Theor. Appl. Genet. 58, 225-235 (1980)

THEORETICAL AND APPLIED GENETICS

Hereditary Variability of Plants Under the Action of Exogenous DNA

V.N. Soyfer

Department of Plant Molecular Biology, Institute of Applied Molecular Biology and Genetics, Moscow (USSR)

Summary. Injection of exogenous barley donor DNA into grains of barley recipient plants at the milk maturity stage, with a specially designed syringe, led to the appearance of transformed plants. The transformation (in rare cases) was caused by the unsheared DNA since the DNA passing through the syringe needle remained relatively stable (10^6 to 10^7 daltons) as was confirmed by DNA sedimentation analysis.

14 plants grown from seeds injected with highly polymeric DNA containing close to 30 per cent protein had transformed pollen grains. In the 2nd generation only 2 plants from the 8 studied preserved these changes. In the progeny of these two plants, i.e., in the 3rd seed generation after injection, 82.1 per cent of plants preserved the transformed characters. The next, 4th generation, preserved a transformed phenotype in 89.6 per cent of plants.

It was also shown that reversion to a recipient-like state was not always constant. We found the reversion of transformed properties (i.e., normal starch and two-rowed spikes) in 40 per cent of the 4th generation descendants of one of the plants which had lost the phenotypical expression of these properties in the 3rd generation but had them in the 2nd generation.

The study of the morphological properties of transformed plants showed that with respect to phenotypic expression some characters were changed towards the donor type, some remained as in the recipients and some were of the intermediate type.

Key words: Genetic transformation – Reversion – Barley – Unfractionated DNA – Pedigree analysis

Introduction

Attempts to induce genetic transformation in higher plants have been undertaken in recent years. Such investigations with exogenous DNA have been performed both at the level of whole plants and of isolated protoplasts. After exogenous DNA is added to germinating seeds a correction of different mutations can be observed. Thus, the treatment of the seeds of a white-flowering pure mutant line of *Petunia hybrida* unable to synthesize anthocyanin with the DNA isolated from a red-flowering true-breeding pure line of the same species resulted in a genetically stable correction of anthocyanin synthesis (Hess 1969). Correction of nutritional mutants of *Arabidopsis thaliana* (deficient in the synthesis of the pyrimidine moiety of thyamine and thiazole) was achieved in experiments with *Arabidopsis* seeds treated with bacterial but not bacteriophage DNA (Ledoux et al. 1974). Similar effects were obtained with seeds of *Capsicum annuum* (Nawa et al. 1975) and with seeds and shoot tips of *Pisum* (Holl et al. 1974), however, the details of these two groups of experiments were not described.

Of some interest was an observation of a simultaneous transformation of more than one character in changed plants. In experiments with *Petunia* a change of the leaf shape was found together with a correction of anthocyanin synthesis (Hess 1970) while in the experiments by Holl et al. (1974) with a *Pisum* mutant which was unable to form nodules or to fix nitrogen, double correction for both characteristics was achieved.

Another interesting result of the above-mentioned works is the absence of an F_2-segregation by selfing of transformed plants both in experiments with *Petunia* and with *Arabidopsis* and *Pisum*.

Earlier we have reported that injection of wild type barley DNA into grains of *waxy* barley mutants at the milk stage maturity induces locus-specific changes (Turbin et al. 1973; Soyfer and Turbin 1974). These changes have been demonstrated only with DNA preparations isolated from milk material (Soyfer et al. 1974), and the DNA from barley leaves, *Escherichia coli* DNA, saline buffer, distilled water, and mechanical puncture of grains were

0040/5752/80/0058/0193/$ 2.20

found to be ineffective (Turbin et al. 1975b). The frequency of changes was the highest in the case of slightly deproteinized highly polymeric DNA (Turbin et al. 1975b), and the isogenic DNA isolated from the *waxy* mutant and then injected into grains of the same strain of barley did not result in transformation (Turbin et al. 1975a). The changes of the *waxy* character were heritable in a portion of the progeny of the initially altered plants (Soyfer et al. 1976a) although many of initially changed plants reverted into a recipient state in the second and later generations. Beginning with the second generation those plants which conserved the *waxy* character (alteration of the starch structure) also revealed such other alterations as the type of spike morphology (hexastichous plants became distichous as it was in the donor plants). Besides, delayed manifestation of the synthesis of normal starch was noted in successive generations. Certain plants which did not show any changes in the first generation yielded wild-type pollen grains in the second generation (Soyfer et al. 1976a). Further analysis of transformed plants have revealed that plants with altered starch and spike morphology are characterized by specific electrophoretic changes in the alcohol soluble fraction of seed storage proteins (hordeins), as was found with polyacrylamide gel electrophoresis (Soyfer et al. 1978).

In the present communication we present the further analysis of the transformed plants.

Material and Methods

Study on Degradation of DNA During its Injection into Grains Through a Syringe

To check up on the possible degradation of the transforming DNA during its injection into barley grains, with the aid of a syringe, we performed experiments with T4D bacteriophage DNA. *Escherichia coli K12* (strain AB 2500) cells were grown in 100 ml of M-9 medium supplemented with 5 g casamino acid (Difco), 10 g casein hydrolyzate (Difco), and 1 μCi/ml of ^{3}H-thymidine (spec. act. 485 mCi/mM, 5-methyl-^{3}H-TdR) to a titer of 10^8 cells/ml. The bacteria were infected with prelabelled phage at a multiplicity of infection of 0.1. After 4 hours incubation bacteria were treated with 3 μg/ml indole, followed 30 min later with chloroform to lyse bacteria. The resultant suspensions were centrifuged for 10 min in a SW-27 rotor at 13,000 r.p.m. in a Spinco L2-65B centrifuge (Beckman); the supernatant was twice centrifuged for 60 min at 17,000 r.p.m. in a SW-27 rotor; the pellet was resuspended in 0.5 ml 0.1 M NaCl at neutral pH and cleaned at 5,500 r.p.m. for 15 min. The phage was purified by gel-chromatography: the phage suspension was added to a column with Biogel P-300 (Calbiochem, 100 to 200 mesh, 1 cm × 10 cm) and eluted with 0.1 M NaCl (pH 6.3). Ten-drop fractions were collected. The phage was found in fractions 5 to 6. In some experiments the purification included not only the gel-filtration procedure but also CsCl centrifugation. 0.5 ml of the purified phage suspension (10^{12} particles/ml) was incubated with 1 mg/ml of pronase (Calbiochem) for 1 hr at 37°C and then transferred into a plastic tube together with an equal volume of freshly recrystallized phenol saturated with 0.15 M NaCl and containing 0.015 M EDTA, pH 8.6. A plastic tube was attached to the axis of a peristaltic pump (LKB, Sweden) and rotated with at a low speed around the axis (gear-box 1:200) for 1.5 hr at 4°C, the angle between the tube and horizontal line being equal to 30°. The water phase was gently removed with a wide plastic tubing (diameter 2.5 mm) connected to the peristaltic pump and poured into a laboratory-made syringe which enabled the slow injection of the DNA (0.5 to 1.0 μl) through the needle into the grains of barley at milk maturity stage. Fractions of these DNA preparations (0.075 ml) were layered at a low speed (0.1 ml/min) either through the syringe needle or without it onto a 5 to 20 per cent gradient of sucrose containing 0.1 M NaCl; 0.01 M Tris and 0.01 M disodium salt of EDTA. One ml of 28.7% sucrose served as a 'cushion'. Centrifugation was performed in a SW-50.1 rotor at 18°C for 55 min. at 35,000 r.p.m. Radioactivity of fractions was measured in a Mark II (Nuclear Chicago) counter using a dioxane scintillator. All syringes, centrifuge tubes, and tubings were pretreated with sodium dodecylsulfate.

