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DETECTION OF GENES DETERMINING THE QUALITY CHARACTERISTICS OF MAIZE GRAIN AND ITS RESISTANCE TO STRESS FACTORS

Two hundred experimental samples of maize (Maize Company) have been examined for the presence of genes determining the quality characteristics of grain (wx and fl-2 genes) and resistance to herbicide (bar (pat), epsps genes) and pests (cry-genes).

The maize DNA has been extracted from living vegetable tissue. The primers to detect the wx, fl-2, bar (pat), mepsps, CP4 epsps, cry1A(b), cry1F, cry1A.105, mcry3A, cry2Ab2, cry3Bb1, cry34Ab1, and cry35Ab1 genes have been selected and designed. The multiplex and the touchdown polymerase chain reaction (PCR) techniques have been developed. The PCR amplification of certain sequences has been carried out. None of transgenes (bar (pat), mepsps, CP4 epsps, cry1A(b), cry1F, cry1A.105, mcry3A, cry2Ab2, cry3Bb1, cry34Ab1, and cry35Ab1) has been found among the 200 analyzed maize experimental samples. At the same time, the fl-2 gene has been detected in 41 samples, whereas the wx gene has been identified in 192 analyzed samples.

Key words: maize, transgenes, grain quality characteristics, primers, polymerase chain reaction (PCR).

Maize is one of the most common cereal, forage, and silage crops in the world. In Ukraine, it is expanding over more and more acreage. According to the State Statistics Committee of Ukraine, in 2012, the area under maize reached 4.7 million hectares. Throughout the world, the transgenic maize occupies more than 50% of acreage allotted for this crop due to its resistance to pests and tolerance to herbicides. In the EU, 12 main maize transformation events (Bt11, Bt176, DAS1507, GA21,MON810,MON863,NK603,T25,DAS59122, MON 89034, MON88017, and MIR604) and their combinations [1] have been registered and authorized according to the EU Regulation 1829/ 2003.

According to various sources 5—10% of maize cultivated in Ukraine contains transgenes or transformational events. In Ukraine, there had been not its own transgenic (hybrid) line to be created till 2007. The majority of transgenic maize in Ukraine contains transformational events created by *Bayer*, *Monsanto*, *Syngenta*, *Pioneer*, and *Dow AgroSciences*.

In 2007, Ukraine adopted the Law on the State Security System for Development, Testing, Transportation, and Use of Genetically Modified Organisms [2]. The producers of crops, including maize, must either remove them from crop rotation system or label as transgenic product.

The aim of this research is to evaluate the experimental forms of maize offered by *Maize Company* farm holding in order to detect the transgenes. Genetically modified maize containing «pure» transgenes mostly does not exist. All the transgenes are a part of transformational events, i.e. the integrated transformants characterized by

unique combination of the elements of genetic maker and genomic DNA of plant with unambiguous place of insertion. Thus, it is necessary to develop a technique to detect the transformation events of maize to which the genes of resistance to stress factors are included. At the same time, to select effectively the maize forms for further selection process we have identified the genes determining the quality characteristics of grain.

TRANSGENES GIVING RESISTANCE TO STRESSFUL FACTORS

Genes *bar* (*pat*), *CP4 epsps*, *mepsps* are highly effective and widely used in the world to create the transgenic plants resistant to herbicides of general purpose, to which the products of these genes provide guaranteed stability. The *bar* (*pat*) genes encode resistance to herbicides based on phosphinothricin (PPT): *Basta*, *Glufosinate*, and *Phosphinothricin*. The *CP4 epsps, mepsps* genes encoding enzyme of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) are not sensitive to harmful effects of glyphosate (Roundup herbicide). The genes *cry1A (b), cry1F, cry1A.105, cry2Ab2, cry3Bb1, cry34Ab1*, and *cry35Ab1* give the plants resistance to pests: namely, the genes *cry1A (b)* and *cry1F* resist the European corn worm or European corn borer (*Ostrinia nubilalis*); the genes *cry34Ab1, cry35Ab1, cry3Bb1*, and *mcry3A* resist the corn beetles and rootworms *(Coleoptera*, *Diabrotica* spp.); and the gene *cry2-Ab2* resists pests of *Lepidoptera* order.

The genes for herbicide tolerance and pest resistance are constituents of transformation events. The *cry1A (b)* gene is a part of the transformation events Bt11, Bt176, MON810; the *cry1F* gene is a constituent of transformational event DAS1507; the genes *cry34Ab1* and *cry35Ab1* are a part of transformation event DAS59122; the *cry3Bb1* gene is a part of transformation events MON863 and MON88017; the *mcry3A* gene is a part of transformation event MIR604; the genes *cry1A.105* and *cry2Ab2* are a part of transformation event MON89034. The *bar (pat)* genes are a part of transformation events Bt11, Bt176, DAS1507, AS59122 and T25, while the *mepsps, CP4 epsps* genes are constituents of transformation event GA21, MON88017, and NK603.

GENES DETERMINING THE GRAIN QUALITATIVE CHARACTERISTICS

Another focus of the research is to find the molecular genetic experimental forms of maize of *Maize Company* farm holding, which contain genetic sequences determining the quality characteristics of grain: the *wx* gene which is responsible for starchy properties of grain endosperm and the *fl-2* gene ensuring increased content of lysine and methionine in grain.

The maize phenotype *floury 2* forms endosperm with reduced quantity of prolamin proteins. These proteins are encoded by group of *zein* genes and are rich in glutamine and proline, but poor in lysine and tryptophan. Their share reaches 60—70 % of the total proteins in seed. Therefore, it is observed an unbalanced aminoacid composition of endosperm, which reduces the grain nutritional value. The *fl-2* mutation disturbs synthesis of *α-, β-,* and *γ-zein* proteins as a result of which loose soft endosperm is formed and the share of lysine- and tryptophan-containing proteins in the grain increases/gets balanced. Therefore, this mutation is highly regarded by the selectioners.

