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Original Article

Preliminary Results of an Attempt to Produce Resistance to *Turnip Mosaic Virus* in Transgenic Canola (*Brassica Napus*)

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Abstract

Background and Aims: *Turnip mosaic virus* (TuMV) has a wide host range and no resistant commercial canola variety to this virus has been reported in Iran. The RNA silencing mechanism was applied to consider the possibility of improvement in resistance to TuMV in spring canola, RGS003 variety.

Materials and Methods: To obtain an effective construct for silencing, based on the bioinformatics analysis, a fragment containing 130 conserved nucleotide sequences of the TuMV coat protein gene was gained as targeting candidate to produce sense, antisense and hairpin constructs and assessed for resistance efficiency in a transient expression system in *Nicotiana benthamiana* by agroinfiltration. The development of symptoms after virus inoculation revealed that the highest efficiency can be obtained by hairpin construct. Therefore, the hairpin construct was applied for the transformation of canola RGS003 via cotyledonary explants using *Agrobacterium tumefaciens* LBA4404. In transgenic and non-transgenic canola plants, the infection and virus titer were assessed by ratio of detection via ELISA and real-time PCR. In addition, severity of disease symptoms was scored four weeks after inoculation with a TuMV isolate.

Results: The results indicated 5-12 days delay in appearance of symptoms in transgenic plants and there was a decrease in severity of symptoms in contrast to non-transgenic plants. The increased virus concentration ratio in non-transgenic compared to transgenic plants was confirmed by qRT-PCR. The ELISA results confirmed the absence of infection on five out of six transformed plants 15 dpi.

Conclusions: These preliminary results proved that transgenic canola plants containing hairpin of 130 nucleotide sequences of TuMV CP gene could resist against TuMV.

Keywords: *Potyvirus*, Iran, transgenic plants, qRT-PCR.

Introduction

Canola (*Brassica napus* L.) is one of the most important oil seed crops in the world that is infected by several viral diseases causing reduction in yield. *Turnip mosaic virus* (TuMV), a member of the *Potyvirus* genus, is the largest genus of the family Potyviridae (1). TuMV has a wide host range and is the only *Potyvirus* known to infect *brassica*s (2, 3). No commercial resistant canola variety to this virus has been reported in Iran and RGS003 line has been reported as one of the susceptible commercial varieties (4). Different surveys have shown that *Turnip mosaic virus* (TuMV) is the most prevalent virus of canola in Iran (5-7) and it has been reported from ornamental crops (8) and *Brassi-ca*ceae weeds (9).

It is difficult to control TuMV because of its wide host range and non-persistent stylet-borne mode of transmission by aphids (3). Due to the ineffectiveness of chemical control of the disease, natural plant resistance is likely to be the most effective method for controlling TuMV (10). RNA silencing is one of the active approaches to alleviate the losses of virus

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infection in plants. In the early 1990s, it was demonstrated that transgenic plants expressing transgenes derived from a particular virus were often immune to infection by the virus from which the transgene sequence was derived (11). There is still a great deal of interest in using RNA interference (RNAi) to manipulate plants for virus resistance as a potentially beneficial method for the immunity of many food and fiber plants (12).

A hairpin viral RNA construct could increase resistance to the virus via RNA silencing (13, 14). There are many reports of transformation of a DNA sequences to induce a hairpin RNA which can increase resistance against viruses belonging to *Potyvirus* genus in transgenic plants. A noticeable increased resistance to *Potyvirus* species has been achieved with this strategy in *Nicotiana benthamiana* that included target sequences from P1 and HC-Pro genes of Plum pox virus (PPV), CP gene of PPV, Potato virus Y (PVY), NIb gene from PVY, and Tobacco etch virus (TEV) which improved resistance to the related virus (15-18). Transgenic watermelon plants containing truncated Zucchini yellow *mosaic* virus (ZYMV) CP and Papaya ringspot virus (PRSV) CP gene sequences showed an increased resistance to ZYMV and PRSV (19). Also, transformed sugarcane showed a significant resistance to Sorghum *mosaic* virus (SrMV) by this technic via using truncated CP gene sequences (20).