Plants Used for Transformation

We used wild type barley (Yuzhny var.) and a *waxy* mutant of spring barley obtained by Dr. G. Eriksson (Sweden) and kindly supplied to Prof. N.V. Turbin by Prof. A. Gustafsson from Lund University, Sweden. The starch of the *waxy* mutant contains only amylopectin. The description of the original lines of donor and recipient plants, the methods of isolation of DNA and their study, as well as the methods of injection of the DNA into grains at milk stage of maturity, the methods of pollen analysis, and other used methodology are described elsewhere (Turbin et al. 1973; 1975).

Study of Transformants

Following DNA injection into all fertile grains, spikes (infertile spikelets were removed) were bagged and left on the field until complete maturation. The seeds of each spike were harvested and stored separately. The following year all the grains were sown and pollen of one anther from each flower was analyzed. After removing the anthers from the flowers of the spike the latter was bagged. The pollen was stained by a solution of iodine in potassium iodide and examined under a light microscope. Mutant pollen became brown after this staining (or red-brown) whereas the pollen from the wild type plants used as a source for DNA isolation was characterized by black staining. In some cases we found black pollen in plants grown from mutant seeds injected with wild type DNA preparations. These plants were referred to as presumably transformed ones (F_0-generation) and we continued their analysis in following generations (Fig. 1). All seeds of changed spikes were grown separately and the resulting F_1-plants were allowed to self-pollinate to produce F_2-seeds. Pollen staining was studied at the stage of flower opening, and the F_2-seeds of each spike were harvested and stored separately. The same method of analysis was used for further seed generations.

Results

Study on Possible Degradation of the DNA When Injected into Plant Seeds

Usually in experiments involving genetic transformation of plants seeds or other plant material, the material is

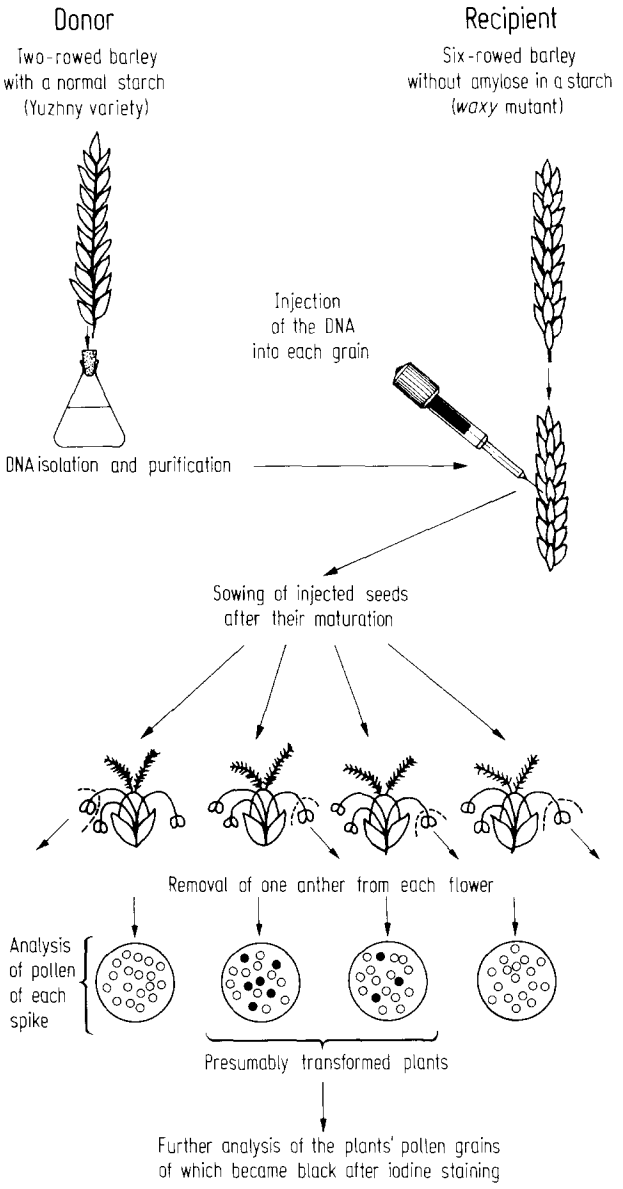

Fig. 1. The outline of transformational experiments

treated in DNA solutions. In these experiments we injected DNA into grains of barley at the milk maturity stage. It was done to facilitate the DNA penetration into embryonic cells since at this stage the embryos contain only a few cells (some dozen), and all these cells are actively dividing. It cannot be excluded also that embryonic cells at this stage might be more competent or susceptible to exogenous DNA due to marked physiological differences between this and other stages of plant development.

Thus, the injection of exogenous material into endosperm at this stage of maturity might lead to more effective transformation but it could also possibly lead to a degradation of the DNA due to hydrodynamic shear in the needle of the syringe or during injection. To avoid such a degradation, a special syringe was designed. This syringe allowed the injection of very small amounts of DNA (0.5 to 1.0 μl) into each grain. Therefore, the speed of DNA injection was markedly reduced and hydrodynamic shear was expected to be eliminated. To check this we studied both double- and single-stranded breaks in DNA by appropriate sucrose gradients. We have found similar sedimentation profiles (Fig. 2) both for the DNA injected through a needle and injected without the needle. This means that the designed syringe either did not increase the amount of breaks in DNA injected or the number of breaks was too insignificant to be revealed by ultracentrifugation. Hence, the transformation capacity of DNA preparations used may relate to the preparations per se but not to degradation products.