The *Waxy* maize has a low amylose content (<5 %) in corn starch and bears a null mutation of *wx* gene. This locus encodes an enzyme, granule-associated-starch synthase-1 which catalyzes the elongation of amylose molecules. The recessive allele of *wx* gene inhibits the activity of this enzyme and reduces the amylose content in starch of endosperm. In grain, only amylopectin is accumulated. As compared with amylose, amylopectin is better digested by amylase. Hence, it is more processable raw material. Nowadays, the selection by wax-like sign is being made.

Identification of genetic sequences determining the grain's quality characteristics is associated with the need to select maize lines to be involved in the further selection process aimed at improving the quality of grain. The maize grain

containing the wax-like gene (*wx*) is more suitable for the production of bioethanol, while the grain with increased content of lysine and methionine (*fl-2*) is preferable for food production.

PROTOCOL OF DETECTION OF TRANSGENES AND GENES DETERMINING THE QUALITATIVE CHARACTERISTICS OF MAIZE

The research deals with study of 200 samples of maize experimental selective seeds. The methods for detection of foreign genes have been elaborated on the reference samples of maize containing the appropriate transformation events. To obtain the purified preparations of maize DNA the seeds have been sprouted. The fresh tissue have been grinded in ceramic mortar with cetyl trimethyl ammonium bromide (20 g/l cetyl trimethyl ammonium bromide, 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na2EDTA, pH 8.0) and 1.4-mercaptoethanol. It has been treated with RNase A and purified from proteins by chloroform. The maize DNA has been salted out by ethanol and flushed out. The precipitate has been dissolved in TE buffer with pH 8.0 [3, 4]. To check availability and quality of the extraction of maize vegetable DNA the separation of nucleic acids by electrophoresis in agarose gel has been used [4]. Thereafter, the spectrophotometric measurement of nucleic acids concentration has been carried out. The purity of maize DNA has been determined by the ratio of absorption factors at wavelengths of 230, 260, 280, and 320 nm. The concentration of DNA samples has been standardized to 30 ng/μl.

The polymerase chain reaction (PCR) has been realized by the Ausubel method [4]. The conditions for annealing have been calculated according to the properties of oligonucleotide primers. The concentrations of primers have been selected empirically in each case individually, depending on the type of bonds with the DNA matrix.

In case of large number of non-specific amplification products or too big difference in melting points of different pairs of primers used in reactive mixture the touchdown polymerase chain reaction method has been developed.

Electrophoresis of 12 μl of each amplification product sample after PCR has been carried out in agarose gel (1.2 %). The molecular ruler was 400 ng of O'GeneRuler™ DNA Ladder Mix (Thermo Scientific) molecular weight markers.

The conditions of electrophoresis for all the samples were as follows: voltage is 5 V/cm, duration is 1—1.5 hours. To visualize the DNA fragments (amplicons) 5 μg / l of ethidium bromide was added to Tris-borate buffer (TBE). The gel was photographed with GelDoc™ (Bio-Rad). The images were processed with the use GIMP graphical editor.

ANALYSIS OF MAIZE EXPERIMENTAL FORMS ON THE PRESENCE OF STRESS RESISTANCE GENES

The structure of each transformation event includes 2-3 transgenes. To identify them the specific primers have been selected and designed for each gene or transformational event (Tables 1 and 2) [5—14]. Therefore, the multiplex PCR (mPCR) that allows one to carry out the amplification of several studied DNA characteristic elements in one reaction has been developed [15]. This helps to decrease reaction time, reagent consumption, and load on device.

To detect the transgenes or transformation events the reactive mixtures having a volume of 20 ml have been prepared. They consist of 1xDreamTaq™ Green buffer, 0.5 units of DreamTaq™ polymerase (Thermo Scientific), 200 mM of dNTF, 30 ng of vegetable DNA, primers to the *zein* reference gene and primers to the studied transformational events / transgenes in respective concentrations according to the data given in Table 1.

In all the cases, the reactive mixture with 1 μl of TE buffer pH 8.0 instead of DNA sample has been used as negative control.

The mPCR program on the presence of transformation events BT176, MON810, MON88017, DAS1507, MIR604, DAS59122, GA21, and NK603 was as follows: denaturation of vegetable DNA during 4 minutes at a temperature of 94 °C; 35 cycles were carried out, each of them included

Table 1

List of primers to transformation events, their melting temperatures, and annealing temperatures in PCR