Coat protein-mediated resistance to TuMV in oilseed rape was achieved, but not RNA-mediated resistance (21). Many attempts have been made to improve the resistance of plants against TuMV via transformation using different mechanisms; Transformation of coat protein gene of TuMV to Arabidopsis (Arabidopsis thaliana) plants (22) and N. *benthamiana* (23) achieved protection against virus inoculation. Over-expressing eIF(iso)4E variants showed broad-spectrum resistance to TuMV in transgenic *Brassica* rapa plants (24). Transgenic *Brassica* juncea L. and L. inshicaoyaozi plants (25) or *Brassica* campestris var. *Pekinensis* (Lour) Olsson (26) which have been transformed with a TuMV-NIb gene fragment in antisense orientation, have shown high levels of resistance to TuMV. Expression of artificial microRNAs conferred resistance in transgenic Arabidopsis (27). Overexpression of a Single-Chain Variable Fragment (scFv) antibody confers TuMV resistance at low frequencies, and that the transgene is presumably frequently silenced (24).

RGS003 has been known as one of the susceptible commercial varieties of canola to infection of TuMV (4). A major goal in this research was to get to a construct and transgenic RGS003 variety with a high efficiency against TuMV in order to transform canola as RNA interference mechanism.

Methods

Viral isolate. A TuMV isolate (Accession no. KU535893) was used for bioinformatics analyses and hairpin RNA design and also this isolate was used for viral inoculation and resistance assays.

RNAi target sequence selection. To select the best target fragment, nucleotide sequences for coat protein gene of 60 TuMV isolates were selected from NCBI. After alignment of CP sequences by MEGA5 software, a highly conserved fragment containing 130 bp (787-916-nt of CP gene) was selected as a target candidate.

The sequence was analyzed via siRNAScan website (http://bioinfo2.noble.org/RNAiScan .htm) for off targets in canola. RNA structure software and RNAfold website (http://rna.tbi. univie.ac.at/cgi-in/RNAfold.cgi) were applied to predict the production of a short hairpin RNA. Two fragments as sense and antisense orientations with four restriction sites (*Nco*I, *Bam*HI, *Xba*I and XhoI) were synthesized in a pGH vector by GeneRay Company. The vectors carrying sense and antisense were named p-GH-TuMV-S and p-GH-TuMV-AS, respectively.

Cloning and construction of viral expression vectors. Vector NTI 8.0 software was used to design constructs and cloning in pFGC5941 vector .The produced constructs were named pFGC-CP-S, pFGC-CP-AS and pFGC-CP-hp as a sense, antisense and hairpin. To gain

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hairpin construct, p-GH-TuMV-S and pFGC5941 were digested by XhoI and *Nco*I enzymes, separately and then the separated fragment from pGH-TuMV-S was cloned in linear pFGC5941 vector and the ligated product was named pFGC-CP-S. In the following, pFGC-CP-S and p-GH-TuMV-S were digested via *Bam*HI and *Xba*I enzymes, separately and then the separated fragment from pGH-TuMV-S was cloned in linear pFGC-CP-S and the ligation product was named pFGC-CP-hp. Also to clone antisense orientation, the cloning of p-GH-TuMV-AS in pFGC5941 was done, like cloning of sense orientation, by XhoI and *Nco*I enzymes and the ligated product was named pFGC-CP-AS. The three obtained constructs as the sense, antisense and hp orientations were transferred to *Agrobacterium tumefaciens* LBA4404 by the electroporation method (28) (Electro-porator eppendorf 2510, Germany, in 200 Ω, 25μF and 2.0 kV).