Study on Genetic Stability of Changes Obtained by Exogenous DNA Injection

Preparations with pollen containing one to three or more iodine black staining grains per slide (containing, on the average, 1240 pollen grains) have been found in some plants of the first generation after injection. Since the expression of the *waxy* gene was investigated at a haploid level, the effect of dominance (complete or partial) was excluded. In total, 82 plants with changed pollen was

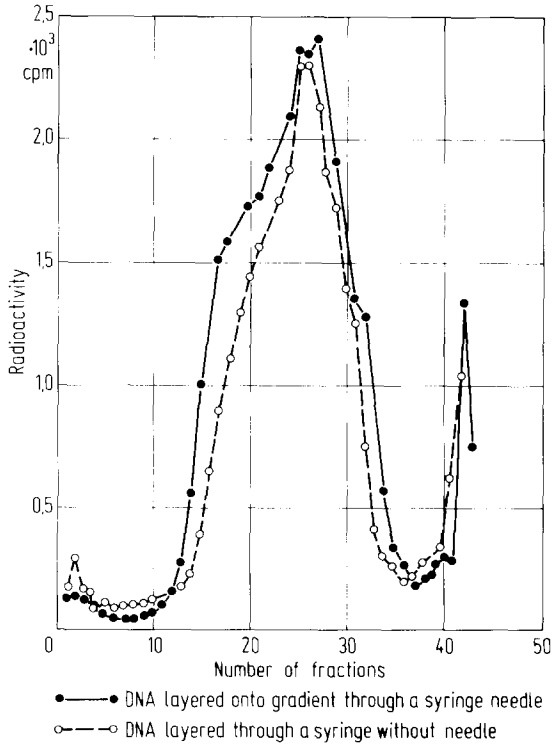

Fig. 2. Linear sucrose alkaline gradient sedimentation of *Escherichia coli* DNA

Table 1. Analysis of characteristics of plants Nos. 506/19 and 506/22 transformed by exogenous wild type DNA

Character	Characteristics of transformed plants and their progeny	
	The 1st seed generation (e.g. plant 506)	The 2nd seed generation (e.g. plant 506/19 and 506/22)
Pollen stained with a solution of iodine	95 to 99.5 per cent of pollen became black; remainder remained red	87 per cent of pollen became black. The distribution of plants is the following: one half of the pollen are black; in one-third there are mixed pollen (99 per cent is black and 1 per cent is red); and about 18 per cent reverted to a recipient (mutant) state
Amount of seed rows in a spike	All plants are hexastichous like a recipient	Transformed plants are distichous while the revertants are hexastichous
Electrophoretic patterns of reserve proteins in grains (hordeins)	Not studied	The patterns of proteins in transformants differed from those in both donor and recipient whereas the patterns of the revertants were similar to those in recipient plants
Molecular structure of starch in grains	Transformed plants had starch similar to that in donor plants	Transformed plants had a starch similar to that in donors while the revertants had a starch similar to the recipient one

found after the DNA injection in the F_0-generation. Among them 14 plants were received after the injection of slightly deproteinized and highly polymeric DNA from endospermal milk. For further analysis 8 plants have been used, and only two plants, 506/19 and 506/22 had a large proportion of changed pollen cells in the first and second generations. We studied the characteristics of these plants in the next generations.

Earlier it has been found (Soyfer et al. 1976a) that many of the 82 plants initially characterized by a small amount of donor-type changed pollen grains lost these changes in the second generation. However, after self-pollination the progeny of the above-mentioned two plants in which 95 to 99.5 per cent of pollen was changed already in the first self-pollinated generation preserved these changes in the second self-pollinated generation (Table 1).

In the first generation of these plants, up to 99.5 per cent of pollen was stained black. In the second generation such black staining pollen amounted to 87 per cent (of 654,710 pollen grains examined 83,543 were of the recipient type), and segregation for the *waxy* character had taken place. In this generation half of all plants produced only wild-type pollen. In about one-third of the plants the proportion of 'black' and 'red' pollen grains was the same as in the first generation (over 99 per cent became black-staining and only individual grains had the colour of the mutant plants, i.e., recipients), and nearly 18 per cent of plants reverted to the original recipient type. Similar results were obtained in the progeny of plant No. 506/22.

Thus, these experiments demonstrated the stable fixation of changed characteristics in a significant proportion of the progeny of those plants which initially showed almost complete transformation of the *waxy* character induced by exogenous wild type DNA.

Pleiotropic Action of Exogenous DNA

Barley plants used for isolation of the donor DNA had distichous spikes. Recipient plants were of the hexastichous type. In the first generation all plants grown from the injected seeds (characterized by both changed and unchanged pollen) had hexastichous spikes. However, in the second generation the progeny that had fully or almost fully altered starch of the pollen became distichous (Table 1). In the next generations all plants which conserved the transformed *waxy* character had distichous spikes, and all plant which lost the *Wx* phenotype had lost the distichous type of spikes (Tables 2, 3).

Besides, it was found that the electrophoretic patterns of the reserve proteins of the grains (hordeins) of donors, recipients and transformants (in the second generation) had marked differences (Fig. 3). The transformants had some patterns similar to the recipient and some similar to the donor. Spectrophotometric studies in a large scale of wavelengths allowed the determination of differences in absorption spectra of grain starch from donor plants, recipients, transformants, and revertants to mutant type. Figure 4 depicts typical absorption spectra of the starch.

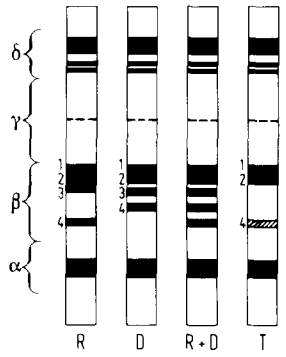

Fig. 3. Gel electrophoresis of storage proteins (hordeins) of barley grains (schematic representation of groups of proteins). R = recipient plant; D = donor plant; T = transformant; R + D = the proteins isolated from the mixture of flour of recipient and donor seeds. The main differences were found in β-zone of electrophoregram. For details see Soyfer et al. (1978)

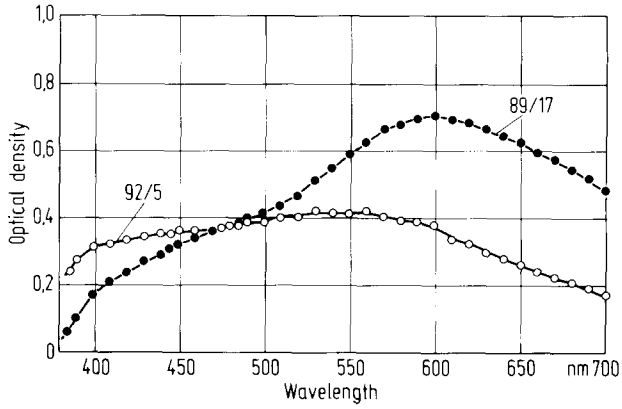

Fig. 4. Absorbance profiles of donor and recipient seed starch. 89/17 is donor; 92/5 is recipient; the transformants had absorbance profiles similar to the donor plants

Spectrophotometric analysis of the starch showed that the transformants contained starch, the structure of which was very similar to that of wild type plants. Furthermore, it was shown that reversion to the mutant type resulted in such changes in starch structure that it was not possible to differentiate them from the mutant ones (see Fig. 2 in Soyfer et al. 1978).