Transformation event (genes)	Primer	Primer concentration in reacting mixture for PCR, μM	Melting temperature, °C	Annealing temperature, °C
Reference gene *adh1*	Adh-F3	0.20	66	55
	Adh-R1	0.20	60	
Reference gene *zein*	Zein3	0.30	57	50
	Zein4	0.30	55	
Bt11 (*cry1A(b)*, *pat*)	IVS2-2	0.35	62	57
	PAT-B	0.35	64	
	Bt11-1	0.45	57	51
	Bt11-2	0.45	56	
Bt176 (*cry1A(b)*/int.9 PEPC, *pat*)	PEPC-C-20	0.35	63	56
	CRYIA4	0.35	61	
MON810 (*cryIA(b)* with intervening sequence int.hsp70)	mg1	0.45	66	59
	mg2	0.45	64	
	VM01	0.35	65	59
	VM03	0.35	64	
DAS1507 (*cry1F*, *pat*)	TC1507 01-5	0.50	61	56
	TC1507 01-3	0.50	61	
	TC1507-1F	0.50	58	59
	TC1507-2R	0.50	57	
MON89034 (*cry1A.105*, *cry2Ab2*)	M8F2	0.25	65	60
	M8R2	0.25	65	
DAS59122 (*cry34Ab1*, *cry35Ab1*, *pat*)	59F1	0.45	67	60
	59R1	0.45	65	
	SEQ ID NO 9	0.45	69	57
	SEQ ID NO 10	0.45	63	
MON863 (*cry3Bb1*, *nptII*)	P863-3F	0.35	55	50
	P863-4R	0.35	56	
	84_18-L	0.35	57	49
	84_18-R	0.35	54	
MON88017 (*cry3Bb1*, CP4 *epsps*)	M7F8	0.45	67	60
	M7R8	0.45	65	
	MON88017-mF	0.45	58	53
	MON88017-mR	0.45	61	
MIR604 (*mcry3A*, *pmi*)	M6F1 01-5	0.35	67	62
	M6R1 01-3	0.35	67	
	E-604-F	0.50	60	55
	E-604-R	0.50	65	
GA21 (*mepsps*)	esGA21-5 ‘F	0.45	63	52
	esGA21-5’ R	0.45	57	
NK603 (CP4 *epsps*)	NK603F	0.45	64	59
	NK603R	0.45	66	
	SEQ ID NO 13	0.45	60	52
	SEQ ID NO 14	0.45	57	
T25 (*bar/pat*)	T25-F7	0.45	59	54
	T25-R3	0.45	63	

Table 2

Nucleotide sequences of primers to transformation events

Transformation event	Name of primer	Nucleotide sequence of primer	Size of amplicon, bp	Specificity
Reference gene *adh1*	Adh-F3	5′-CGTCGTTTCCCATCTCTTCCTCC-3′ [5]	231	to *adh1* gene
	Adh-R1	5′-GACAGAGGAGAAACAAGGCG-3′ [5]		
Reference gene *zein*	Zein3	5′-AGTGCGACCCATATTCCAG-3′ [5]	277	to *zein* gene
	Zein4	5′-GACATTGTGGCATCATCATTT-3′ [5]		
Bt176	CRY04	5′-GGTCAGGCTCAGGCTGATGT-3′ [6]	186	to GM
	PEPC-C-20	5′-ATCTCGCTTCCGTGCTTAGC-3′ [7]		
MON810	mg1	5′-TATCTCCACTGACGTAAGGGATGAC-3′ [7]	401	to GM
	mg2	5′-TGCCCTATAACACCAACATGTGCTT-3′ [7]		
	VM01	5′-TCGAAGGACGAAGGACTCTAACG-3′ [8]	170	to TE
	VM03	5′-TCCATCTTTGGGACCACTGTCG-3′ [8]		
MON88017	M7F8	5′-CGCCAAGTCCAAGGCCCTGG-3′ [8]	313	to gene *cry3Bb1*
	M7R8	5′-CGCCAAGTCCAAGGCCCTGG-3′ [8]		
	MON88017-mF	5′-ATCGTGTGACAACGCTAGCA-3′ [9]	150	to TE
	MON88017-mR	5′-CATATTGACCATCATACTCATTGCT-3′ [9]		
DAS1507	TC1507 01-5	5′-GCTTCAACAGGGCTGAGTTTG-3′ [7]	103	to GM
	TC1507 01-3	5′-CCCCACACAGTTTGGGATCTA-3′ [7]		
	TC1507-1F	5′-CTTGTGGTGTTTGTGGCTCT-3′ [7]	279	to TE
	TC1507-2R	5′-TGGCTCCTCCTTCGTATGT-3′ [7]		
DAS59122	59F1	5′-GCACCTCCCCGACCAACGTG-3′ [7]	148	to GM
	59R1	5′-CCGGCGAACGGGTTGTCGAA-3′ [7]		
	SEQ ID NO 9	5′-CTCCTTCAACGTTGCGGTTCTGTCAG-3′ [10]	313	to GM
	SEQ ID NO 10	5′-TTTTGCAAAGCGAACGATTCAGATG-3′ [10]		
MIR604	M6F1	5′-CGCCATCAGCGGCTACGAGG-3′ [7]	268	to gene *mcry3A*
	M6R1	5′-GGTCATCTCGCGGCGGTAGC-3′ [7]		
	E-604-F	5′-TGGACGCCAGATCACACATG-3′ [7]	133	to TE
	E-604-R	5′-GGTCATAACGTGACTCCCTTAATTCT-3′ [7]		
GA21	esGA21-5’ F	5′-CGTTATGCTATTTGCAACTTTAGAACA-3′ [11]	101	to TE
	esGA21-5’ R	5′-GCGATCCTCCTCGCGTT-3′ [11]		
NK603	NK603F	5′-ATGAATGACCTCGAGTAAGCTTGTTAA-3′ [12]	108	to TE
	NK603R	5′-AAGAGATAACAGGATCCACTCAAACACT-3′ [12]		
	SEQ ID NO 13	5′-AATCGATCCAAAATCGCGACTG-3′ [9]	501	to TE
	SEQ ID NO 14	5′-TTCACTTTGGGCCACCTTTAT-3′ [9]		
Bt11	Bt11-1	5′-TATCATCGACTTCCATGACCA-3′ [7]	207	to TE
	Bt11-2	5′-AGCCAGTTACCTTCGGAAAA-3′ [7]		
	IVS2-2	5′-CTGGGAGGCCAAGGTATCTAAT-3′ [13]	189	to GM
	PAT-B	5′-GCTGCTGTAGCTGGCCTAATCT-3′ [13]		
MON863	P863-3F	5′-GGCGATGAATAAATGAGAAATA-3′ [9]	200	to TE
	P863-4R	5′-TAGCCAGTTCATTGCGAGTA-3′ [9]		
	84_18-L	5′-GATGACCTGACCTACCAGA-3′ [7]	234	to GM
	84_18-R	5′-GCACACACATCAACCAAATT-3′ [7]		
MON89034	M8F2	5′-TTGGGGTGGAAGCACCGGGA-3′ [7]	713	to gene *cry1A.105*
	M8R2	5′-GCACACGTTTGTCTGCGGCG-3′ [7]		
T25	T25-F7	5′-ATGGTGGATGGCATGATGTTG-3′ [14]	209	to GM
	T25-R3	5′-TGAGCGAAACCCTATAAGAACCC-3′ [14]		

Note: GM = genetic maker; TE = transformation event.