To confirm hairpin construct cloning, PCR was carried out with FGC-F and FGC-R primers and also, digestion was applied by *Hind*III to show the differential size between cloned and non-recombinant pFGC5941 vector. Finally, to improve the right orientation, recombinant constructs were sequenced.

Transient expression of *N. benthamiana* and evaluation of constructs efficiency. N. *benthamiana* plants were infiltrated by A. *tumefaciens* LBA4404 containing pFGC-CP-S (sense construct), pFGC-CP-AS (antisense construct), pFGC-CP-hp (hairpin construct) and an empty vector (pFGC5941) as negative control. Three test plants were analyzed for each construct and empty vector. Transformed A. *tumefaciens* cells were grown overnight at 28°C in YEB media containing 50 mg/l rifampicin and 100 mg/l kanamycin. After that cells were centrifuged at 5,000× g for 5 min and precipitated cells were re-suspended in infiltration medium containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 10 mM MgCl2, 100 μM acetosyringone, adjusted to optical density 0.1 at A600 and incubated at room temperature for 2–3 h before agro-infiltration (30). *Agrobacterium* cell culture carrying the sense, antisense and hairpin constructs was infiltrated into the secondary leaves of healthy *N. benthamiana* plants, separately using a 1 ml syringe. Six days after infiltration, virus inoculation was carried out.

Agrobacterium-mediated transformation of canola plants and regeneration of transgenic plants. Transgenic lines of canola RGS003 variety were produced using tissue culture procedures and transformation of *A. tumefaciens* LBA4404 strain carrying pFGC-CP-hp to cotyledony leaves (31). Transgenic callis were selected on MS medium containing 10 mg/ml Basta (200 SL, Bayer CropScience) and 500 mg/ml Cefatoxime.

After regenera-tion, stem development and rooting, young transgenic plantlets were transplanted to soil and gradually adapted to greenhouse conditions.

Selection of transgenic plants. Under greenhouse condition, to select transgenic from non-transgenic plants, whole plants were assayed by resistance to Basta. Before this assay, it was necessary to optimize the efficiency concentration. Finding the minimum Basta concentration that causes leaf necrosis is important to use as selected concentration. Half leaf of non-transgenic RGS003 canola was treated at eight different concentrations (2, 1, 0.5, 0.1, 0.05, 0.01, 0.005 and 0.001 mg/ml). Plants were treated with the least determined effective concentration of Basta on half leaf and water on another half leaf, as a negative control. Then, resistant plants were selected to extract DNA and PCR assay was performed by CP-130-F and CP-130-R primers to amplify transgene and by Accf and Accr primers for amplifying Acc gene as an internal control (Table 1).

Inoculation of plants and resistance evaluation to TuMV. Transgenic and non-transgenic canola variety RGS003 grown under greenhouse condition, were inoculated with an TuMV isolate (GenBank accession number. KU535893). One leaf of each plant was inoculated mechanically.

The symptom severity and virus infectivity of transgenic plants were assessed by system scoring and indirect ELISA assay (TuMV antibody and Anti-rabbit IgG conjugate were received from DSMZ and Promega companies,

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Table1. Properties of primers used in this study.

Primer	Sequence (5'-3')	Product (bp)	Reference
FGC-F	ACA ATC CCA CTA TCC TTC GCA	1700	This study
FGC-R	CGC ATATCTCATTAAAGCAGG		This study
CP-130-F	GAGAACTCCAATACGTGCGAG	130	This study
CP-130-R	GTCGTATGCCTTTCCGTGTTC		This study
Accf	CATATGCTGGGGTCAATGACAAC	800	(28)
Accr	GTCGACAGAAGAATGATCGCGAA		(28)
TuMV-F	GGAAGTAAACGCTGGAACCT	96	(29)
TuMV-R	GCCACTCTTTGCTCGTATCT		(29)
Actin-F	CTGGAATTGCTGACCGTATGAG	145	(29)
Actin-R	ATCTGTTGGAAAGTGCTGAGGG		(29)