Segregation of Transformed Characters in Further Generations

Taking into account that a number of the transformants reverted to a mutant state, we analyzed in detail the third and the fourth generations of primary transformed plants. The results of this analysis are shown in Table 2. All plants

Table 2. Characteristics of the third and the fourth generations of transformed plants[a]

Plant Nos.	The 3rd generation		The 4th generation					
	Iodine staining of pollen grains	Type of spikes	Type of spikes	Quantity of studied plants	Number of pollen grains[b]		Frequency of appearance of changed pollen	
					Black	Red	Red among black	Black among red
12	Black	Two-rowed	Only two-rowed	14	665,000	5	7.5×10^{-5}	
16	„	„	„	15	45,500	16	3.5×10^{-4}	
21	„	„	„	20	129,200	24	1.9×10^{-4}	
22	„	„	„	38	148,200	21	1.4×10^{-4}	
25	„	„	„	18	41,400	5	1.2×10^{-4}	
12	Black	Two-rowed	Two-rowed	24	186,200	244	1.3×10^{-3}	
		-	Six-rowed	9	167	40,000		3.8×10^{-3}
14	„	„	Two-rowed	25	46,900	8	1.7×10^{-4}	
			Six-rowed	1		Unmature pollen		
15	„	„	Two-rowed	20	41,400	1	2.4×10^{-5}	
			Six-rowed	1	7	2,300		3.0×10^{-3}
19	„	„	Two-rowed	28	101,500	0	$< 10^{-5}$	
			Six-rowed	1	0	1,672		$< 10^{-3}$
20	„	„	Two-rowed	29	66,700	8	1.2×10^{-4}	
			Six-rowed	3	11	6,900		1.6×10^{-3}
28	„	„	Two-rowed	3	20,900	24	1.1×10^{-3}	
			Six-rowed	1	4	2,300		1.7×10^{-4}

[a] All plants indicated in this table are the descendants of the 506/19 plant

[b] In each case two preparations of pollen were scored

analyzed were the descendants of the two initially stable transformed plants, 506/19 and 506/22. The progeny of plants which had no revealed transformation phenotype in the first seed generation have not been analyzed in the next generations. The left column of Table 2 represents the number of these plants in the second generation, each line represents the results of pedigree analysis of each plant. In the third generation, all plants which gave black staining pollen had distichous spikes. As was mentioned, the segregation analysis of the second generation revealed (Soyfer et al. 1976a) that 82.1 per cent of the plants preserved the transformed characters unchanged; of these 32.1 per cent were stable since all their pollen grains stained black.

In the fourth generation, the segregation was continued. However, in a predominant number of the plants with black-staining pollen one could find a small amount of red pollen. The segregation in the fourth generation was related not with one but with two characters at once, namely, with the prevalence of a certain pollen type (black over red, or vice versa) and linked with it, row numbers in spikes (distichous and hexastichous, resp.) A part of plants (105 of 249 studied, or 42.2 per cent of the total plant number) did not show segregation regarding spike type. These 105 plants were the progeny of 5 two-rowed plants. The frequency of red pollen among black was relatively constant in these plants (the mean value was 1.75×10^{-4}).

In the progeny of the other 6 plants segregation in spike morphology was seen. Of 144 plants of the fourth generation, 129 preserved the two-row character and predominantly the black staining of the pollen while 15 plants reverted to the recipient type regarding both starch staining and spike type. There were 89.6 per cent of segregating plants which preserved their genotype unchanged. The mean frequency of red pollen grains among black ones in these two-rowed plants was 5.4×10^{-4}. Among the plants of this variant, 10.4 per cent became six-rowed; a great proportion of the pollen in revertants stained red by iodine. Nevertheless, the reversion was incomplete since the frequency of black pollen among red pollen was relatively high (the mean value is 2.1×10^{-3}).

Recovery of Transformational Properties in a Part of Plants of the Fourth Generation

Similar to the events occurring in the second generation, in the third one about 10 per cent of all the plants reverted to a recipient, i.e., the mutant state. Plant No. 27 can serve as a good example. Its ancestor in the second generation (plant No. 506/19) was distichous and had pollen stained black by iodine, while in the third generation the phenotypical expression of both these characteristics was simultaneously lost.

One could think that the loss of the transformational properties was irreversible. However, the analysis of the fourth generation of plant No. 27 showed that the exogenous genetic material introduced into the plant grains which was expressed in the first and second seed generations, but not in the third was not completely lost. In the fourth generation, 40 per cent of the daughter plants manifested the properties which were seemingly lost earlier. Sixteen plants became distichous again, and at the same time their pollen again contained normal starch (Table 3). The frequency of red pollen among black pollen was equal to 8.0×10^{-4}. The remaining 60 per cent of plants preserved a phenotype similar to that in recipient plants although they also contained a larger quantity of pollen grains (2.3×10^{-4}) with normal starch when compared to control plants.

