



SZENT ISTVÁN UNIVERSITY

**EXPRESSION OF DNA SEQUENCES RESULTING RESISTANCE
AGAINST ABIOTIC AND BIOTIC STRESSES IN WHEAT**

Ph.D. Theses

ZOLTÁN ÁY

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The doctoral school's

Name: Szent István University Doctoral School of Plant Sciences

Head: Dr. Lajos Helyes, D.Sc.
professor
Szent István University Faculty of Agricultural and
Environmental Sciences,
Institute of Horticulture Sciences

Scientific branch: crop and horticultural sciences

Supervisor: Dr. János Pauk, D.Sc.
deputy scientific director
Cereal Research Non-Profit Ltd. Co.

.....
Dr. Lajos Helyes
head of doctoral school

.....
Dr. János Pauk
supervisor

1. BACKGROUND AND OBJECTIVES

Thanks to the development of biotechnology and chemical industry, newer and newer compounds play role in weed management of cereals. Some of them are so called wide range herbicides targeting cultivated and weed species as well. We should interpret this toxic impact as abiotic stress but there are resistance genes, which can make these herbicides selective. In the first part of my thesis, I studied such a gene's impact in a model wheat genotype under greenhouse conditions.

Amino acid biosynthesis is one of the pathways targeted most by herbicides. The discovery of the peptide antibiotic PTT (phosphinotricin-tripeptide) produced by the actinomycetes *Streptomyces viridochromogenes* and *S. hygroscopicus* was reported several decades ago. The bioactive component of the PTT molecule is the PT which, as a structural analogue of glutamic acid, interferes with amino acid synthesis through the irreversible inhibition of GS (glutamine synthetase), the key enzyme of nitrogen metabolism. Using ammonia, GS catalyses the glutamic acid → glutamine conversion. The inhibition of the enzyme triggers ammonium ion and glutamic acid accumulation to levels up to 100-fold higher than in control cells. PT influences other pathways, too. It modifies the membrane transport, reduces protein as well as nucleotide concentration, and increases phosphoglycolate, glycolate and glyoxalate levels. These lead to lower photosynthetic activity (Schinko et al., 2009). It follows from the foregoing that PT has bactericide, fungicide and herbicide properties. In case of plants, photosynthesis slows down 2-4 hours after spraying, then chlorosis of leaves, desiccation and necrosis are visible within 2-5 days, and finally, the plants die (Berzsenyi, 2011). Both crops and weeds share the processes mentioned above. Consequently, selectivity must be based on the different ways herbicides act upon weeds and crops. So the resistance is going to be dependent on the genotype, not on the species.

Glufosinate ammonium is a proherbicide which is converted by plant cells into PT. The strategy to develop PT resistant crops is based on the mechanism used by PTT-producing actinomycetes. This pathway is mediated by the enzyme PAT (phosphinotricin-N-acetyltransferase) which acetylates the free amino group of PT, thereby causing its detoxification. The PAT-encoding *bar* (bialaphos resistance) and *pat* genes were isolated from *Streptomyces hygroscopicus* and *S. viridochromogenes* Tü494, respectively. Engineering tolerance to glufosinate ammonium in crops including wheat has been studied by many research groups (Tan et al., 2006) but the extent of herbicide resistance is less studied yet as well as the effect of extremely high concentrated glufosinate ammonium on different yield parameters in a *bar*⁺ wheat genotype.

Plant viruses as biotic stresses can cause significant losses in wheat production. Breeding for resistance is not too easy since suitable genes are limited and can be found usually in wild species. In case of crossings between common wheat and its wild relatives many undesirable features can be transferred to the breeding stock

which makes the selection more difficult. Crossings can be eliminated with utilizing pathogen derived resistance. Incorporation of viral sequences into wheat germplasm can induce RNAi (RNA interference), the ancient gene silencing machinery of the plant cell. In the second part of my theses I studied the efficiency of this system in model wheat genotypes under greenhouse conditions. My further aim was to establish whether the primer pairs developed for polymerase chain reactions are suitable for detection of natural virus infections.

Triticum species are sensitive to more than fifty plant viruses. According to investigations of many years, BSMV (Barley stripe mosaic *hordeivirus*), BYDV (Barley yellow dwarf *luteovirus*), WDV (Wheat dwarf *mastrevirus*) and WSMV (Wheat streak mosaic *tritimovirus*) are the most frequent viral pathogens of wheat in Hungary. The most efficient way of protection is breeding for resistance. It was proven in the last decade, that RNAi an ancient gene silencing system typical of eukaryotes induced by dsRNAs (double stranded RNA) can be very useful for creating virus resistant plant materials. dsRNAs are degraded into pieces as short as 21-25 nt by DICER, an RNAaseIII like nuclease. DICER generated siRNAs (short interfering RNA) incorporate into different silencing complexes and guide them to the complementary nucleic acids for specific degradation. As a result, genetic information of the targeted mRNA can not to be expressed (Bapat, 2013).

