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DEVELOPING WHEAT DWARF VIRUS RESISTANCE IN BARLEY USING ARTIFICIAL miRNA

Theses of PhD dissertation

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INTRODUCTION

Even the time being, the battle against plant viruses imposes severe challenge to crop production. As plant breeders do not always possess adequately efficient resistance genes due to the constantly shrinking gene pools and the rapid genetic adaptability of viruses, the only measure against them is to combat the vectors using insecticides. Wheat dwarf virus (WDV) is the main viral pathogen of barley (*Hordeum vulgare* L.) and wheat (*Triticum* a*estivum* L.) causing dwarfing and yellowing of the plants. The disease may result up to 80% yield loss if the conditions are favourable for the virus. Field tests performed over several years in Hungary have proved that WDV was present in 88-100% of the infected plants, in both cereal species bearing the yellowing symptoms.

Insect vector of WDV is the leafhopper *Psammotettix alienus* Dahlbom (*Hemiptera, Cicadellidea*), which transmits the pathogens in a circulative, persistent manner. Chances of viral diseases can be partly reduced by choosing the right sowing time and by applying insecticides at the right time. However, it cannot be left unmentioned that this method is not entirely efficient due to the large number of vectors and their mobility. This problem could be solved by applying virus resistant cereal species; however, up to the present day, only a few WDV-tolerant cereal lines have been reported in the literature.

The discovery of RNA interference has opened a new chapter in the battle for plant virus resistance. From among the different RNA silencing methods, regarding efficiency and reliability, miRNA technology is one of the most outstanding. The structure of the miRNA precursors isolated from the plant genome, which provides the basis of the method, defines the quantity of miRNA that can develop from the precursors. Therefore, the

investigation of new miRNA precursors also has great importance in this topic.

Aims of the Research

I. Creating RNA interference-based wheat dwarf virus (WDV) resistance in barely by artificial miRNA technology.
   - To achieve this: searching for barley miRNA precursor ensuring high miRNA expression and transforming them into WDV-specific precursors containing miRNA,
   - improving a rapid, transient system for the analysis of biological activity of miRNAs,
   - genetic transformation of barley plants using polycistronic artificial miRNA created from the effective artificial miRNAs.
   - Designing a reliable WDV-inoculating system for the testing of the created barley lines.

II. Detecting miRNA precursors from wheat small RNA libraries that could be used for creating artificial miRNA.

MATERIALS AND METHODS

Plant material

For the agroinfiltration experiments, 2 or 3-day-old *Nicotiana benthamiana* plants were used. They were grown in plant growth chambers (Versatile Environmental Test Chambers; Sanyo, Tokyo Japan) under 14-h light (50 μE $m^{-2}s^{-1}$) and 10-h dark cycle, at 23 °C, in Jiffy pellets.

Barley plants (*Hordeum vulgare* cv. 'Golden promise') used for the *Agrobacterium*-mediated transformation were also grown in Sanyo plant growth chambers under 15 °C daytime and 12 °C night-time temperatures with a 16-h light (50 μE/m2/s) and 8-h dark period.

Plants (*Triticum aestivum* cv. 'Bánkúti B35 and B52'; *Triticum spelta*) used for creating wheat small RNA libraries were grown in greenhouse at 23-28 °C temperature. Time of fertilisation was calculated from the appearance of the first anthers. 10-20-30-day-old seeds were collected and stored at -80 °C until processing.

Rearing of Virus Vectors

A pooled population of *P. alienus*, originally collected from a barley field, was maintained for several generations on barley (*H. vulgare* cv. 'MV Jubilant'), kept in growth chambers under a 14-h light (50 μE/m2/s) and 10-h dark cycle at 23 °C and 30%–40% relative humidity. Leafhoppers were maintained on potted plants covered with a fine mesh (mesh size: 0.1 mm).

Agroinfiltration of **Nicotiana benthamiana** *plants*

Agrobacterium tumefaciens strain C58C1, harbouring the binary vectors, was infiltrated into the leaves of *Nicotiana benthamiana* plants. Bacterium solution diluted to OD_{600}=1 concentrate was injected into the leaf blade of

the tobacco plant with a 1 ml syringe. GFP expression was examined in the infiltrated leaves after 3 days, under UV lamp.

Agrobacterium-mediated transformation of barely

For barley transformation, immature 'Golden promise' barely embryos of 1,5-2-mm-diameter were used. After removing the axis, the binary vector (pCUbiVirusBuster171) harbouring the target gene was transferred into the plant cells with the help of *A. tumefaciens* bacterium. *Agrobacterium* was used in OD_{600}=1 concentration. In the course of calli formation, transformed cells were selected by hygromycine (50 mg/l).

