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Development of Transgenic Sorghum Plants with Improved *In Vitro* Kafirin Digestibility

Lev A. Elkonin, Julia V. Italyanskaya,
Valery M. Panin and Nikolai Yu. Selivanov

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Abstract

Improvement of nutritional value of crops is one of the main goals of plant biotechnology. These studies are extremely important for sorghum—a unique drought-tolerant cereal crop that is of special importance for sustainable grain production in the arid regions. The major cause of relatively low nutritive value of sorghum grain is the resistance of one of its seed storage proteins, γ-kafirin, to protease digestion. Using *Agrobacterium*-mediated genetic transformation, we have obtained transgenic sorghum plants harboring a genetic construct for RNA interference (RNAi) silencing of the γ-kafirin gene. In T_1 generation, transgenic plants with modified endosperm texture were found. These plants had lowered level of the 28-kDa γ-kafirin protein and kafirin oligomers, which are formed by natural kafirin polymerization. *In vitro* protein digestibility analysis showed that the amount of undigested protein in transgenic plants was reduced by 2.9–3.2 times, in comparison with the original line, the digestibility index reached 85–88% (60% in the original line). HPLC analysis showed that total amino acid content in transgenic plants was reduced, while the lysine proportion was increased by 1.6–1.7 times. PCR analysis confirmed inheritance of the genetic construct up to T_4 generation.

Keywords: transgenic plants, *Agrobacterium*-mediated genetic transformation, gamma-kafirin, *in vitro* protein digestibility, RNA silencing, endosperm, *Sorghum bicolor* (L.) Moench

1. Introduction

Development of plant varieties and hybrids that possess the necessary traits and properties is the main goal of plant breeding. With the accumulation of knowledge in the field of genetics,

physiology and molecular biology of plants, the ability of breeders and geneticists to create valuable varieties and hybrids has significantly expanded. Development of genetic engineering approaches have allowed creating a significant number of cultivars and lines, resistant to biotic and abiotic stresses, with improved quality of final products, increased photosynthetic rate and nutrient-use efficiency [1].

Creation of transgenic plants with the changed composition of proteins and improved nutritional value is one of the most promising areas of genetic engineering. These investigations are particularly relevant for cereals being the main source of food and feed protein. It is known that humans receive from cereals up to 50% of proteins (or up to 70% in developing countries) and up to 65% of calories, in which the storage proteins account for up to 80% of the total protein content in the mature seed [2]. To solve this problem, various genetic engineering technologies had been developed. These technologies allow the introduction of new genes and thereby modulate the synthesis of new proteins with higher nutritional value or in a highly specific way downregulate genes that control the synthesis of proteins with a low nutritional value or reducing the digestibility or assimilation of other proteins [3–5]. Genetic engineering techniques are quite promising for enrichment of cereal grain with essential amino acids, i.e., lysine, tryptophan and methionine [6]. To date, transgenic lines with a modified composition of seed storage proteins, with increased lysine content and with improved baking properties have already been obtained in all most important species of cereals—maize, rice and wheat [7–9].

These studies are extremely important for sorghum—a unique drought-tolerant cereal crop having special importance for sustainable grain production in the arid regions. Today, sorghum is one of the five most widely cultivated cereal crops, and with the increase of climate aridity, observed in many regions of the globe, demand for sorghum will increasingly grow. However, the majority of sorghum cultivars and hybrids have relatively poor nutritive value in comparison with other cereals [10, 11]. One of the reasons of relatively low nutritive value of sorghum grain is resistance of its seed storage proteins (kafirins) to protease digestion [12]. The causes of the poor sorghum protein digestibility were studied extensively [10, 13, 14]. Among the factors that cause or may affect this phenomenon, there are chemical structures of kafirin molecules, some of which (α- and β-kafirins) are abundant with sulfur-containing amino acids capable to form S–S bonds, resistant to protease digestion; interactions of kafirins with non-kafirin proteins and non-protein components such as polyphenols and polysaccharides; spatial organization of different kafirins in the protein bodies of endosperm cells; endosperm structure (vitreous or floury).

It is generally accepted that the peripheral disposition of γ-kafirin in protein bodies reduces digestibility of α-kafirin—the major sorghum seed storage protein located central position in protein bodies and comprising up to 80% of total endosperm kafirins [13, 14]. This hypothesis is supported by studies of protein bodies of the mutant with improved protein digestibility. In this mutant, protein bodies shape has been changed from spherical to invaginate; the γ-kafirin was located at the bottom of invaginations where it should not interfere with the digestion of the α-kafirin [15]. Recent study also showed that a sorghum mutant with high digestibility

of kafirins has a point mutation in the signal sequence of the α-kafirin gene, which apparently disrupts its deposition in protein bodies [16]. One of the main characteristic features of kafirin proteins is their ability to form oligomers or polymers of high molecular weight. These oligomers comprise α- and γ-kafirins that are linked together by disulfide (S–S) bonds [17, 18]. They are resistant to protease digestion and occur more in the vitreous endosperm fraction [14, 19].