Virus infections activate the RNA silencing machinery of the host because both replication intermediaries of RNA viruses and overlapping sense-antisense transcripts of ssDNA viruses can be identified as dsRNA in plants. According to Watson-Crick base pairs, siRNAs generated from viral dsRNAs by DICER can recognize the nucleic acid of the pathogen which will be destructed by the silencing complexes (Ding and Voinnet, 2007). Even though silencing is an effective antiviral system, many viruses can infect plants because plant viruses express RNA silencing suppressor proteins as well and because silencing system is activated only when the virus started to replicate (Méraï et al., 2006). If a viral sequence is introduced into a plant as an inverted repeat separated by an intron, the derived transcripts will form a hairpin structure, which will be processed into dsRNA. Such a DNA sequence can cause total silencing of the targeted molecule. If the inverted repeat constructs are regulated by a constitutive promoter, the viral nucleic acids will be degraded before replication and the plant will be immunized (Fusaro et al., 2006). So it is possible to engineer wheat lines resistant to the viruses frequently infect wheat in Hungary.

Development of serological tests was a milestone in the history of virus detection. ELISA (enzyme linked immunosorbent assay) is used since the 1960's. Antigen-antibody reaction can be followed by an enzyme bound to the antibody. Colour changing of the solution is directly proportional to the concentration of the given virus. There are indirect and direct tests like DAS-ELISA (double antibody sandwich) which is the most popular, specific and accurate serological method in plant virus diagnosis. It is able to detect at once half nanogram virus (Tatineni et al., 2013). Methods of virus detection as well as virus taxonomy have been

increased with the development of nucleic acid based *in vitro* techniques. Both DNA and RNA viruses can be detected and characterised by hybridisation and amplification assays if the sequence of the given virus is known at least partially. PCR (polymerase chain reaction) is a good choice for sequence specific amplification since some pikogram nucleic acid is enough for the success. On the other hand, higher sensitivity of PCR than ELISA could be a disadvantage because an ambiguous conclusion can be easily drawn from the results of gel electrophoresis. It is difficult to estimate the volume of virus infection since PCR gives only a yes/no answer instead of quality result. The above disadvantage can be eliminated with using the improved qRT-PCR (quality real time PCR) which makes nucleic acid amplification one of the most accurate methods of virus detection (Jarosova and Kundu, 2010).

Our aims were:

1. To evaluate herbicide resistance of a wheat line showing broad spectrum herbicide resistance gene expression in comparison to wild type wheat.
2. To study the complex effect of extremely high glufosinate ammonium concentration on the yield parameters.
3. To incorporate DNA sequences into wheat providing resistance against viruses frequently infecting wheat in Hungary by the induction of RNA interference.
4. To confirm the efficiency of the engineered resistance at least against one of the pathogens.
5. To establish whether the primer pairs developed for detection of incorporated DNA sequences are suitable for detection of natural virus infections. To compare of sensitivity and effectiveness of polymerase chain reaction with the classical serological detection.

2. MATERIALS AND METHODS

2.1. Examination of resistance to a broad spectrum herbicide

A wheat line expressing a detoxifying gene was challenged with glufosinate ammonium. The lethal dose of this agent was defined in a preliminary experiment. Mature embryos were excised from surface sterilized seeds of CY-45 wheat genotype and were *in vitro* germinated in tubes, containing 5 ml of half-strength MS₀ medium supplemented with 0, 1, 2, and 4 mg·l⁻¹ of glufosinate ammonium, respectively. The resistance test was carried out with the wheat line 'T-124' expressing the gene *bar*. Mature embryos were germinated on media supplemented with fourteen different concentrations of glufosinate ammonium as follows: 2, 4, 8, 16, 32, 64, 128, 200, 400, 600, 800, 1000 and 5000 mg·l⁻¹. Medium in the control tube contained no herbicide. Every treatment was repeated eight times. After three weeks of culture, plantlets were transferred to pots filled with soil and grown to maturity in the greenhouse. Spikes were harvested individually and sorted into two groups termed *well-filled* and *low-filled* according to visual qualification. Yield components as number of spikes per plant, number of grains per spike, and yield per plant were measured while thousand kernel weight was calculated after harvesting. One out of the eight plants was randomly chosen in each herbicide treatment for analysis of the expression of the gene *bar* with RT-PCR using total RNA as template. Results of well-filled and low-filled groups were analysed separately by one way ANOVA. Data of partially and totally sterile spikes were also included in the statistical analysis. Plant material was destroyed after evaluation in the presence of NÉBIH (National Food Chain Safety Office) experts. According to the Permission No. 103613/4/2006, this was documented in the Minutes No. 162/1/210 which was introduced to the competent authority.