WDV infection through leafhopper vector

WDV-infected, 4-5-week-old plants were equipped by micro-isolators and then both ends were blocked by sponge disks entwining the leaf. In the isolators, 3-3 leafhoppers were placed. The insects were left to feed upon the WDV-infected plants for one week. Afterwards, they were placed onto the healthy transgenic lines for one week. Then the presence of the virus both in the plants and leafhoppers was revealed by PCR.

RNA isolation, Northern and small RNA Northern blot hybridisation

Total RNA was extracted from agroinfiltrated tobacco leaves and transgenic barley plants using a TRI® Reagent RNA Isolation Reagent according to the manual of the manufacturer (Sigma-Aldrich, St. Louis,MO, USA).

To isolate total RNA, grains (~100 mg) were ground to fine powder in liquid nitrogen and Direct-zol™ RNA MiniPrep kit (Zymo Research Corp, Tustin, USA) was used according to the manufacturer's protocol.

RNA of WDV replicase was detected by northern blot hybridisation according to own protocol. From all samples, 5 μg of total RNA were

separated on formaldehyde–1.2% 1x MEA agarose gels. Then RNA blotted on nitrocelluloide membrane was hybridised by radioactively labelled PCR (replicase gene) product in 65 °C Church puffer.

For small RNA Northern blot hybridisation of agroinfiltrated tobacco leaves, transgenic barley and wheat seed samples, 10 µg total RNA was used and fractionated on denaturing 12% polyacrylamide 1x TBE gels containing 8 M urea. RNA was blotted on nitrocelluloide membrane using capillary blotting. Then RNAs were fixed to the membrane by applying chemical cross linking.

After hybridisation (in ULTRAHyb-Oligo puffer), radioactive signals emitted by the probe was detected by X-RAY roentgen film.

Creating wheat (Triticum aestivum L.) *small RNA libraries*

Wheat seeds 10, 20 and 30 days after pollination were used in two-fold biological replication to create the small RNA libraries with Illumina TruSeq® Small RNA Library Prep Kit (Illumina, San Diego, USA). The libraries were created by two methods: from total RNAs (T libraries) (UD-GenoMed created by Debrecen) or from purified (15-30 nucleotides) small RNA fractions (P libraries). Overall, 36 libraries were sequenced and qualitative characterisation was performed in two procedures.

Bioinformatics analysis of small RNA libraries

FastQC program was used for the quality control of the sequenced small RNA libraries. Adapters (Cutadapt 1.2.1) were removed from raw data, 16-28 bp nucleotides were filtered, sequences of low abundance (<3), transfer and ribosomal RNAs were removed (Python scripts, Rfam database). Remaining sequences were aligned to the wheat genome (Bowtie). Secondary structures of the hypothetic miRNAs were predicted by

ViennaRNA Package program. Then, potential pre-miRNAs were sorted by miDeep-P program. Already known and potentially new miRNAS were identified in 21 databases of miRBase Release.

The following formulas were used for normalising the small RNA readings: number of readings/total number of raw readings × 10^6 or number of readings/number of total filtered readings × 10^6.

RESULTS

Isolation and testing of the used miRNA precursor

Barley precursor *hvu-MIR171* (miRBase accession number: MI0016461; NCBI accession number: JX195502, 138 nucleotides) was isolated from 'Golden promise' barley variety and cloned into CaMV 35S expression cassette (pC61K vector). By generating point mutations, unique cleavage sites (BstEII-MluI) were created on the precursor in order to facilitate the transformation of amiRNA into precursor. *Nicotiana benthamiana* plants were infiltrated and Northern blot hybridisation was used to investigate the quantity of miRNA originating from the overproducing precursor (hvu-miR171MOD).

Selection of amiRNA target sequences

Based on the WDV h07 barley strain sequence (NCBI: FM210034), we searched for potential target sites of the *replicase* and *movement protein* genes. 136 and 31 potential target sites, respectively, were identified.

Previous criteria of the above process were the following:

- Presence of 5'- U initial nucleotide is required for the incorporation of amiRNA into the corresponding (AGO1) effector complex.
- G and C nucleotides have to be present on the 3'-end of amiRNA. At this location, more intensive base pairing is required with the stair strand.
- Base pairing with the target RNA is important in the 5'- 2nd and 12th position.
- Base pairing with the target RNA is also important in the 5'- 10th and 11th position.

Unwanted (OFF-target) effects of amiRNAs on barley transcriptome were examined using an internet-based application (http://plantgrn.noble.org/psRNATarget). OFF-target-free amiRNAs were subjected to sequence alignment with the WDV barley and wheat strains (5-5) and 8 and 2 amiRNAs were chosen for *replicase* and *movement protein* genes, respectively.