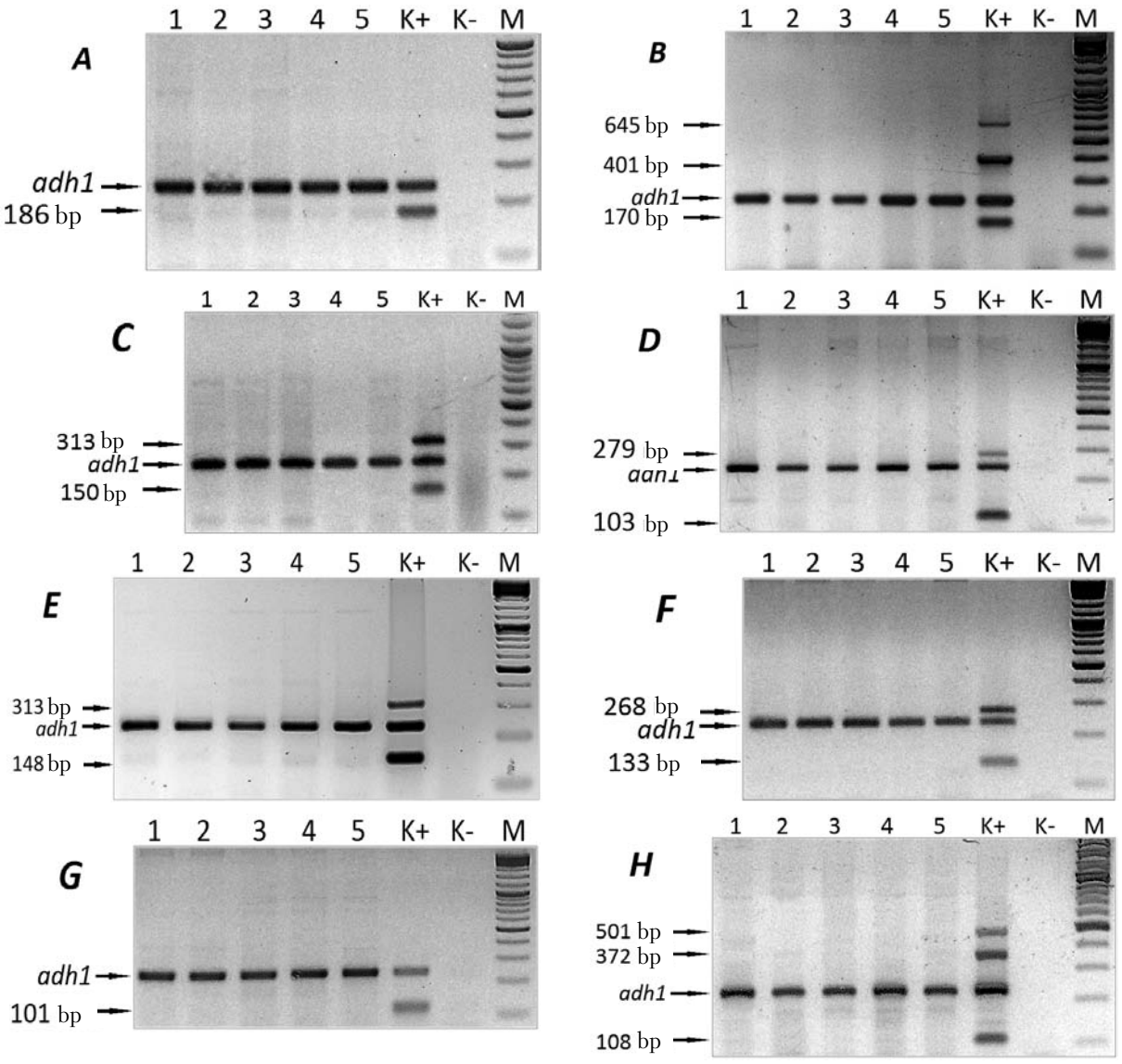

Fig. 1. Electrophoretograms of amplification products of multiplex PCR for detection of transformation events: tracks 1–5 correspond to samples; K+ is the positive control (reference sample); K- is the negative control (the TE buffer instead of the DNA sample); M is O'GeneRuler ™ DNA Ladder Mix (Thermo Scientific) molecular weight marker. Amplicon *adh1* (231 bp) is the reference one for all the reactions

denaturation of DNA during 30 seconds at a temperature of 94 °C.

The temperature of renaturation of DNA with oligonucleotide primers and the time of synthesis of fragments of target genes for each transformation event were selected individually (Table 3). The DNA renaturation time was 30 seconds, temperature of synthesis of fragments of target genes was 72 °C. The final synthesis in all the reactions lasted 10 minutes, at a temperature of 72 °C.

The mPCR products are the fragments of *adh1* maize reference gene having a size of 231 base pairs (bp) and the target fragments of DNA genes /

transformation events / genetic makers with a certain characteristic length (see Table 2, Fig. 1).