2.2. Development and examination of virus resistant wheat lines

RNA Biology Group of Biological Research Centre provided us seven version of pBAJEN vector. They cloned intron separated inverted repeat sequences providing resistance to the four most important cereal viruses (BSMV, BYDV, WDV and WSMV) via RNAi into the MCS. These pathogen derived sequences were developed with cloning of the most conservative regions of the viral nucleic acids according to partial sequencing of some isolates. Four plasmids contained sequences only from one virus. Two others could be effective against two viruses while the seventh could provide complex resistance to all of the four pathogens. CY-45 and GK Tavasza wheat genotypes (both of them are common wheat with CYMMIT origin) were used in our experiment. Immature embryos were isolated onto D₂ media 12-14 days after flowering. Dedifferentiated calli were placed to media completed with mannitol 4 hours before gene transfer. The induced osmosis helped the vector molecules to achieve their purpose by making the cell walls

permeable. Plasmids were transferred to the cells by particle bombardment. Since all vectors contained the gene *bar* regulated by a constitutive promoter, putative transgenic plantlets were selected with glufosinate ammonium. Survivors were grown to maturity in greenhouse (closed system). Genomic DNA was isolated from the leaves for confirmation of integration of the transferred sequences by PCR while the expression of the gene constructs was proven by RT-PCR using total RNA as template. We got primer stocks from the Biological Research Centre. Yield of PCR-positive variants were harvested.

Haploid technique was used in order to have the transferred sequences in homozygous form. Donor diploid plants were sprayed with Finale 14 SL at 3-4 leaf stage since genetic segregation was not eliminated in this generation yet. *In vitro* callus induction and plant regeneration were done on W14_f and 190-2Cu media, respectively. Vigorous plantlets at least with 10 cm shoots and strong roots were transferred into plots filled with soil in greenhouse. Spontaneous doubled haploids and haploids were separated according to visual qualification, and only the TDH (transformed doubled haploids) plants were grown to maturity. At waxy stage of ripening, 2-8 individuals promising the highest yield were chosen from each line for checking the expression of the introduced viral sequences by RT-PCR.

Seeds of TDH lines positive in molecular tests were multiplied and those expressing sequences of four different virus species were challenged with barley stripe mosaic virus. Artificial inoculation was done in greenhouse (closed system) by an emulsion derived from young barley shoots showing strong symptoms. In a preliminary experiment, 4 ten-day-old seedlings of untransformed CY-45 and GK Tavaszi genotypes were inoculated with BSMV while 4 other individuals (mock control) with emulsion derived from virus free barley. Efficiency of virus transfer was evaluated three weeks later by scoring of symptoms on the second leaf over the inoculated one with a five-rate-scale where 1 meant no symptoms and 5 meant very severe symptoms. Resistance test was carried out with the following wheat lines: 243-1, 243-3, 243-4 (GK Tavaszi origin) and 249-3, 249-4, 249-8 (CY-45 origin). Wild genotypes were also involved into the experiment as sensitive controls. Twenty seeds were sown per lines. Four plants were mock inoculated while sixteen others were challenged with BSMV. Symptom scoring was done as above but on the fourth leaf over the inoculated one in every week 12-18 weeks after sowing. SPAD value (Soil Plant Analysis Development – a unitless index correlating well with chlorophyll content) of the same leaves was measured as well. For detection of virus titer by DAS-ELISA method, one mock inoculated and four BSMV-inoculated individuals were randomly chosen from each genotype on the 18th week after sowing. Samples were considered virus infected if the extinction value was higher than 0.7. Plant height, length of spikes (the main one if there were more), thousand kernel weight and yield were documented at harvesting time. Data of chlorophyll content were statistically evaluated by linear regression analysis. Estimated value of correlation coefficient was also determined and the regression equation was set up. Difference between regression coefficients of the mock inoculated and virus inoculated groups was evaluated, too.

Concerning the agronomical parameters measured after harvesting, differences between wild type controls and TDH plants were analysed by one way ANOVA both in mock inoculated and virus inoculated groups while differences between mock inoculated and virus inoculated individuals of the same genotype were evaluated by mean analysis. Data of sterile plants were also included in the statistical analysis.

2.3. Detection of virus infections by serological and molecular methods

Our aim were to establish whether the primer pairs developed in the scope of the virus resistance project are suitable for detection of viruses and to compare the sensitivity of serological and molecular methods. Virus infections under field conditions in twelve registered wheat varieties (Élet, Garaboly, Kalász, Verecke, Ati, Tisza, Békés, Csillag, Petur, Hattyú, Holló and Piacos) were tested. Earlier and later sowing times were used (first ten days of October and November, respectively). Each genotype was sown in small plots containing two rows with 25 cm of row space. Two individuals were randomly chosen from each parcel and their leaves were collected in April. Leaf samples were divided for detection of virus infections by the two methods: DAS-ELISA and PCR. The first experiment served only with partial results, therefore further sample collection was needed in the middle of May.