Development of amiRNA precursors

Firstly, during the designing process, given amiRNA sequence was inserted in the place of 171 miRNA, then the sequence of miR171* strand was modified in a way that the secondary structure of amiRNA precursor became identical to the original one.

The amiRNA precursors were developed by PCR mutagenesis with the use of pC61Khvu-miR171MOD vector as a template. Then the 110-nucleotide-long PCR product was cloned into the pC61Khvu-miR171MOD vector using restriction enzymes.

Polycistronic amiRNA expression vectors

The three selected amiRNA precursors aligned immediately next to each other (amiR1-amiR6-amiR8) were synthetized (GeneArt®, Life Technologies) and cloned into the 35S cassette of the pC61K vector (pC61KVirusBuster171).

The construction (pCUbiVirusBuster171), which is adequate for monocot transformation, was created in the pCUbiNOS vector that contained the monocot-specific promoter of maize *Ubi1* polyubiqitine gene.

Validation and testing of amiRNAs in a transient system

Vectors harbouring the amiRNA precursors (amiR1-10) were transformed into *Agrobacterium*, then, after infiltration into tobacco leaf, the maturing

amiRNA from miRNA precursors were examined by Northern blot hybridisation. AmiRNA accumulation could be detected seven in ten cases and no accumulation could be detected in three cases (amiR4, amiR5, amiR7).

GFP sensor construction was created for examining the biological activity of amiRNAs. Target sequences of amiRNAs were ligated in group of 5s in the untranslated but transcribed region of *mgfp5* gene 3'. The constructions containing the amiRNAs were expressed in tobacco plant leaves. Infiltration was performed at 15 and 23 °C in 2-2 replications. Six in 10 amiRNAs were found to be biologically active. At both temperatures, the same results were obtained, which can confirm that the amiRNAs positively develop on the miRNA instead of the siRNA pathway.

Barley transformation with polycistronic amiRNA construction

In the course of *Agrobacterium*-mediated, immature embryo-based barley transformation, 365 embryos were inoculated by the *Agrobacterium* harbouring the polycistronic amiRNA construction. Altogether, 77 transformants were collected from 20 independent calli. Plants grown from one callus were considered to be belonging to the same line.

Testing of transgenic barley lines by WDV inoculation

Four lines differently expressing amiRNAs were selected. VB8 line displayed high expression, VB9 and VB10 displayed moderate expression and VB20 produced far less amiRNAs than the other 3 lines. The plants were inoculated by WDV h07 strain via leafhopper vectors. The insects collected the virus from the artificially (by agro-virulent construction) constructed, WDV-inoculated plants. Plant growth temperature (12-15 °C) was set to block the plant's endogenic, RNA interference-based (siRNA) defence system, thus adjusting climatic conditions characteristic for fields in early

autumn. After the 1-week-long inoculation period, PCR technique was used to reveal the WDV DNA in the leafhoppers. Then, at four different time points [42, 56, 77 and 112 days post-inoculation (DPI)], WDV presence in the plants was investigated by molecular tools. Both the results of PCR-based and Northern blot hybridisation virus diagnostics confirmed that the WDV-specific amiRNAs have an impact on virus reproduction.

While in the first time point (42 DPI), relatively high level of WDV replicase could be detected in the control inoculated plants, in the case of transgenic lines, the presence of the virus could be revealed by PCR only in VB20, which, regarding its phenotype, rather resembles to the control plants without inoculation (Mock) than to the inoculated controls (WDV1, WDV2). In later time points (56, 77, 112 DPI), the *replicase* RNA could also be detected in the plants and their phenotype also markedly showed its presence. VB9 and VB10 lines, which expressed amiRNAs approximately up to similar extent, showed similar reactions to virus infection as well. Although WDV could be detected in them by PCR technique already after 56 days, Northern blot hybridisation could not reveal the *replicase* RNA even after 112 days (at heading stage). Compared to VB9 and VB10 strains, VB8 produced approximately two times higher amiRNA expression and showed complete resistance to WDV infection. During the whole vegetation period, neither the virus DNA, nor the virus replicase RNA could be revealed in the plants. Regarding its phenotype, the plant was completely identical to the wild type control without inoculation. VB9 and VB10 plants could grow their first 2-3 heads but later, due to the virus infection, the new heads could not ripen or poorer grain filling was observed.

Expression of amiRNAs was witnessed in the VB8 strain offspring as well.

Creation of small RNA library from total RNA and purified small RNA fraction

The libraries created from the total RNA (T) and the purified small RNA (P) did not show huge difference in terms of the number of total reads during sequencing (average 9,192,098 (T), 9,244,612 (P) reads). The variability of the data already showed greater differences between the two libraries in the case of crude data and after normalisation as well. Following the different selection steps, about 30% more reads were obtained in the case of "P" libraries. In the case of the already known miRNAs, substantially more reads were obtained by the alignment to miRBase database. In the T and P libraries, 1934 and 3247 unique potential miRNA sequences were aligned to the wheat genome, respectively.