respectively). The scoring system (0 to 8) to assess symptoms severity of TuMV in canola was used in plants. The symptom reactions were assessed four weeks after inoculation using grading of symptom scales (4): 0: Symptomless; 1: Mottling on top leaves; 2: *mosaic* on top leaves; 3: systemic mosaic; 4: Severe *mosaic* and vein clearing on top leaves; 5: Ring spot-like; 6: green islands; 7: Growth reduction; 8: Growth reduction and yellowing. Systemic infection was recorded every day at least until 45 days. Fifteen days post-inoculation, one tender leaf, just above the inoculated leaf, was used for ELISA and real-time RT-PCR assays for detection of early infections and virus accumulations. The valid date time for real-time RT-PCR was chosen as in previous TuMV assays (32).

RNA extraction. Total RNA was extracted from leaf samples, using RNx-Plus buffer (Sinaclon, Tehran) and was used from Dnase I, Rnase-free (Fermentas, Lithuania) according to the manufacturer's instruction. In following, 1µg RNA was reverse transcribed to cDNA (RT master mix 2X, HyperScriptTM).

Relative real-time RT-PCR and detection of CP gene ratio in transgenic plants compared to non-transgenic. qRT-PCR was performed on the step one, applied Biosystem. The reaction system included 10µL SYBR Green PCR Master Mix, 0.5µL of each upstream and downstream primer (10 µmol/L), 1 µL cDNA, 8 µL ddH2O. The reaction conditions were carried out in two steps with 40 cycles. In the first step it was at 95 ºC for 20s and in second step at 54 ºC for 40 s. After the detection of Ct reactions for actin and CP genes for transgenic and non-transgenic plants, the ratio of CP gene was calculated in non-transgenic to transgenic plants, 15 days post-inoculation by TuMV.

Results

RNAi target sequence selection. The alignment of TuMV-CP sequences for 60 isolates gained from GenBank, was conducted to identify a conserved region close to 3' CP terminal. A fragment containing 130 bp and showing the highest identity from 787 to 916 TuMV-CP sequence (Accession no. KU535-893) was selected. Bioinformatics analysis with siRNA-Scan and RNA structure software and RNA fold website confirmed no proof off targets in canola plant and successfully predicted induction of a short hairpin RNA by pFGC-CP-hp vector.

Cloning and constructions of plant expression vectors. Final Product of cloning were named pFGC-CP-hp, hairpin construct (Fig 1). The digestion of pFGC-CP-hp using *Hind*III enzyme confirmed inserted sense and antisense fragments in pFGC5941 vector (Fig 2-A). Also, PCR assay by FGC-F and FGC-R primers, indicated the presence of inserted sense and antisense fragments in pFGC-CP-hp vector (Fig 2-B). Finally, the results of nucleotide sequencing definitely confirmed the

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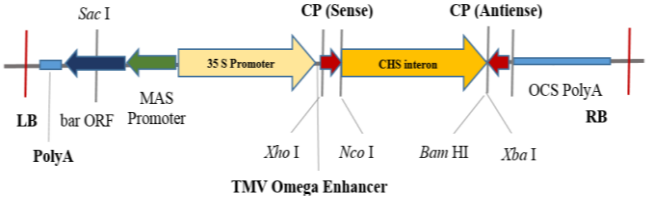


Fig. 1. The cloning products of 130 nucleotides target fragment of TuMV coat protein gene via XhoI, NcoI, BamHI and HindIII restriction sites as sense and antisense orientations.