During serological tests, antisera specific to BSMV, BYDV, WDV and WSMV were used. Samples were considered virus infected according to the chapter before. Before molecular examinations, genomic DNA and total RNA were isolated from the divided leaf segments. WDV was detected by PCR using DNA template while BSMV, BYDV and WSMV were detected by one step RT-PCR using RNA template. The following primer pairs were chosen: (BSMV Bb 156 for + BSMV Bb 1178 rev), (PAV Sal2 S for + PAV Sal2 S rev) (WDV S for + WDV S rev) and (WSMV 1000 for + WSMV 1565 rev).

3. RESULTS

3.1. Examination of resistance to a broad spectrum herbicide

In a preliminary experiment, we defined the lethal dose of glufosinate ammonium. Embryos excised from the non-transgenic spring wheat variety CY-45 were germinated *in vitro*. Only those embryos germinated which were placed onto medium without any glufosinate ammonium while 1-4 mg·l⁻¹ effective medium concentration resulted in neither shoots nor roots. This revealed that the lethal dose of glufosinate ammonium must be less than 1 mg·l⁻¹ in this experiment.

In the course of the test for herbicide resistance of the transgenic wheat line 'T-124' genetic segregation of the *bar* gene was not observed in the experimental plant population. This fact was confirmed by RT-PCR as well. Every embryo germinated under herbicide pressure; consequently, the resistance test was done with 112 transgenic wheat plants. Embryos germinated with the same intensity but, noticeably, the presence of 5000 mg·l⁻¹ glufosinate ammonium in the medium led to slower germination and prolonged the growing period by three weeks. In spite of these observations, every plantlet grew to maturity and developed 773 spikes in total (100%). According to visual qualification of the seeds, 311 spikes (40.2%) were considered as well filled while 462 others (59.8%) proved to be low filled. Obviously, partial and total sterility occurred only among the low-filled ones [19 spikes (2.4%) and 7 spikes (0.9%), respectively]. The number of spikes per plant varied between 2.38 and 3.13 in the well-filled group. These data represent the same level of significance. By contrast, this parameter was similar in the case of low-filled spikes but strongly increased at the three highest concentrations of glufosinate ammonium. Plants treated with 5000 mg·l⁻¹ herbicide showed the most intensive shoot development causing a bushy phenotype. Data in this group corresponded to three levels of significance.

The highest value of the number of grains per well filled spikes was 21.09 while the lowest was 17.43. The latter one was a result of application of 5000 mg·l⁻¹ glufosinate ammonium and it is significantly lower than the other values. Compared to this, the number of grains per spike was lower in the low-filled group and varied between 21.56 and 12.65. These data correspond to three levels of significance. Obviously, drastic differences were found between the two main groups in the thousand kernel weight. Representing three levels of significance, weight values of the well filled spikes varied from 37.19 g to 28.21 g. Contrary to this, data of the low-filled spikes indicated four levels where the weight value changed between 29.84 g and 16.92 g. Yield per spikes showed similarity between the two groups since values in the well-filled group varied from 2.15 g to 1.43 g and in the other case from 2.18 g to 1.03 g. The total yield per plant varied between 4.32 g and 2.64 g. Compared to the control plants, total yield of those treated with 128–5000 mg·l⁻¹ glufosinate ammonium – except the 1000 mg·l⁻¹ one – significantly decreased below 3 grams.

3.2. Development and examination of virus resistant wheat lines

For introduction of seven versions of pBAJEN plasmid into wheat cells, immature embryos were excised from wheat genotypes CY-45 and GK Tavaszi (12250 pc and 300 pc, respectively). After *in vitro* selection based on the marker gene *bar*, 673 plantlets were transferred into soil in greenhouse and 486 of them were grown up. After spraying with Finale 14 SL, 117 plants remained which were analysed by PCR. All integrated sequences proved to be expressed and both sense and antisense oriented sequences were detectable. It suggested that RNAi could be induced by the developed dsRNAs. According to the molecular tests, 44 plants were positive and grown to maturity. Totally 628 seeds were harvested. To have a view on the resistance of the plants, TDH lines were created (21 with CY-45 origin and 1 with GK Tavaszi origin) by selective anther culture. *In vitro* regenerated plantlets were transferred into soil in greenhouse. Haploids and spontaneous doubled haploids were separated by visual qualification and 381 TDH individuals were grown up. Some plants from each line were randomly chosen in wax stage and transcription of the introduced sequences was checked. According to the molecular characterisation, selection based on the marker gene *bar* was successful since 97 % of the plants expressed the inverted repeats.