Changes in Morphological Characters of Transformed Plants

The exogenous DNA induced changes not only of the above-mentioned characters but also of some morphologi-

Table 3. Recovery of transformed properties of plant No. 506/19/27 in the 4th generation[a]

The 2nd generation			The 3rd generation			The 4th generation				
Plant no.	Iodine staining of pollen grains	Type of spike	Plant no.	Iodine staining of pollen grains	Type of spike	Type of spike	Quantity of progeny plants studied	Number of pollen grains[b]		Frequency of appearance of changed pollen
								Black	Red	Red among black / Black among red
506/19	Predominantly	Two-rowed	27	Predominantly	Two-rowed	Two-rowed	16	43,600	35	8.0×10^{-4}
						Six-rowed	24	19	81,400	2.3×10^{-4}

[a] The genetic linage in these experiments is as follows: the 1st generation is plant No. 506; the 2nd generation is the plant No. 506/19, the descendant of plant No. 506; the 3rd generation is the plant No. 27, the descendant of plant No. 506/19, i.e., 506/19/27 plant; the 4th generation is the descendant of plant No. 27

[b] In two preparations of pollen grains

Table 4. Morphological characteristics of transformants in the 4th generation

Characteristic	Recipient '*Waxy*-mutant' (six-rowed)	Donor 'Yuzhny cultivar' (two-rowed)	Transformants				
			The 2nd generation		Two-rowed		
			The 3rd generation	Two-rowed		Six-rowed	
			The 4th generation:	Two-rowed	Six-rowed	Two-rowed	Six-rowed
Height of plants	72.0 ± 9.1	53.5 ± 12.3		50.7 ± 13.4	74.9 ± 9.5	54.7 ± 11.5	83.2 ± 16.0
Length of upper internode	14.6 ± 3.9	11.7 ± 3.4		11.1 ± 2.9	13.5 ± 4.2	14.0 ± 4.1	18.9 ± 5.1
Length of upper leaf	8.0 ± 1.6	6.4 ± 3.0		6.3 ± 2.1	9.4 ± 1.7	3.7 ± 0.8	9.7 ± 2.2
Width of upper leaf	0.7 ± 0.1	0.6 ± 0.2		0.4 ± 0.1	0.9 ± 0.2	0.4 ± 0.01	0.8 ± 0.2
Length of spike	8.4 ± 1.3	7.4 ± 2.3		7.8 ± 1.8	9.0 ± 1.1	5.7 ± 2.6	6.6 ± 2.0

cal patterns, namely, height of plants, amount of internodes, length of upper internode, length of upper leaf, tillering, length of spikes, amount of grains per one plant, etc. The results concerning the transformants of the third seed generation of plants (I_4-generation) were published earlier (Soyfer et al. 1976b). The study of these morphologic characters was continued in the fourth seed generation. The analysis was performed with two- and six-rowed descendants of plants which had two- and six-rowed spikes in the third generation. The results showed that many characters of the two-rowed transformants resemble those in the donor plants more than those in the recipient ones (Table 4). However, some of the characters, namely, the quantity of internodes, grain number per one plant, weight of 1,000 seeds, more closely resemble the characters of recipient plants. In the plants where such transformational properties as starch structure and number of spike rows remained unchanged for four generations we have found morphological expression of some donor-like characters. On the contrary, in the plants which had the donor-like phenotype in the second generation and then lost it in the third one, we have not found such donor-like peculiarities and moreover we have observed an intermediate pattern between the donor and the recipient of a number of quantitative characters, for instance, for such a character as the length of the upper internode.

Discussion

A. Genetic Transformation in the World of Higher Organisms Including Higher Plants

The phenomenon of genetic transformation in the world of prokaryotes is well studied and does not give rise to any doubts. In relation to higher animals such distinct evidence for the occurrence of transformation was not obtained for a long time (cf. Kraus 1961; Szybalska and Szybalski 1962; Merril et al. 1971; and negative data by Volkova et al. 1969; Otroshenko and Lavrentjev 1968). However, the induction of hereditary changes of cells of animals has now been shown for various DNA preparations, both vector DNA and unfractionated DNA (Horst et al. 1975; Wigler et al. 1979).

Concerning higher plants such distinct information has not been obtained up to now, and extreme precautions in the interpretation of the results in transformation of plants are necessary. If the question of hereditary variability of such lower plants as fungi is definitely and positively solved (Struhl et al. 1979), the data on transformation of higher plants are contradictory. There is obvious evidence for easy induction of crown-gall calli by both plasmid and total DNA preparations of *Agrobacterium tumefaciens* containing appropriate plasmids (Schell and Montague 1978; Murton et al. 1979). But, although some authors have obtained positive data on possible changes of hereditary traits of higher plants after the treatment of seeds by exogenous plant DNA (Hess 1969; Holl 1975; and some others, see a review by Hess 1977) or bacterial DNA (Ledoux et al. 1974), in other experiments negative results have been obtained (Coe and Sarcar 1966; Carlson 1972; Scowcroft 1977).

In our experiments with barley (Turbin et al. 1973; Soyfer and Turbin 1974) we found that the transformation of the *waxy* character took place after the injection of exogenous DNA into the caryopses of barley. However the transformation effect was obtained only with DNA isolated from endospermal milk and only in those cases when the DNA was injected into grains at the milk maturity stage. Several experiments of genetic transformation of the *waxy* character in barley, wheat and maize, or other biochemical and morphological characters in grass pea (*Lathyrus sativus L.*), red beet and sugar beet applying different kinds of exogenous DNA gave negative re-

sults (our unpublished data). Similarly negative results were obtained in experiments dealing with necrosis genes in spring wheat (Soyfer and Titov 1980).

In this communication we present new data on the characteristics of DNA injected into barley, type of inheritance of changed characters in the successive generations and discuss new and earlier published data on the rare positive effect of transformation in barley.

B. Stability of Transformed Characters

Black-staining pollen was found in an F_1-generation among the pollen preparations of 82 plants grown from DNA-injected seeds (Turbin et al. 1973). Fourteen changed plants were revealed in the experiment using the injection of highly polymeric but slightly deproteinized DNA. In the second generation we analyzed 8 plants from these 14 initially transformed plants and found that only 2 plants preserved their transformation characteristics. A similar picture was found in other variants of the experiments (Soyfer et al. 1976a) when we discovered that from 17 studied descendants of F_1-changed plants only four had preserved their changed phenotype. But it should be emphasized here that these four plants carried only rare black-staining pollen grains, as was also found in the parents of these plants, while the two plants obtained in the variant using highly polymeric slightly deproteinized DNA were characterized by a high per cent of wild type pollen grains.

Thus, a large proportion of changed plants had lost their transformed characteristics already in the first seed generation. But all these initially changed plants had only solitary changed pollen grains. At the same time, two plants which had a high proportion of changed pollen had preserved these changes at a relatively high level: 87 per cent of their pollen grains were stained black in the second generation. Therefore, we may say that in spite of a marked loss of changed features in a great number of the plants under study, some of the relatively stable changed plants retained their transformed character. Unfortunately, the rate at which stable-transformed plants arose in experiments using unfractionated DNA preparations was very low.