Improving of sorghum genetic transformation technology [20, 21] makes it possible to solve this problem by using RNA interference (RNAi) that allows targeted downregulation of individual genes. In recent years, RNAi technology has become widely used for changing the composition of the storage proteins and starch in different cereal species [3–5].

In maize, with using of genetic constructs harboring inverted repeats of genes of α-zeins (19 and 22 kDa), transgenic lines with suppressed synthesis of these proteins were obtained [22, 23]. It was found that repression of the synthesis of zeins possessing a relatively low nutritional value leads to accumulation of other proteins with a higher nutritional value. Maize plants with gene silencing of α-zeins were characterized by doubled content of essential amino acids tryptophan and lysine in the kernels. These experiments showed that gene silencing of 22 kDa α-zein resulted in the formation of the floury endosperm. Such a modification in the type of endosperm was associated with abnormalities in the formation of the structure of protein bodies, namely the violation of deposition of 19 kDa α-zein into the center of a protein body, or a modification of its interaction with β- and γ-zeins [22].

In sorghum, transgenic lines with genetic constructs capable of RNAi silencing of different kafirin classes were obtained [24–27]. Transgenic plants harboring these constructs were characterized by improved *in vitro* protein digestibility (IVPD) that was accompanied by opaque floury endosperm. Unfortunately, the floury endosperm reduces the practical value of these lines, because the reduction of the vitreous layer increases the fragility of kernels and increases the susceptibility to fungal infection.

In our experiments, we obtained transgenic sorghum plants with genetic construct for silencing of the gamma-kafirin gene [28]. These plants retained sectors of vitreous endosperm in their kernels and were characterized by high level of *in vitro* kafirins digestibility. In this chapter, we review these experiments and present new data, confirming inheritance of the genetic construct and its effect on endosperm protein spectrum and endosperm texture.

2. Obtaining of transgenic plants with genetic construct for RNA silencing of the γ-kafirin gene

To obtain transgenic plants with silencing of gamma-kafirin gene, the binary silencing vector, pNRKAFSIL, has been designed. This vector contained a hairpin insert that consisted of an inverted repeat of the fragment of the γ-kafirin gene and *ubi1* intron as the spacer between the arms of the inverted repeat (**Figure 1**). The 307-bp fragment of the γ-kafirin gene was
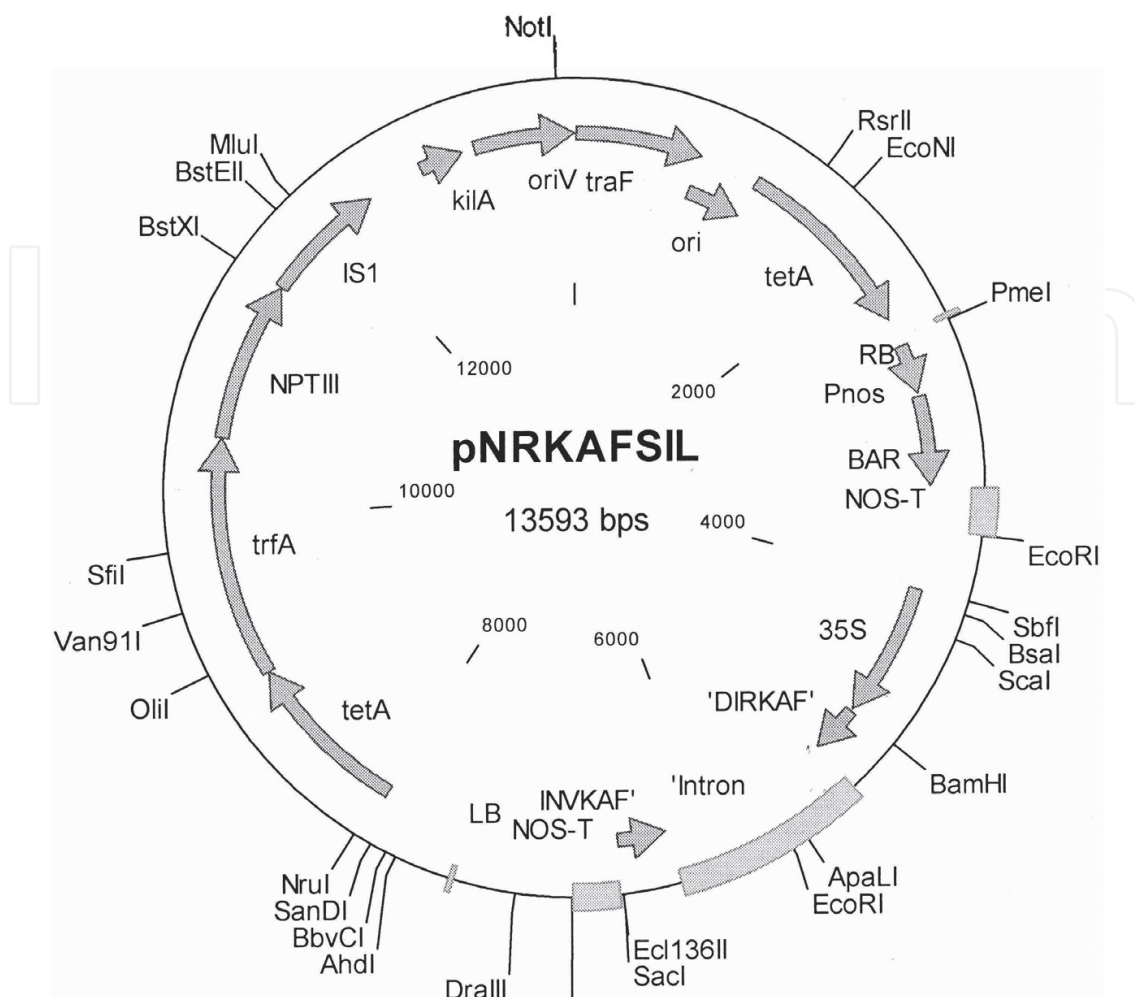
Figure 1. Map of the pNRKAFSIL vector containing hairpin insert consisted from inverted repeat of the fragment of the γ-kafirin gene ("INVKAF" and "DIRKAF") and *ubi1* intron as the spacer between the arms of the inverted repeat (published with the permission of the publishing house "Nauka").