TDH progenies of two T₀ plants (243 and 249) transformed with nucleic acid sequences of four different viruses were challenged by BSMV. In a preliminary experiment, efficiency of the artificial infection system was tested on wild type CY-45 and GK Tavaszi genotypes. Three weeks after inoculation, all mock inoculated individuals were symptom free while each plant inoculated with the virus showed typical symptoms. Consequently, our method proved to be suitable for BSMV transmission to both wheat varieties and made possible the objective examination of the TDH plants. The following TDH lines were involved into resistance test: 243-1, 243-3, 243-4 (GK Tavaszi origin) and 249-3, 249-4, 249-8 (CY-45 origin). Symptoms were scored weekly 12-18 weeks after sowing. Mock inoculated plants remained symptom free. Concerning those inoculated with BSMV, very severe symptoms were observed on the leaves of the wild genotypes. In a five-rate scale, the 16 GK Tavaszi individuals had scored average 1.75 points at the 12th week and after, their condition has worsened and finally, only 4.44 points were recorded at the 18th week. CY-45 plants had 1.81 points at the 12th week but 4.69 points at the 18th week. Counter to these, all of the TDH plants were seen to be healthy at the beginning of the examination period and 85 % of them remained symptom free until the end. Average scores barely surpassed the mock inoculated control's values; they varied between 1.06 and 1.44 at the 18th week. Photosynthetic activity of the previously scored leaves was measured with a quick tester. SPAD values were in harmony with the symptoms. Chlorophyll content of the mock inoculated controls was about 40 and changed in the slightest degree during the 7-week-long period. By contrast, chlorophyll content of the wild type wheat varieties inoculated with BSMV was lower than the mock ones at the 12th week and the difference between them became more considerable till the 18th week.

SPAD values dropped to 17 and 16.1 in GK Tavasza and CY-45, respectively. Compared to their own mock inoculated controls, photosynthetic activity of the TDH lines changed not at all or only a bit. According to linear regression analysis, the regression coefficient value of the TDH lines decreased significantly smaller than in case of GK Tavasza or CY-45. In other words, wild type controls gave statistically different answer to BSMV inoculation than the TDH plants. One mock inoculated and four virus inoculated individuals were randomly chosen from each genotype for checking BSMV concentration by DAS-ELISA test. Extinction values were in harmony with symptom scores and SPAD values. All the mock inoculated plants proved to be uninfected because their values varied between 0.367 and 0.533. BSMV inoculated wild wheat genotypes were strongly infected. Virus concentration was over 1.000 in these samples. None of the TDH lines with CY-45 origin was infected. On the other hand, one individual of the line 243-1 and 243-4 (GK Tavasza origin) had 0.882 and 0.662, respectively. The former one refers to virus infection while the latter one approaches the infection limit 0.7 from below. Furthermore, two other individuals of 243-4 just exceeded the limit. Directly before harvesting, plant height was measured. Mock inoculated GK Tavasza and CY-45 plants were 40-50 cm high while those inoculated with BSMV were backward and achieved only 20-30 cm. Height and vigour of TDH plants inoculated with the virus were very similar to the mock inoculated wild type controls but noticeably, those showing symptoms and considered as infected at the 18th week, were shorter than the average of their group but not so much like the BSMV inoculated wild wheat genotypes. Before threshing, length of spikes was recorded. In case of GK Tavasza and CY-45, BSMV inoculation decreased the length of spikes from 55 mm to 30 mm but differences between mock inoculated and virus inoculated TDH plants were not higher than 12 mm. Average yield of the mock inoculated GK Tavasza was dramatically decreased by the virus from 539 mg below 60 mg. Its reasons are that 6 out of the 16 individuals proved to be totally sterile and yield of the fertile others were even under 200 mg. BSMV inoculated TDH lines with GK Tavasza origin provided about 400 mg of yield. Among 243-1 and 243-4 individuals, yield lower than 200 mg occurred in those cases where the plant was infected according to serological test. Yield of wild type CY-45 was 457 mg in our experiment and it was decreased by the virus below 25 mg since eight totally sterile plants were found in this genotype and almost every fertile others yielded below 100 mg. Both BSMV and mock inoculated TDH lines with CY-45 origin had about 400 mg yield. After threshing, thousand kernel weights were calculated which indirectly referred to the quality of the yield. Generally the tendencies learned at yield prevailed in this parameter, too. Compared to the mock inoculated plants, BSMV inoculated GK Tavasza and CY-45 had thousand kernel weights decreased with 18 g and 23 g, respectively while in case of TDH lines, the difference between the two groups was only 1-5 g. According to one way ANOVA, differences in the yield components between the mock inoculated wild genotypes and TDH lines were not significant, so the examined properties of GK Tavasza and CY-45 did not change because of introduction of the inverted repeat viral sequences. On the other

hand, we demonstrated that there were significant differences between BSMV inoculated wild genotypes and TDH lines. It means that TDH lines expressing the inverted repeats gave statistically different answers to virus infection than the original genotypes. Within the same genotype, differences between the mock and virus inoculated plants were also evaluated. In case of wild genotypes, BSMV caused significant losses in each agronomical parameter. Similar result was found in the length of spikes of 249-4 and 249-8. Leaving this out of consideration, there were no significant differences between the mock and virus inoculated TDH lines.