C. Segregation in the Successive Generations

As is known, the *waxy* character is coded by one gene located in the barley chromosome 1 (Nilan 1964). Therefore, it was reasonable to expect that the segregation of characters in the successive generations would follow the Mendelian laws. However, a non-mendelian segregation for the *waxy* character was revealed in our experiments (Tables 2, 3). The reason for such a segregation may be connected with the abnormal character of realization of exogenous information in the cells of recipient plants. In this respect the data obtained by us differ from those of Hess and Ledoux who didn't find any segregation among the descendants of transformed plants.

D. Pleiotropic Action of Exogenous DNA

Beginning with the F_2-seed generation, the progeny of plants transformed with respect to the *waxy* character have revealed another donor-like characteristics, namely, two-rowedness of spikes. All plants preserving the wild-type phenotype of the *wx* gene in the second and successive generations showed two-rowed spikes. Those plants which lost their transformed *waxy* character in the second (or successive generations) reverted to the six-rowed spike character.

A similar phenomenon of co-ordinated origin and loss of transformed characters was revealed in relation to another characteristic studied, i.e., electrophoretic mobility of reserve proteins of seeds (hordeins) (Soyfer et al. 1978). The transformants had a spectrum of hordein similar in some bands to both that of the recipient and donor plants (Fig. 3). But under the reversion of the *waxy* character to the recipient state the spectrum of hordeins in some of descendants of transformed plants completely reverted to the recipient type.

Some additional changed characteristics, besides the above-mentioned, were found in the third generation. These changes were related to those morphological characteristics which had a quantitative type of inheritance (Soyfer et al. 1976b). The data presented in this communication demonstrate that all these changes were found in the fourth generation as well (Table 4). Thus, the pleiotropic action of exogenous DNA was revealed in the offspring of the constantly transformed plants.

E. Multiple Character of the Realization of Foreign Genetic Information and Arguments Against Seed or Pollen Contamination

The simultaneous appearance of several donor characteristics in transformed plants is a most important but also a most questionable feature of our results since alleles of *wx* and row-number lie in different chromosomes (Nilan 1964). Hence, it might be thought that the simplest explanation of these data is seed contamination or accidental outcrossing. However, some reasons speak against such an interpretation.

First, the study of genetical control of rowedness in barley revealed a complex inheritance of this character-

istic. At least two loci appear to be involved in the inheritance of two- and six-rowedness in barley: the *V* locus on chromosome 2 and the *I* locus on chromosome 4; and both loci are represented by multiple allelic series (Woodward 1949). These are four well studied alleles of one locus and three of another. Therefore, 'there are some 60 theoretical combinations; of these, six would produce six-rowed phenotypes and six would produce deficient phenotypes; the rest would be phenotypically two-rowed or show various degrees of fertility of the lateral spikelets' (Harlan 1979). Besides, we cannot exclude the possibility of the presence of any modificator(s) of the character of rowedness in the first chromosome which might manifest their action, under the influence of exogenous DNA on the *wx* locus, for example, in the form of specific position effect.

Second, it was very important that we observed in the progenies of transformed plants the transfer from six-rowedness to two-rowedness. The fact is that the mutations from two-rowed to six-rowed barley have been reported by Gustafsson (Nybom 1954) and many other investigators but 'no good cases of six-rowed to two-rowed mutations are reported in the literature' (Harlan 1979). Thus, this result, too, indicates that an unusual genetic process has appeared in these experiments.

Third, the strongest argument against the supposition of seed contamination is the restoration of a set of transformed patterns in the fourth generation of changed plants, the third generation of which had lost the phenotypical expression of these patterns. So, the plant No. 506 was six-rowed and after DNA injection had a high proportion of wild type pollen grains. Part of the progeny of the plant No. 506 became two-rowed, with black-staining pollen in the second generation after self-pollination, as indicated in Table 1. Then, in the third generation, a number of the descendants of these transformed plants (namely, plant No. 27, i.e. 506/19/27) became six-rowed with red-staining pollen, or, in other words, they lost the transformed phenotype. But, in the next, the fourth, generation a proportion of the progeny of this 506/19/27 plant, also after self-pollination, restored the two-rowedness and the black-staining of pollen, i.e., restored a previously lost transformed phenotype (Table 3). Such a reversion is impossible if we had seed contamination or accidental outcrossing. And this is, in my point of view, of great interest and importance. Taking this into account, we may draw the conclusion that the genetic information injected into maternal plants is not absolutely excluded from the cells of this and further generations of the cells which lost this information but may be temporarily supressed in its expression, although later the transcription and the translation of the whole set of this information may be restored.

Finally, another argument against simple contamination is the non-mendelian type of segregation of the monogenically-determined *waxy* character in the progenies of the second (Table 2 in: Soyfer et al. 1976a), the third and the fourth generations in all variants of the experiments (Tables 3, 4). Such quantitative data on segregation which we have in our experiments cannot be explained by simple seed contaminations.

F. Possible Molecular Explanations for the Transformant Appearance

The molecular nature of the transformation effect in barley still remains unclear. Earlier, in discussing the reasons of hereditary variability caused by the exogenous DNA treatment, we suggested (Soyfer and Turbin 1974; Soyfer et al. 1976a) that this phenomenon might be explained in several ways: 1. a regulator effect due to external DNA action in situ or on regulator genes and modifiers of definitive characters; 2. mutagenic action of foreign DNA; 3. specific DNA action both at the chromosomal and the cytoplasmic level; or 4. due to a result of unspecific action of DNA treatment. The sum total of the results in the transformation of barley cannot be explained by a stable insertion of exogenous DNA into chromosomal DNA as suggested by Ledoux et al. (1974) and Hess (1969).

This exogenous DNA may have a regulatory effect on gene activity in the cells of barley (Hess 1977) but the mechanism of such reversible effect of exogenous DNA introduced into the cells of recipient plants (a simultaneous 'switch-on and switch-off' of the whole set of transformed characters which take place) is as yet unknown.

It does not seem probable that the data presented in this paper and in our other publications could be explained by the mutagenic action of exogenous DNA due primarily to the observation of pleiotropic action and reversible type of manifestation of the transformed characters in the successive generations.

The discovery of insertion elements entering the chromosomal DNA and relatively easily leaving it, as well as jumping genes (see Starlinger and Saedler 1972; Rasmuson and Green 1974; see also Cohen 1976; Klecker 1977) may serve as a basis for further studies of transformation phenomena in plants but we have no distinct information on the role of these elements in higher plants.

The study of different extrachromosomal elements acting on such different levels, as exosomes (Fox et al. 1971), episomes and plasmids (Cohen 1976), minichromosomes (Struhl et al. 1979) have opened the possibilities for further analysis of transformation.