A. BT176. The DNA fragment having a size of 186 bp consists of a part of RERS promoter and a part of cry1A (b) gene of genetic maker.

B. MON810. The DNA fragment of 170 bp consists of flanking fragment of vegetable DNA and DNA fragment of genetic maker; the DNA fragment of genetic maker has a size of 401 bp; the fragment having a length of 645 bp is formed as a result of the synthesis of fragment limited by primers from different pairs (VM01 and mg2), which testifies to the transformation event MON810 in DNA sample.

C. MON88017. The DNA fragment having a size of 150 bp consists of flanking fragment of vegetable DNA and DNA fragment of genetic maker; the gene encodes sequence of *cry3Bb1* gene having a size of 313 bp.

D. DAS1507. The DNA fragment having a size of 279 bp consists of flanking fragment of vegetable DNA and DNA fragment of genetic maker; the DNA fragment of genetic maker has a size of 103 bp.

E. DAS59122. The DNA fragment having a size of 148 bp consists of a part of cry34Ab1 gene; the DNA fragment having a length of 313 bp consists of 5'-flanking fragment of vegetable DNA and DNA fragment of genetic maker.

F. MIR604. The DNA fragment having a size of 133 bp consists of flanking fragment of vegetable DNA and DNA fragment of genetic maker; the *cry3A* gene fragment of genetic maker has a length of 268 bp.

G. GA21. The DNA fragment having a size of 101 bp consists of fragment of vegetable DNA and fragment of actin promoter of genetic maker.

H. NK603. The DNA fragment having a size of 501 bp consists of 5'-flanking fragment of vegetable DNA and DNA fragment of genetic maker; the DNA fragment having a size of 108 bp consists of 3'-flanking fragment of vegetable DNA and DNA fragment of genetic maker; the fragment having a length of 372 bp is formed as a result of synthesis of fragment limited by primers from different pairs (SEQ ID NO 13 and NK603F), which bears additional witness to transformation event NK603 in DNA sample.

Fig. 1 shows that for the reference samples the obtained amplicons correspond to the expected ones given in Table 2. In the studied samples of maize experimental forms there has been observed only *adh1* reference gene of maize indicating the presence and quality of vegetable DNA in the studied samples and the absence of transgenes or transformation event in genetic material of plants. In the reference samples there have been observed both the *adh1* maize marker gene and the amplicons of expected sizes. This fact proves the adequacy of mPCR conditions and the presence of transformation events. The negative control does not contain any fragments, which proves the absence of contamination of DNA reagents and the adequate quality of reaction.

In some cases the technique for detection of transformation events with the use of mPCR did not yield precise and reliable results. Therefore,

Table 3

Renaturation temperature of DNA with oligonucleotide primers and time of synthesis of fragments of target genes in mPCR

Transformation event	DNA renaturation temperature, °C	Time of synthesis of fragments of target genes, s	Transformation event	DNA renaturation temperature, °C	Time of synthesis of fragments of target genes, s
BT176	55	18	DAS59122	59	22
MON810	58	27	MIR604	58	19
MON88017	55	22	GA21	56	17
DAS1507	55	20	NK603	56	33

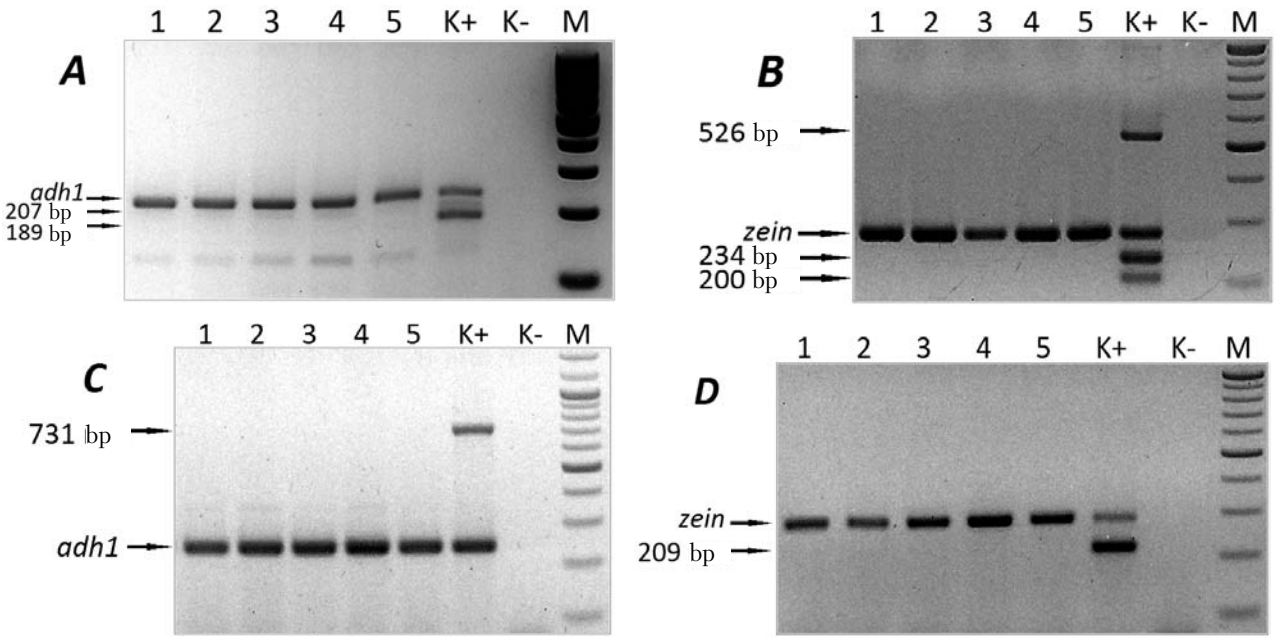

Fig. 2. Electrophoretograms of amplification products of touchdown PCR for detection of transformation events: tracks 1—5 correspond to samples; K+ is the positive control (reference sample); K- is the negative control (the TE buffer instead of the DNA sample); M is O'GeneRuler™ DNA Ladder Mix (Thermo Scientific) molecular weight marker. Amplicons of *adh1* (231 bp) and *zein* (277 bp) are the reference ones