3.3. Detection of virus infections by serological and molecular methods

One part of the divided leaf samples was examined by DAS-ELISA. From the late-sown plant material, only one variety ('Élet') showed detectable BYDV infection but BSMV, WDV and WSMV were undetectable. By contrast, the early-sown varieties were strongly infected. BYDV was detected in 46 % of the plants. Both individuals contained the virus in the following varieties: 'Garaboly'; 'Kalász' and 'Ati' while in case of 'Élet'; 'Verecke'; 'Békés'; 'Holló' and 'Piacos' only one of the two samples showed infection with BYDV. 'Tisza'; 'Csillag'; 'Petur' and 'Hattyú' proved to be uninfected by BYDV. The other three viruses were detected at much lower frequencies even in the early-sown parcels. WSMV infection was found in 'Holló' and 'Piacos' whereas WDV infection was detected only in one sample of 'Piacos'. BSMV was not detected at all. 'Holló' and 'Piacos' showed mixed virus infection. BYDV occurred together with WSMV in the former variety, while WDV, BYDV and WSMV were presented in the latter one. Since almost only BYDV occurred in the samples, we collected other symptomatic leaves definitely from the early-sown parcels three weeks later. BSMV, BYDV, WDV and WSMV were found in 1, 16, 7 and 6 samples, respectively. Mixed infections were detected in 9 cases. Other part of the leaf samples were examined by PCR assays. From the late-sown parcels, only the same 'Élet' sample showed detectable virus infection which was also positive for BYDV in ELISA. According to the PCR results, 58 % of the tested plants proved to be infected by BYDV in the early-sown plant material. Both samples of 'Garaboly'; 'Kalász'; 'Ati'; 'Békés'; 'Holló' and 'Piacos' contained the virus while only one sample showed infection with BYDV in the case of 'Élet' and 'Verecke'. The same four varieties: 'Tisza'; 'Csillag'; 'Petur' and 'Hattyú' which were negative in ELISA proved to be uninfected in PCR, too. Moreover, in the early-sown parcels, WSMV infection was found only in one variety ('Ati'). BSMV and WDV were undetectable in each case. All the four pathogens were detectable from the strongly symptomatic leaves collected later. BSMV, BYDV, WDV and WSMV were found in 1, 18, 13 and 14 samples, respectively. Except only one case, PCR results confirmed the serological data. On the other hand, molecular detection proved to be much more sensitive, since 19 samples positive in PCR were uninfected according to ELISA.

NEW SCIENTIFIC RESULTS

1. It was concluded that wheat line 'T-124' survives 5000 mg·l⁻¹ of glufosinate ammonium during germination. Despite of the expression of detoxifying *bar* gene, extremely high concentration of glufosinate ammonium blocks the apical dominance which leads to a bushy phenotype and can cause sterile spikes.
2. It was found that yield of the wheat line 'T-124' constitutively expressing the *bar* gene does not change significantly even if it is challenged by glufosinate ammonium 64 times the lethal dose.
3. Using two donor genotypes, 38 fertile plants were created which express RNA interference inducing inverted repeat DNA sequences thus can be resistant to Barley stripe mosaic *hordeivirus*, Barley yellow dwarf *luteovirus*, Wheat dwarf *mastrevirus* and Wheat streak mosaic *tritimovirus*. Out of these 38 plants 13 contain such plasmid construction which can result in resistance to all of the above mentioned pathogens.
4. Using selective anther culture, 22 transformant doubled haploid wheat line versions were created. Genetic segregation of the inverted repeat sequences is eliminated in their progenies.
5. Six doubled haploid wheat lines were artificially inoculated with Barley stripe mosaic *hordeivirus* under greenhouse conditions. It was statistically verified that the virus had no significant effect on photosynthetic activity, height, thousand kernel weight and yield of the plants. It was serologically confirmed that replication of the virus was inhibited in the doubled haploid plants thus RNAi was successfully induced in two different wheat genetic backgrounds.
6. It was proven that four primer pairs (BSMV Bb 156 for + BSMV Bb 1178 rev; PAV Sal2 S for + PAV Sal2 S rev; WDV S for + WDV S rev; WSMV 1000 for + WSMV 1565 rev) are suitable for detection of natural virus infections by PCR based techniques.