Identification of new wheat miRNA for amiRNA use

In the libraries, a 21 nt, so far unknown miRNA (tae-2187, 5'-CGCGGCTCCGTCGACTGGTGC -3') was found that showed the highest or the second highest reading values in all samples. The precursor of miRNA was present on the two wheat genomes (B and D genomes) as well, one of them was 63 and the other one was 89-nucleotiode-long. Small RNA Northern blot hybridisation was also used to reveal miRNA.

New results:

1. It was the first time that virus resistance (to WDV) in barley could be developed by miRNA technology, which was created in polycistronic construction.
2. An artificial, barley-origin miRNA precursor (hvu-miR171MOD) was created, which is perfectly adequate for gene silencing in barley (or perhaps in other plants as well), due to its efficient procession.
3. It was proved that substantial information gain can be obtained during the development of small RNA libraries if the libraries are created from purified small RNA fractions than immediately from total RNA.
4. In the course of the wheat small RNA sequencing, a so far unknown miRNA with large reading number was identified and its precursors were found in the wheat B and D genomes as well. Their structure makes them adequate for using them as miRNA precursors.

CONCLUSIONS AND SUGGESTIONS

Barley hvu-miR171 as an artificial miRNA precursor

The fundamental point of using amiRNA technique against viruses is the selection of miRNA precursor.

Based on our results, the barley *hvu-MIR171* miRNA gene's precursor proved to be excellent for using it as an artificial miRNA. In the case of 7 out of 10 transformed precursors, measurable amiRNA signal could be revealed by Northern blot hybridisation. In 6 cases, even biological activity of amiRNAs could be proved. However, it must be mentioned that not all working amiRNAs could be revealed by Northern blot hybridisation.

Our results confirm that the barley hvu-miR171 miRNA precursor is perfectly adequate for the simultaneous expression of even three different amiRNAs in ploycistronic construction, which is an important aspect regarding its use against viruses.

Barriers of the resistance provided by amiRNAs

Based on the results we can conclude that the expression of amiRNAs developing from the polycistronic construction shows negative correlation to the extent of WDV systemisation. As the system cannot degrade the virus DNA genomes that are already infiltrated, only the *replicase* RNAs generated from them, therefore, after a while, the virus may be able to systemise even if the initial amiRNA level was higher (VB9, VB10 lines).

It would be worth investigating the plants in field trials under natural weather and infection conditions. However, regarding the fact that these lines are to be treated as GMOs for several reasons (polycistronic amiRNA and hygrmoycin resistance gene), it is very likely that the implementation of the above experiment will remain prohibited in Hungary, in the near future.

Development of wheat miRNA libraries

Wheat (*Triticum aestivum* L.) provides excellent material for discovering new miRNAs as its genome (2n=6x=42, AABBDD) consists of the chromosome sets of three species; therefore, it contains potentially more different miRNA precursors as well.

Between the small RNA libraries developed by the two, above mentioned methods [from total RNA (T) and from purified small RNA fraction (P)], there are fairly huge differences regarding quality. These differences mainly stem from the fact that most of the small nucleic RNAs (>60 nucleotides) and the scrap RNA were removed from the system by purify of the 15-30-nucleotide-long small RNAs from gel and they binded the adapter sequences used during the development of libraries. This is reflected by the fact that 68% more potential, unique miRNA sequences could be identified in the case of the libraries developed by purification (P) than by directly from total RNA (T).

New wheat miRNA precursor for amiRNA expression

The expression of amiRNAs can be influenced by two factors: the promoter sequence specifying the level and place of transcription and the used miRNA precursor sequence that specifies the procession of the given amiRNA. Therefore, it is worth choosing such miRNA that ensures higher amiRNA level through effective procession even with a given transcription.

The level of tae-2187 miRNA reached 2nd or 3rd place in every library, which suggests that it developed from a well-processioning precursor. Precursors of miRNA were identified on the two wheat genomes (B and D). In the future, we plan to isolate two precursors at DNA level and study the level of small RNA accumulation provided by the two precursors

in 35S promoter-driven construction. Also, we plan to study their adequacy for amiRNA transformation.

At the same time, it is important to note that the miRNA sequence from the precursor possesses ‘C’ initial nucleotide that has inherently greater chance to incorporate into the AGO5 protein in the PTGS pathway (in *Arabidopsis thaliana*), which plays important role mainly in the antiviral gene silencing, but also in the regulation of different development processes. In amiRNA technology, such approach has not been reported yet.

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