the touchdown PCR technique (for transformation events Bt11, MON863, MON89034, and T25 and the constituent transgenes) has been developed. The distinctive feature of touchdown PCR technique is that the initial annealing temperature is up by 15 °C than the predicted melting temperature of primers. During 15 consecutive cycles the annealing temperature gradually decreases by 1 °C to the optimal one for given pair of primers. The further series of 21 runs takes place at the optimal temperature providing an exponential increase in quantity of target amplicon only. The initial growth of annealing temperature ensures only the specific binding of primers to the DNA matrix, at almost the total absence of synthesis of nonspecific fragments. Due to a wide range of temperatures used the touchdown PCR can provide the optimal course of reaction even for the matrices which are too complicated for amplification [16].

When detecting the majority of transgenes or transformational events the *adh1* gene is used as reference gene. Amplicons of events MON863 and T25 have a size of 234 bp and 209 bp respectively, i.e. they are similar in size to the amplicon *adh1* (231 bp), which complicates their identification and separation by gel electrophoresis. In this regard, it has been decided to use the *zein* gene (277 bp) as reference one (see Table 2).

The touchdown PCR program for transformation events BT11, MON863, MON89034, and T25 was as follows: denaturation of vegetable DNA during 4 minutes at a temperature of 94 °C; 15 cycles were carried out, each of them consisted of denaturation of DNA during 30 seconds at a temperature of 94 °C. The temperature of renaturation of DNA with oligonucleotide primers and the time of synthesis of fragments of target genes for each transformation event were established individually (Table 4).

Thereafter 21 cycles were performed, each of them consisted of denaturation of DNA during 30 seconds at a temperature of 94 °C. The temperature of renaturation of DNA with oligonu-

cleotide primers and the time of synthesis of fragments of target genes for each transformation event were established individually. The time of renaturation of vegetable DNA with oligonucleotide primers specific for the two stages was 30 seconds, and the temperature of synthesis of fragments of target genes was 72 °C. The final synthesis lasted 10 minutes at a temperature of 72 °C.

The products of touchdown PCR fragments are the fragments of *adh1* maize reference gene having a size of 231 bp or the *zein* reference gene having a size of size 277 bp and the target fragments of DNA genes / transformational events / genetic makers having a characteristic length as given in Table 2 and Fig. 2.

A. BT11. The DNA fragment having a size of 189 bp consists of fragment of *adh1*- enhancer and fragment of *pat* gene in genetic maker by which the transformation is realized; the DNA fragment having a size of 207 bp consists of 5'-flanking fragment of vegetable DNA and fragment of DNA of genetic maker by which the transformation is realized.

B. MON863. The DNA fragment having a size of 200 bp consists of flanking fragment of vegetable DNA of genetic maker; the DNA fragment of genetic maker has a size of 234 bp; the fragment having a length of 526 bp is formed from the synthesis of fragment limited by primers from different pairs (84_18-L and P863-4R), which bears additional witness to transformation event MON863 in selected DNA sample.

C. MON89034. The DNA fragment having a size of 713 bp consists of part of *cry1A.105* gene of genetic maker.

D. T25. The DNA fragment having a size of 209 bp consists of part of *pat* gene and 35S fragment of genetic maker terminator.

In Fig. 2 one can see that the obtained amplicons correspond to the expected ones, as illustrated in Table 2. In the studied samples of maize experimental forms there have been observed only *adh1* or *zein* reference genes, which indicates the presence and sufficient quality of vegetable DNA in the samples and the absence of transgenes or transformative events in the genetic material of plants. The reference samples contain both the *adh1* or *zein* maize marker genes and the amplicons of expected size, which means that the selected conditions of touchdown PCR have been set adequately and that the transformational events take place. The negative control does not contain any fragments, which testifies to the absence of DNA reagent contamination and the adequate quality of reaction.

ANALYSIS OF MAIZE EXPERIMENTAL FORMS FOR THE PRESENCE OF GENES DETERMINING GRAIN QUALITY CHARACTERISTICS (WX AND FL-2)

Detection of *wx* and *fl-2* genes has been performed according to the protocol outlined above. For this the selection and design of specific primers (see Tables 3 and 4) have been made. The *wx* gene has been detected by multiplex PCR while

Table 4

Renaturation temperature of DNA with oligonucleotide primers and time of synthesis of fragments of target genes in touchdown mPCR

Transformation event	Number of cycles	DNA renaturation temperature, °C	Time of synthesis of fragments of target genes, s	Transformation event	Number of cycles	DNA renaturation temperature, °C	Time of synthesis of fragments of target genes, s
BT11	1—14	65	17	MON89034	1—14	65	46
	15—35	51	17		15—35	50	46
MON863	1—14	66	27	T25	1—14	65	20
	15—35	52	27		15—35	50	20

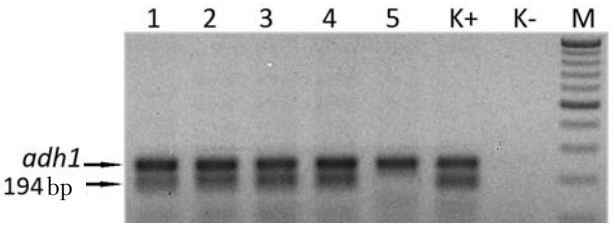

Fig. 3. Electrophoretograms of wx gene amplification products: tracks 1—5 correspond to samples; K+ is the positive control (reference sample); K- is the negative control (the TE buffer instead of the DNA sample); M is O'GeneRuler™ DNA Ladder Mix (Thermo Scientific) molecular weight marker. Reference amplicon of *adh1* gene (231 bp)