4. DISCUSSION

4.1. Examination of resistance to a broad spectrum herbicide

In the preliminary experiment, we found that less than $1 \text{ mg} \cdot \text{l}^{-1}$ of glufosinate ammonium in the culture medium is enough to inhibit CY-45 (wild-type) embryo germination. Similarly low concentrations of PT-like herbicides made possible the successful selection of transgenic tissues according to pioneer wheat transformation studies (Vasil et al., 1992). Throughout the first three weeks of their life cycle, wheat plantlets derived from the transgenic plant line 'T-124' constitutively expressing the *bar* gene were challenged by 14 different concentrations of glufosinate ammonium. Those treated with higher concentrations showed significant differences in the examined parameters compared to the controls. We also recorded the length of the growing period of the plants which had been prolonged strikingly by the influence of the highest concentration ($5000 \text{ mg} \cdot \text{l}^{-1}$) of the herbicide. It is probable that in spite of the constitutive production of the PAT enzyme, plants could detoxify the herbicide only at the expense of slowed down metabolism. Plants tried to compensate for this lag after the transfer into soil made manifest not in the strengthening of the main shoot but in developing several lateral shoots. Those individual plants treated with 800 and $1000 \text{ mg} \cdot \text{l}^{-1}$ of glufosinate ammonium showed a similar stool phenotype at harvest time. Certain studies reported that PT applied in sublethal dose stimulates *in vitro* shoot regeneration in the case of grape (Hébert-Soulé et al., 1995), snapdragon (Hoshino and Mii, 1998) and rice (Liu et al., 2005). Our results reveal that increased ammonium ion level within the plant cell might act as a source of abiotic stress. Therefore, according to the apical dominance theory, inhibition of the apical tissues can lead to more intensive lateral shoot growth. However, this kind of escape was coupled with a weaker condition, which developed low filled spikes without exception. The decrease in the number of grains per spike was caused mainly by the shortening of spikes but in some cases, this was supplemented with partial or total sterility of low-filled spikes. Thousand kernel weight decreased with almost the same intensity in both groups. However, changes in the values of this index did not manifest themselves in the summarized yield of spikes in the well-filled group because the stable number of spikes and number of grains per spike offset them. Lower thousand kernel weights began to cause a decrease in the summarized yield of low-filled spikes at $128 \text{ mg} \cdot \text{l}^{-1}$ of glufosinate ammonium, but this tendency was reversed at $800 \text{ mg} \cdot \text{l}^{-1}$ and higher concentrations. This decrease can be traced back unambiguously to the negative changes in thousand kernel weight and number of grains per spike while the increase was caused by the higher number of spikes. Total yield per plants fluctuated similarly. We did not check the quality of the grains in this experiment but we must make it absolutely clear that in the case of the three highest concentrations of glufosinate ammonium, the yield was restored

definitely by the increased number of low filled spikes representing a visibly poor quality.

If we take plant death as a basis we can't say by how much more resistant our transgenic plants are compared to the control ones since all of them survived the 5000 mg·l⁻¹ treatment, thus the lethal dose remained unknown. If we take the slightest significant change in the examined parameters we can see that the 8 mg·l⁻¹ treatment was the highest which caused no significant difference. We think that both of these approaches are misleading; therefore we chose the total yield, the most important trait of cereal crops, as a benchmark. We found that 64 mg·l⁻¹ was the highest concentration of herbicide which caused no significant loss in the yield. Consequently, a threshold value of resistance to glufosinate ammonium must be between 64 and 128 mg·l⁻¹ according to this experiment. Since the lethal dose of the herbicide proved to be less than 1 mg·l⁻¹, 'T-124' plants therefore achieved at least 64-fold resistance. This value is undoubtedly higher than or similar to those published in articles (Gopalakrishnan et al., 2000; Manickavasagam et al., 2004). We suggest that this kind of high herbicide resistance should be utilized in practice cautiously because it can lead to the rapid natural development of resistant weed populations. However, we set a high theoretical value on our results as the gene *bar* can be substituted with other resistance genes (drought or cold stress). If the results will be similar to ours, many agricultural problems could be solved in the future.

4.2. Development and examination of virus resistant wheat lines

We have done an experiment with seven plasmid vectors which can induce resistance to simple or complex infections of BSMV, BYDV, WDV and WSMV through RNAi. Transformation efficiency was 1.02 % and 0.33 % related to *bar*⁺ T₀ plants and fertile inverted repeat⁺ T₀ plants, respectively. These values correspond to the internationally published 0.1-5.0 % (Bhalla et al., 2006). Since our aim was to test the resistance of the created plants as well, wheat lines were made homozygous by anther culture in order to eliminate genetic segregation. Our laboratory is well experienced in haploid technique of cereals. We had confirmed for the first time that selection based on the *bar* gene can be used during *in vitro* androgenesis without any loss of green plant production (Mihály et al., 2002). Plant regeneration capacity depends mostly on the genotype but both of the two wheat varieties used in our experiment proved to be excellent. Totally 381 plants of 22 doubled haploid lines (21 with CY-45 and 1 with GK Tavaszi origin) were created. Expression of inverted repeats was checked by molecular assay in 91 plants. Selective anther culture was successfully used since viral sequences were detectable in 97 % of the examined individuals.