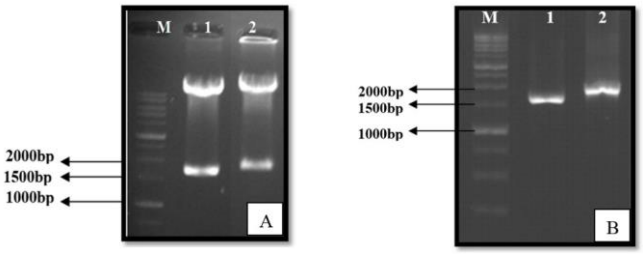


Fig. 2. The cloning confirmation of pFGC-CP-hp vector. M: Size marker, (GenRuler™ 1kb DNA ladder, Fermentas). Line 1: Empty vector pFGC5941. 2: pFGC-CP-hp. A: Enzyme digestion with HindIII enzyme released inserted sense and antisense orientation DNA fragment contrast digested of empty vector pFGC5941 (line 2). B: Amplified increased PCR production by FGC-F and FGC-R primers, because of inserted DNA fragment (line 1) contrast PCR production in empty vector pFGC5941 (line 2).

cloning of sense, antisense and hairpin constructs with certainty.

Evaluation of constructs efficiency by transient expression in N. benthamiana. Symptoms development revealed that the differential efficiency of the three constructs (sense, antisense and hairpin) were not the same. Symptoms appearance in plants infiltrated with sense construct or empty vector, occurred shortly (5 days after inoculation) and the virus caused quick death of plants, whereas in plants infiltrated with antisense and hairpin constructs, symptoms were observed with a delay of two and five days respectively and with the reduction in symptom severity. Although, plants infiltrated with antisense construct showed disease symptoms, yet they survived. Also, the hairpin construct confirmed the best efficiency of protection against TuMV, as these plants survived and grew strongly 30 dpi. Results showed that hairpin construct could be used for production transgenic plants with improved resistance and protection against TuMV.

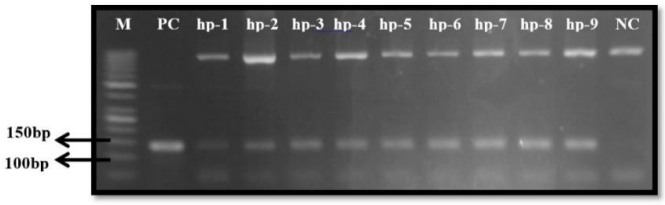


Fig. 3. Electrophoretic of PCR products of 130 nucleotide fragment of TuMV CP gene in transgenic canola RGS003 plants. 130 nucleotide fragment at the below and 800 nucleotide fragment of Acc gene of canola as PCR control at the above. (M: GenRuler™ 50bp DNA ladder, Fermentas; pFGC-CP-S as a pFGC-CP-S, pFGC-CP-AS and pFGC-CP-hp as a sense, antisense and hairpin.

Selection of transgenic plants. The least determined effective Basta concentration was 0.1 mg/ml for non-transgenic RGS003 canola plants. Use of this concentration helps to select transgenic plants from non-transgenic with certainty. In PCR assay A 130 bp fragment were amplified using CP-130-F and CP-130-R primers from plants that showed resistant reaction to Basta (Fig 3).

positive control; hp (1-9): extracted DNA of transformed RGS003 canola plants; NC: Extracted DNA of non-transgenic RGS003 canola as a negative control.

Evaluation of resistance to TuMV. Results showed symptom development was delayed for 5 days in transgenic canola plants compared to non-transgenic plants. Systemic symptoms were observed in non-transgenic plant 9 dpi, whereas in transgenic plants symptoms were identified between 14-21 dpi. The hp-7 line showed 12 days delay in symptoms appearance in contrast to non-transgenic plants (Fig 4-A). This line grew vigorously abundant and produced good pods in contrast to non-transgenic plants (Figs 4-B and 4-C).

Maximum and minimum scores (8 and 2) were assigned to non-transgenic and hp-7 transgenic line. There was a different range of resistance among the transgenic plants, too. Hp-7 line had a minimum symptom severity and did not show symptoms for 20 days. ELISA results confirmed no infection of upper leaves just above inoculated leaves 15 dpi for all of the transgenic plants, except for hp-2 as non-transgenic plant (Table 2).