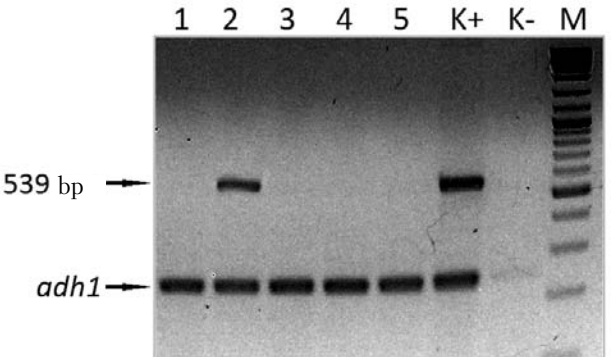

Fig. 4. Electrophoretograms of fl-2 gene amplification products: tracks 1-5 correspond to samples; K+ is the positive control (reference sample); K- is the negative control (the TE buffer instead of the DNA sample); M is O'GeneRuler™ DNA Ladder Mix (Thermo Scientific) molecular weight marker. Reference amplicon of *adh1* gene (231 bp)

the *fl-2* gene has been detected with the use of touchdown PCR.

To identify the *wx* gene 20 ml of reactive mixture has been prepared. It consists of 1.DreamTaq™ Green buffer, 0.75 units of DreamTaq™ (Thermo Scientific) polymerase, 200 μM of dNTF, 30 ng of vegetable DNA sample, 0.2 μM of primer ADH F3, 0.2 μM of primer ADH R1, 0.35 μM of primer W4F, and 0.35 μM of primer W4R (Tables 5 and 6). The negative control is the reactive mixture of 1 μl of TE buffer with pH 8.0 instead of the DNA sample. The positive control is the maize DNA with *wx* gene.

The composition of reactive mixture having a volume of 20 ml to detect the *fl-2* gene is as follows: 0.35 μM of primer FL2F, 0.35 μM of primer FL2R, 0.2 μM of primer ADH F3, 0.2 μM of primer ADH R1, 1.DreamTaq™ Green buffer, 0.75 units of DreamTaq™ polymerase (Thermo Scientific), 200 μM of dNTF, and 30 ng of DNA sample. The negative control is the reactive mixture of 1 μl of TE buffer with pH 8.0 instead of the DNA sample. The positive control is the maize DNA containing the *fl-2* gene.

The mPCR program for detecting the *wx* gene is as follows: denaturation of vegetable DNA during 4 minutes at a temperature of 94 °C; 35 cycles are carried out, each of them involves DNA denaturation during 30 seconds at a temperature of 94 °C, renaturation of vegetable DNA with oligonucleotide primers during 30 seconds at a temperature of 50 °C, the synthesis of fragments of target genes during 20 seconds at a temperature of 72 °C, and the final synthesis during 10 minutes at a temperature of 72 °C.

The touchdown PCR program for the presence of *fl-2* gene is as follows: denaturation of vegeta-

Table 5

List of primers, their melting temperatures, and annealing temperatures in PCR

Gene	Primer	Primer concentration in reacting mixture for PCR, μM	Melting temperature, °C	Annealing temperature, °C
Reference gene *adh1*	Adh-F3	0,20	66	55
	Adh-R1	0,20	60	
wx	W4F	0,35	56	51
	W4R	0,35	56	
fl-2	FL2F	0,35	68	63
	FL2R	0,35	69	

Table 6

Nucleotide sequences of primers to the *wx* and *fl-2* genes

Gene	Primer	Nucleotide sequence of primer	Amplicon size, bp	Specificity
Reference gene *adh1*	Adh-F3 Adh-R1	5′-CGTCGTTTCCCATCTCTTCCTCC-3′ [5] 5′-GACAGAGGAGAAACAAGGCG-3′ [5]	231	To *adh1* gene
wx	W4F W4R	5′-AATAATCCCTGCTGTTCGGT-3′ [17] 5′-CAGCTTTTGGTGGCCAGA-3′ [17]	189	To *wx* gene
fl-2	FL2F FL2R	5′-GCCCTTTTAGTGAGCGCAACAAATGTG-3′ [18] 5′-GCAGGGTTTGCCATAGCTAGCTGATG-3′ [18]	539	To *fl-2* gene

ble DNA during 4 minutes at a temperature of 94 °C; 14 cycles are carried out, each of them involves DNA denaturation during 30 seconds at a temperature of 94 °C, renaturation of vegetable DNA with oligonucleotide primers during 30 seconds at a temperature of 66 °C (during each cycle the temperature decreases by 1 °C), and the synthesis of fragments of target genes during 36 seconds at a temperature of 72 °C. 21 cycles are carried out, each of them includes DNA denaturation during 30 seconds at a temperature of 94 °C, renaturation of vegetable DNA with oligonucleotide primers during 30 seconds at a temperature of 52 °C, and the synthesis of fragments of target genes during 36 seconds at a temperature of 72 °C; the final synthesis of target gene fragments lasts 10 minutes at a temperature of 72 °C.

The PCR products are the fragments of target genes with the following characteristic length: the fragment of *adh1* maize gene has a size of 231 bp; the fragment of *wx* maize gene's own allele has a size of 194 bp (when detecting the *wx* gene); the DNA fragment having a size of 539 bp is a part of 24-kDa α-*zein* gene (when detecting the *fl-2* gene) (see Table 4 and Figs. 3, 4).