However, we must again emphasize that the effect of stable transformation was revealed in our experiments 1. only in relation to a few plants; 2. only with DNA prepa-

rations isolated from milk endospermal material; 3. under the injection of this DNA into grains at milk maturity stage, and 4. with slightly deproteinized highly polymeric DNA containing no less than thirty per cent protein. Many attempts to find transformation with other preparations of DNA, or with using the model of seeds soaking in the DNA (the model used by Ledoux, Hess and some other investigators) were unsuccesful in our hands (Soyfer and Titov 1980). We must study the physical-chemical structure of different preparations of DNA giving positive transformation effects, the competence stage of plants and organs, the process of elimination of exogenous material from cells and its manifestation in the phenotype before we will be able to receive a high proportion of transformed plant cells. We strongly believe that the difficulties in interpretation of the data on transformation call for repetition of the experiments on this phenomenon in higher plants and we are currently planning just such experiments.

In conclusion we must state that, in spite of how and in what manner exogenous DNA penetrates the plant cells, whether or not it is inserted into the chromosomal DNA (Kleinhofs et al. 1975; Kleinhofs, 1977; Hemleben et al. 1975), whether it has a biological action itself, or whether the observed phenomena are a consequence of biological contamination (see Cocking 1977 and the controversal view of Ledoux et al. 1977), there are no doubts that this interesting phenomenon requires further experimental analysis.

Acknowledgments

I wish to thank Dr. Y.B. Titov from my department for collaboration and participation in the experiments, Mrs. G.D. Kurova for technical assistance, Dr. J.D. Shaposhnikov from this department and Dr. T.R. Troost from Georgetown University, U.S.A. for critical reading of the English version of the manuscript, and Prof. N.V. Turbin for his interest to this study. The studies were supported by the U.S.S.R. Academy of Agricultural Sciences. The data presented in this communication were presented in XIV International Genetic Congress in Moscow, August, 1978.

Literature

Carlson, P.S. (1972): Attempts to detect DNA-mediated transformation in a higher plant. Genetics **71**, No. 3, 9-10

Coe, E.H., Jr.; Sarkar, K.R. (1966): Preparation of nucleic acids and a genetic transformation attempt in maize. Crop Sci. **6**, 432-435

Cocking, E. (1977): Genetic modification of plant cells: a reappraisal. Nature **266**, 13-14

Cohen, S.N. (1976): Transposable genetic elements and plasmid evolution. Nature **263**, 731-738

Fox, A.S.; Yoon, S.B.; Duggleby, W.F.; Gelbart, W.M. (1971): Genetic transformation in *Drosophila*. In: Informative molecules in biological systems (ed.: Ledoux, L.G.H.) 313-333. Amsterdam: North-Holland Publ.

Harlan, J.R. (1979): On the origin of barley. In: Barley: origin, botany, culture, winter hardiness, genetics, utilization, pests. USDA Agric. Handbook **338**, 10-36

Hemleben, V.; Ermisch, N.; Kimmich, D.; Leber, B.; Peter, G. (1975): Studies on the fate of homologous DNA applied to seedlings of *Matthiola incana*. Eur. J. Biochem. **56**, 403-411

Hess, D. (1969): Versuche zur Transformation an höheren Pflanzen: Induktion und konstante Weitergabe der Anthocyansynthese bei *Petunia hybrida*. Z. Pflanzenphysiol. **60**, 348-353

Hess, D. (1970): Versuche zur Transformation an höheren Pflanzen: Möglische Transformation eines Gens für Blattform bei *Petunia*. Z. Pflanzenphysiol. **63**, 461-467

Hess, D. (1977): Cell modification by DNA uptake. In: Applied and fundamental aspects of plant cell, tissue and organ culture (eds.: Bajaj, Y.P.S., Reinert, J.) 506-535; 563-577. Berlin, Heidelberg, New York: Springer

Holl, F.B. (1975) Molecular Genetic Modification of Legumes. In: Workshop on Uses of Molecular Genetic Modification of Eukaryotes, 19-21 March 1975, University of Minnesota, St. Paul

Holl, F.B.; Gamborg, O.L.; Ohyama, K.; Pelcher, L. (1974): Genetic transformation in plants, In: Tissue culture and plant science (ed.: Street, H.E.), 301-325. London, New York: Acad. Press

Horst, Y.; Kluge, F.; Bejreuther, K.; Gerok, W. (1975): Gene transfer to human cells: transducing phage λ plac gene expression in GM_1-gangliosidosis fibroblasts. Proc. Natl. Acad. Sci. (Wash.) **72**, 3531-3535

Klecker, N. (1977): Translocatable elements in prokaryotes. Cell **11**, 11-23

Kleinhofs, A. (1977): Physical and biological studies of DNA uptake by plants. In: Molecular genetic modification of eukaryotes (eds.: Rubenstein, I.; Phillips, R.L.; Green, C.E.; Desnick, R.J.), 137-147 New York, San Francisco, London: Acad. Press

Kleinhofs, A.; Eden, F.C.; Chilton, M.D.; Bendich, A.J. (1975): On the question of the integration of exogenous bacterial DNA into plant DNA. Proc. Natl. Acad. Sci. (Wash.) **72**, 2748-2752

Kraus, L.M. (1961): Formation of different haemoglobins in tissue culture of human bone marrow treated with human DNA. Nature **192**, 1055

Ledoux, L.; Huart, R. (1975): Importance of DNA size for integration in plant materials. Arch. Int. Physiol. Biochim. **83**, 194-196

Ledoux, L.; Huart, R.; Ryngaert-Adriassens, A.; Matagne, R.F.; Schlösser, J.P.; Jacobs, M. (1974): Does DNA correct *Arabidopsis thaliana* mutants? Arch. Int. Physiol. Biochim. **85**, 992-994

Merril, C.R.; Geier, M.R.; Petricciani, J.C. (1971): Bacterial virus gene expression in human cells. Nature **233**, 298-300

Murton, L.; Willems, G.J.; Molendiyk, L.; Schilperoort, R.A. (1979): In vitro transformation of cultured cells from *Nicotiana tabacum* by *Agrobacterium tumefaciens*. Nature **277**, 129-131

Nawa, S.; Yamada, M.K.; Ohta, Y. (1975): Hereditary changes in *Capsicum annuum L.* 3. Induced by DNA treatment. Jpn. J. Genet., **50**, 341-344

Nilan, R.A. (1964): Cytology and Genetics of Barley. 1951-1962, Monogr. Sup. 3. Research Studies. Pullman: Washington State Univ.

