E-mediated recessive resistance to plant viruses and its utility in crop improvement. Mol Plant Pathol 13:795–803. https://doi.org/10.1111/j.1364-3703.2012.00791.x

Wang X, Kohalmi SE, Svircev A, Wang A, Sanfaçon H, Tian L (2013) Silencing of the host factor *eIF(iso)4E* gene confers *plum pox virus* resistance in plum. PLoS ONE 8:e50627. https://doi.org/10.1371/journal.pone.0050627

Weeks JT, Ye J, Rommens CM (2008) Development of an in planta method for transformation of alfalfa (*Medicago sativa*). Transgenic Res 17:587–597. https://doi.org/10.1007/s11248-007-9132-9

Xu M, Xie H, Wu J, Xie L, Yang J, Chi Y (2017) Translation initiation factor eIF4E and eIFiso4E are both required for *Peanut stripe virus* infection in Peanut (*Arachis hypogaea* L.). Front Microbiol 8:338. https://doi.org/10.3389/fmicb.2017.00338