In Figs. 3 and 4 one can see the amplicons corresponding to the *adh1* reference gene of maize having a size of 231 bp. This proves the high quality of extraction of maize DNA and PCR reaction. On the track of the positive control there is showed an amplified fragment of the target sequence having a size of 194 bp (Fig. 3) and 539 bp (Fig. 4). The amplicon having a size of 194 bp has been observed in the majority of samples (Fig. 3, tracks 1—4), while the amplicon having a size of 539 bp (Fig. 4, track 2) is much rarer. The *wx* gene is very common in maize experimental samples: it has been found in 192 cases of analyzed samples, whereas the *fl-2* gene has been detected in 41 selective samples.

CONCLUSIONS

Hence, the methods of multiplex PCR and touchdown PCR developed within the framework of this research allow the researchers to detect the genes of resistance to stress factors (*pat (bar), CP4 epsps, mepsps, cry1A (b), cry1F, cry1A.105, cry2Ab2, cry3Bb1, cry34Ab1, cry35Ab1,* and *mcry3A*), which are the constituents of transformational events (Bt11, Bt176, DAS1507, GA21, MON810, MON863, NK603, T25, DAS59122, MON89034, MON88017, and MIR604) in a safe, reliable, efficient, and quick manner. The methods have been used for analyzing a huge amount of maize samples.

Among the 200 maize experimental forms of *Maize Company* farm holding studied with respect to the presence of genes determining resistance to stress factors, none of transgenes (*bar (pat), mepsps, CP4 epsps, cry1A (b), cry1F, cry1A.105, mcry3A, cry2Ab2, cry3Bb1, cry34Ab1, cry35Ab1*) has been found. The study of these forms with respect to the presence of genetic sequences determining the quality characteristics of grain the

fl-2 gene has been detected in 41 selective samples, while the *wx* gene has been identified in 192 analyzed samples.

The research has been carried out within the framework of research and technological project II-4-12, «Detection of Genetic Sequences Determining the Grain Quality Characteristics and Resistance to Stress Factors in Maize» (registration number 0112U002802, 2012). Upon the results of research the patents of Ukraine for useful model have been obtained [19, 20].

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ОБНАРУЖЕНИЕ ГЕНОВ, ДЕТЕРМИНИРУЮЩИХ КАЧЕСТВЕННЫЕ ХАРАКТЕРИСТИКИ ЗЕРНА И УСТОЙЧИВОСТЬ К СТРЕССОВЫМ ФАКТОРАМ, У КУКУРУЗЫ

Осуществлена оценка 200 экспериментальных форм кукурузы НПФХ «Компания "Маис"» относительно наличия генов, детерминирующих качественные характеристики зерна (гены *wx* и *fl-2*), устойчивость к гербицидам (гены *bar (pat), epsps*) и насекомым *(cry*-гены). Проведено выделение общей ДНК из живой растительной ткани кукурузы, подбор и дизайн праймеров для обнаружения генов *wx*, *fl-2*, *bar (pat)*, *mepsps*, CP4 *epsps*, *cry1A(b)*, *cry1F*, *cry1A.105*, *mcry3A*, *cry2Ab2*, *cry3Bb1*, *cry34Ab1*, *cry35Ab1*. Разработаны методики мультиплексной и нисходящей (Touchdown) полимеразной цепной реакции (ПЦР) и проведена амплификация определенных последовательностей. Среди проанализированных 200 экспериментальных форм кукурузы НПФХ «Компания "Маис"» ни одного трансгена (*bar (pat)*, *mepsps*, CP4 *epsps*, *cry1A(b)*, *cry1F*, *cry1A.105*, *mcry3A*, *cry2Ab2*, *cry3Bb1*, *cry34Ab1*, *cry35Ab1*) обнаружено не было, в то время как ген *fl-2* найден в 41-м образце, ген *wx* выявлен в 192-х проанализированных образцах.

Ключевые слова: кукуруза, трансгены, качественные характеристики зерна, праймеры, полимеразная цепная реакция (ПЦР).

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ВИЯВЛЕННЯ ГЕНІВ, ЯКІ ДЕТЕРМІНУЮТЬ ЯКІСНІ ХАРАКТЕРИСТИКИ ЗЕРНА ТА СТІЙКІСТЬ ДО СТРЕСОВИХ ФАКТОРІВ, У КУКУРУДЗИ

Здійснено оцінку 200 експериментальних форм кукурудзи НВФГ «Компанія "Маїс"» щодо наявності генів, які детермінують якісні характеристики зерна (гени wx та fl-2) та стійкість до гербіцидів (гени bar (pat), epsps) і комах (cry-гени). Проведено виділення загальної ДНК з живої рослинної тканини кукурудзи, добір та дизайн праймерів для виявлення генів wx, fl-2, bar (pat), mepsps, CP4 epsps, cry1A(b), cry1F, cry1A.105, mcry3A, cry2Ab2, cry3Bb1, cry34Ab1, cry35Ab1. Розроблено методики мультиплексної та низхідної (Touchdown) полімеразних ланцюгових реакцій (ПЛР) та проведено ампліфікацію визначених послідовностей. Серед проаналізованих 200 експериментальних форм кукурудзи НВФГ «Компанія "Маїс"» жодного трансгена (bar (pat), mepsps, CP4 epsps, cry1A(b), cry1F, cry1A.105, mcry3A, cry2Ab2, cry3Bb1, cry34Ab1, cry35Ab1) виявлено не було, у той час як ген fl-2 знайдено у 41-му зразку, ген wx — у 192-х проаналізованих зразках.

Ключові слова: кукурудза, трансгени, якісні характеристики зерна, праймери, полімеразна ланцюгова реакція (ПЛР).

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