Nybom, N. (1954): Mutation types in barley, Acta Agr. Scand. **4**, 430-456

Otroshenko, V.A.: Lavrentiev, G.A. (1968): Incorporation of polymeric DNA into nuclei of human cells in vitro. Molec. Biol. (Russ.) **2**, 498-507

Rasmuson, B.; Green, M.B. (1974): Genetic instability in *Drosophila melanogaster*. A mutable tandem duplication. Molec. Gen. Genet. **133**, 249-260

Schell, J.; Montague, M. (1978): On the transfer and expression of prokaryotic DNA in plant cells transformed by *A. tumefaciens*, In: Integration and Expression of DNA Molecules (eds.: Hofschneider, P.H.; Starlinger, P.), 127-134. Berlin, Heidelberg, New York: Springer

Scowcroft, W.R. (1977): Crop improvement through plant tissue culture, In: Genetic diversity in plants (eds.: Muhammed, A.; Aksel, R.; von Borstel, R.C.), 417-430. New York, London: Plenum Press

Soyfer, V.N. (1974): Repair of Genetic Damage and Radiation Mutagenesis. Thesis of Dissertation of Doctor of Biology, 46 pp. Odessa University. Odessa

Soyfer, V.N.; Kartel, N.A.; Chekalin, N.M.; Titov, Y.B.; Cieminis, K.K.; Turbin, N.V. (1976a): Genetic modification of the *waxy* character in barley after an injection of wild-type exogenous DNA. Analysis of the second seed generation. Mutat. Res. **36**, 303-310

Soyfer, V.N.; Morozkin, A.D.; Bogdanov, V.P.; Titov, Y.B.; Chekalin, N.M. (1978): Starch composition and hordein electrophoretic patterns of grains of genetically transformed barley plants. Environ. Exper. Bot. **18**, 105-111

Soyfer, V.N.; Titov, Y.B. (1980): Negative results in the study of genespecific transformation in wheat after seed treatment with exogenous DNA (in press)

Soyfer, V.N.; Turbin, N.V. (1974): Genetic transformation of the *waxy* character in barley. In: 2nd Europ. Meet. Transformation and Transfection in Microorganisms, Cracow, pp. 120-123

Soyfer, N.V.; Turbin, N.V.; Chekalin, N.M.; Kartel, N.A. (1976b): Characteristics of some quantitative characters of transformed plants in I_4-progeny compared to recipient and donor cultivars. In: Abstr. 3rd Ukrainian Congr. Genet. and Breeders, Naukowa Dumka, Kiev, pp. 48

Struhl, K.; Stinchcomb, D.T.; Scherer, S.; Davis, R.W. (1979): High-frequency transformation of yeast: autonomous replication of hibrid DNA molecules. Proc. Natl. Acad. Sci. (Wash.) **76**, 1035-1039

Szybalska, E.; Szybalski, W. (1962): Genetics of human cell lines. IV. DNA-mediated heritable transformation of a biochemical trait. Proc. Natl. Acad. Sci. (Wash.) **48**, 2026-2034

Starlinger, P.; Saedler, H. (1972): Insertion mutations in microorganisms. Biochimie **54**, 177-185

Turbin, N.V.; Soyfer, N.V.; Kartel, N.A. (1975a): Investigation of genetic transformation of plants under using isogenic DNA. Proceed. USSR Acad. Agric. Sci. No. **12**, 4-5

Turbin, N.V.; Soyfer, N.V.; Kartel, N.A.; Chekalin, N.M.; Dorohov, Y.L.; Titov, Y.B.; Cieminis, K.K. (1973): Modification of the *waxy* character in barley under the action of wild type DNA. (in Russian with engl. summary) USSR Agric. Academy Publications: Moscow

Turbin, N.V.; Soyfer, N.V.; Kartel, N.A.; Chekalin, N.M.; Dorohov, Y.L.; Titov, Y.B.; Cieminis, K.K. (1975b): Genetic modification of the *waxy* character in barley under the action of exogenous DNA of the wild variety. Mutation Res. **27**, 59-68

Volkova, L.V.; Lukjanov, S.Y.; Otroshenko, V.A. (1969): Search for genetic transformation of human amnyon cells cultivated in vitro. Genetica **5**, 159-168 (In Russian)

Wigler, M.; Pellicer, A.; Silverstein, S.; Axel, R.; Urlaub, G.; Chasin, L. (1979): DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. (Wash.) **76**, 1373-1376

Woodward, R.W. (1949): The inheritance of fertility in the lateral florets of the four barley groups. Agron. J. **41**, 317-322

Received December 27, 1979
Communicated by Å Gustafsson

Dr. V.N. Soyfer Chertanovskaya 39, Build. 2, Apt. 131
Moscow 113519 (USSR)

Note added in proof

To examine the possibility of gene specific, directed and heritable changes in higher plants during the course of the foreign DNA treatment, experiments were performed with spring wheat. The plants used differed in two heritable marker characters: spikelets of Balaganka var. had awns and the plants were characterized by the Ne_2-gene of necrosis; the Marquillo var. was awnless and was characterized by the Ne_1-gene. The Ne_1 and Ne_2 genes, being additive and united in one genome, were complemented and led to the development of necrotic spots in leaves, a stop in plant development and the death of plants. The DNA was isolated from 5 to 7 day seedlings of wheat without using chloroform or phenol and was subjected to ultracentrifugation at 20,000 g.

Seeds of ricipient (awnless) plants were grown in the DNA solution isolated from awned plants and sown in the ground. Plants having spikelets with reduced and normal awns were found among a portion of the plants growing from the seeds treated with the exogenous DNA. However, the awned spikes were found both after the treatment of Marquillo seeds with homologous (Marquillo) DNA (9 complete and 3 incomplete awned plants among 542 studied) and after treatment with heterologous donor DNA when the Marquillo seeds were germinated in the Balaganka DNA (11 complete and 7 incomplete awned among 606 plants under study). At the same time, the observed changes of awn character were heritable, i.e. awned plants, after the treatment with a homologous and a heterologous DNA, retained the changed characters up to the F_2-seed generation.

The attempt to reveal a complementation between the Ne_1-genes of plant chromosomes and the Ne_2-genes introduced into cells with the exogenous DNA were unsuccessful. In several experiments using DNA preparations of different purity and polymerity we studied thousands of treated plants but no examples of an interaction between Ne_1 and Ne_2 was found, although some nonspecific effects (the decrease in number of germinating plants, the increase in height of plants and length of spikes, etc.) were revealed. In many instances the changes induced were similar to the character observed in the donor.

Dr. V.N. Soyfer