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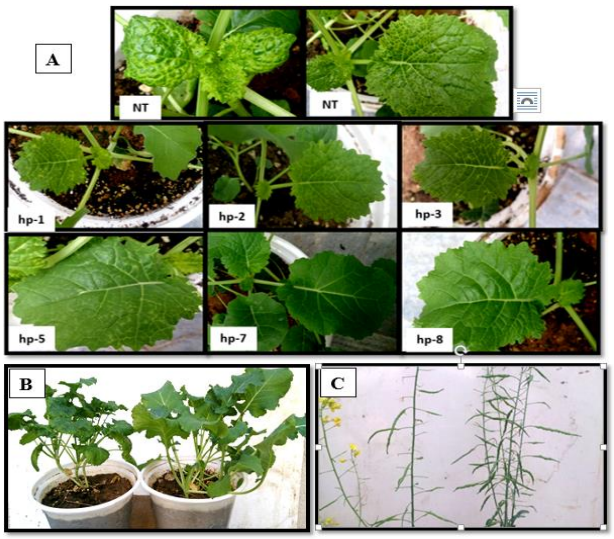


Fig. 4. Different symptoms development in transgenic canola RGS003 plants with non-transgenic plants A: 15 days post TuMV inoculation. NT-1: non-transgenic plant with severe mosaic and apical leaf malformation. NT-2: vein clearing in developmental leaves of non-transgenic plant. Hp- 1, 2, 3, 5, 7 and 8: RGS003 canola plants transgene vector express 130 nucleotide sequences of CP TuMV gene as a short hairpin RNA. the beginning of mild vein clearing in hp-1,2,3,5 and 8 plants and no symptoms in hp-7 plant.

B: Increased resistance in transgenic RGS003 canola hp-7 line after 35 days post TuMV inoculation. Non-transgenic plant with 8 scale symptoms severity (left) and transgenic plant (hp-7 line) with 2 scale. C: lower seed production in quantity at non-transgenic plant (left) contrast more seed production in transgenic plant (right).

Table 2. The evaluation of transgenic canola RGS003 lines to TuMV inoculation. ELISA results 15 days post inoculation, the difference in the time of symptoms appearance (days) in transgenic plants with non-transgenic plants and symptom severity four weeks post inoculation (NT: non-transgenic plant, hp1, 2, 3, 5, 7, 8: Transgenic plants.

Line	ELISA	The difference in the time of symptoms appearance (days) in transgenic with non-transgenic plants	Symptom severity (0-8)
NT	+	-	8
hp-1	-	5	4
hp-2	+	5	7
hp-3	-	5	6
hp-5	-	5	5
hp-7	-	12	2
hp-8	-	5	5

Relative real-time PCR and detection of CP gene ratio in non-transgenic plant to transgenic plants. Relative real-time PCR confirmed higher concentration of TuMV in non-transgenic plant compared to transgenic plants. The concentration of TuMV was 1947 fold in non-transgenic to hp-7 transgenic canola after 15 dpi.

Discussion

In summary, here, we report for the first time that transgenic canola plants expressing a short hairpin RNA, obtained from a conserved part of TuMV-coat protein gene, were protected against TuMV. Symptoms were appeared with at least five days delay in transgenic canola plants compare to non-transgenic canola plants. Furthermore, transgenic canola plants showed a minimum scores of symptom severity. Moreover, ELISA results showed no systemic infection in inoculated transgenic plants (exception in a line) and relative RT-PCR of coat protein of TuMV in non-transgenic compared to four assessed transgenic plants hp lines confirmed the increased virus concentration.

Although, we improved the resistance of a commercial canola line, RGS003, against TuMV, but environmental risk assay and comparative products assess of T1 and T2 resistant lines remain to be addressed. Resistance stability against other viruses infecting canola simultaneity, is more important too. Therefore, it was suggested that the inoculation of resistance canola lines with other TuMV isolates and also other viruses infecting canola such as *Cauliflower mosaic* virus or *Cucumber mosaic virus* to determine TuMV resistance stability. It has been revealed that the transgenic resistance of Arabidopsis plants carrying TuMV full length CP gene showed susceptibility reaction after inoculation of a mild strain of CMV (33).