Doubled haploid lines 243 and 249 (GK Tavaszi and CY-45 origin, respectively) containing sequences of four different viruses were chosen for resistance test. We have proven that barley stripe mosaic virus can be efficiently transmitted to both

wheat genotypes which are sensitive to the used strain of the virus. In our greenhouse experiment, 16 individuals of GK Tavasza, CY-45 and TDH lines 243-1, 243-3, 243-4, 249-3, 249-4 and 249-8 were inoculated with BSMV while 4 other individuals of the same genotypes were mock inoculated. Symptoms became visible 15-18 weeks after sowing. Stronger than medium symptoms were observed on the wild type plants whereas 85 % of TDH plants were symptom free and 15 % of them showed only slight symptoms. SPAD value of the leaves was also measured which provided information about photosynthetic activity. According to statistical analysis, chlorophyll content of the wild type control plants was decreased significantly by BSMV while in case of TDH lines, difference between mock and virus inoculated plants was not significant. It suggests that virus replication could be inhibited by RNAi in the plant cells. This hypothesis was confirmed by DAS-ELISA. Extinction values were considerably over the infection limit in case of all GK Tavasza and CY-45 leaves but virus concentration in TDH plants was under or just over the limit. Agronomical parameters like plant height, length of spikes, thousand kernel weight and yield were analysed at harvesting time. Differences between wild type and TDH plants were not significant so introduction of viral sequences had no statistical effect on the evaluated parameters. It was also proved that TDH plants expressing inverted repeats were more resistant to BSMV than wild type controls since significant differences were found between these two groups. Within the same genotype, as it was expected, there were significant differences between mock inoculated and virus inoculated plants in case of GK Tavasza and CY 45, whereas in case of TDH plants, the differences between the two groups were not significant except the shortening of spikes of 249-4 and 249-8 lines. Data of certain genotypes showed big spread. We think this was an epigenetic effect or an aftermath of inhibition of RNAi generated by the virus. Such kind of ability of viruses is widely studied (Bragg és Jackson, 2004).

RNAi was successfully utilized for development of resistance to virus infections in many dicotyledonous species but there are much less results in monocots. To the best of our knowledge, pathogens relevant to our thesis only BYDV and WSMV were published as target of induced RNAi (Wang és mtsai, 2000; Fahim és mtsai, 2010). Vector constructs carrying intron separated inverted repeat sequences make possible the induction of multiple virus resistance even in case of simultaneous inoculation (Bucher és mtsai, 2006; Zhang és mtsai, 2011). Our experiment achieved its purpose. We managed to induce virus resistance based on RNAi in two different wheat genetic backgrounds. The induced resistance proved to be effective against BSMV since neither photosynthetic activity nor yield of the challenged plants changed significantly after virus inoculation. Because of the used vector construct, our plants should be resistant to BYDV, WDV and WSMV as well but this needs further verification.

4.3. Detection of virus infections by serological and molecular methods

We got an affirmative answer to our question since the primer pairs proved to be useful for PCR based virus detection. ELISA data were confirmed by PCR, so we found similar results like Robertson et al. (1991) in wheat, Figueira et al. (1997) in barley and Mumford et al. (2004) in oat. Optimised reaction conditions made possible simultaneous detection of the three RNA-viruses. PCR gave positive results in 22 samples which were negative in ELISA. This reveals that the molecular assay is more sensitive than the corresponding serological method. For more accurate and quantitative detection, we suggest to use qRT-PCR with the same primer pairs. Infections were not detectable by PCR in four samples positive in ELISA. It was supposed to be a manual mistake or the plant was infected by a virus strain with nucleic acid sequences inhomogeneous to the primers. BYDV was the dominant virus in the experimental season. Certainly the extra mild autumn allowed the aphid vector species to feed on winter wheat until late October. Counter to this, very few cicada and mite species were observed. This explains why WDV and WSMV occurred only in few samples. The mechanically transmitted BSMV was detectable only from one sample by both methods. Occurrence of BYDV was significantly higher in the early sown population which all goes to show that early sowing is risky because it is favourable for development of epidemic (Perry et al., 2000). We can't characterize the susceptibility of the examined wheat varieties since we used very few samples. This was not an aim of our thesis. We just wanted to compare the different methods for virus detection.

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