In this research, transient transformation assays of sense, antisense and hairpin constructs designed from a 130-nt fragment of TuMV-CP gene released no efficiency of the sense

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construct. Previous researches have shown that other TuMV genes such as HC-Pro and NIb are good candidates for RNA silencing target (26, 27).

In our research, according to bioinformatics studies, we could not identify a highly conserved region in TuMV-HC-Pro in the isolates recorded in NCBI Gene Bank (data not shown). The spectrum of the RNA-mediated virus resistance is generally restricted to viral strains with greater than 90% sequence homology to the introduced transgenes (34-38), though, in some cases, the viral resistance of transgenic plants is overcome by the different potyviral strains or isolates that share more than 96% sequence identity (36). Transgenic plants showed different levels of protection against TuMV in other researches. For example in transformation of *N. benthamiana* plants with the CP nucleotide sequence, just 5 of 29 tested lines confirmed protection in more than 50% of their progeny (23).

Symptoms appearance delay is a factor to select for increased resistance in transgenic plants against TuMV. A categorized reactions to TuMV, in CP transgenic *N. benthamiana* involve into three phenotypes; susceptible (typical systemic symptoms were observed 5-8 days after inoculation, and delayed (systemic symptoms were delayed 3-16 days compered to control susceptible plants and resistance (the plants grew and developed normally throughout their life cycles) (23).

A disease indexing system was used to assess the reaction of transgenic Chinese cabbage carrying antisense NIb gene of TuMV against virus inoculation. Assessed transgenic Chinese cabbage plants were grouped into immune and highly resistant plants, resistant plants and, mildly resistant plants (26). In our research, we introduced scoring system for TuMV in canola plants by infectivity screening. Plants showed a range of disease symptoms that were assigned scores of 2-8 for minimum and maximum symptoms severity of the assessed plants.

The type of selective marker in transgenic plants is very important, too. Transgenic plants resistant to TuMV having Kanamycin and NPT-II resistance selective markers have been produced (21-23). Chines cabbage marker-free plants with high level of resistance to TuMV were produced (26). In the present study, we applied Basta resistant marker to select transgenic plants from non-transgenic plants that help to control weeds in canola fields. Transgenic resistant canola were one of the earlier goals for weed control in farms (39, 40). Transformation of coat protein gene of TuMV conferred broad-spectrum resistance in transgenic Arabidopsis (22), similarly, canola lines carrying the CP gene with functional start codon synthesized coat protein have shown variable levels of resistance whereas lines carrying the CP with a mutated start codon were as susceptible to TuMV (21). In our study, the small hairpin RNA with 130 nucleotide sequence convey resistance to canola plants.

Comparative analysis of the CP genes among the 17 TuMV isolates revealed that the 380-nt in the 3' region was highly conserved. The inoculation of all 17 isolates showed the same resistance as first candidate isolate to design RNA silencing target, suggesting the importance of the 380-nt in the 3' region for broad-spectrum resistance (22). Therefore, we predicted the designed hp construct in this research which would protect the transgenic canola plants against other TuMV isolates, because firstly, the selected 130 nucleotide sequence fragment designed according of 60 isolates alignment showed maximum identity and secondly the selected 130 bp fragment as a target region is located close to 3' region of TuMV CP gene (787-916-nt from 980-nt full length of CP gene). However, the inoculation of obtained transgenic canola plants with other TuMV isolates remain to be done.

One of the innovations about the design of selected target and nucleotide sequence fragment in our research is a minimum fragment size, 130-nt, improving the minimum probability off-target in transgenic canola. Therefore, this introducing hp construct could be used to transform to other TuMV hosts.

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