isolated by PCR from genomic DNA of sorghum. The sequence corresponded to bases 280–588 of GeneBank accession number M73688 [29]. This construct was driven by the CaMV 35S-promoter. The T-DNA region of this vector contained selectable marker *bar* gene driven by *nos*-promoter. The binary vector pNRKAFSIL was introduced in *Agrobacterium tumefaciens* GV3101.

To obtain transgenic plants with genetic construct for RNA silencing of the γ-kafirin gene, cocultivation of immature embryos of sorghum cv. Zheltozernoe 10 (Zh10) (15–17 days after pollination) with cell suspension of the *A. tumefaciens* strain GV3101/pNRKAFSIL was performed.

Activation of *vir*-genes was made according to the published protocol [30] with some modifications. *A. tumefaciens* strain GV3101/pNRKAFSIL vector was grown on an Agrobacterium (AB) minimal medium [31] with the antibiotics for 3 days at 28°C. After that a loop of the Agrobacterium cells were transferred into the flask with 20 ml of liquid yeast extract peptone (YEP) medium with the antibiotics and grown for 9 h under continuous shaking (220 rpm) at

28°C. Then, the cells were collected by centrifugation and suspended in a small volume (5–6 ml) of modified AB medium without phosphates with the addition of 200 μM acetosyringone (Sigma-Aldrich, USA) and were incubated for 18 h under gentle shaking (60–70 rpm) at 22–23°C. After incubation, the cells were collected by centrifugation and suspended in inoculating medium PHI-I [32] with the addition of 200 μM acetosyringone to a final OD_{600}=0.6. This suspension was used for inoculation of immature embryos.

Agrobacterial transformation was based on previously published protocols [20, 32] with some modifications. Immature embryos after pre-cultivation for 3 days on the agar M11 medium [33] were placed onto sterile filter paper wetted with inoculating medium and were inoculated with an agrobacterial cell suspension in PHI-I medium for 10 min at room temperature. The Agrobacterium inoculum was then removed, and the filter with embryos was transferred into another Petri dish on a dry filter and was wetted with cocultivation medium (M11 medium supplemented with 200 μM acetosyringone). The cocultivation step was performed for 3 days at 23 ± 1°C in the dark. After cocultivation, the embryos were transferred to the M11 medium with the addition of 200 mg/l timentin solidified with 2.5 g/l phytagel and were cultured at 27 ± 1°C in the dark for 7 days. Then, the embryos with developing embryogenic calli were subcultured to the fresh medium of the same composition with the addition of 2.5 mg/l glufosinate ammonium (GA) and were cultivated at 28°C in the dark for 3–4 weeks.

From two experiments on cocultivation of immature sorghum embryos of Zh10 with *A. tumefaciens* strain GV3101/pNRKAFSIL 35 embryogenic calli survived after selection on the medium with 2.5 mg/l GA (**Table 1**; **Figure 2A**). For plant regeneration, the herbicide-tolerant calli were transferred onto regeneration medium (murashige and skoog (MS), 1.0 mg/l kinetin, 1.0 mg/l Indole-3-Acetic Acid (IAA)) and maintained at 25°C under a photoperiod of 16 h light and 8 h dark. Initiation of shoot development was observed in 13 calli transferred to the regeneration medium, but in the majority of the cultures, shoot development was arrested at early stages. Nevertheless, few regenerants were obtained (**Figure 2B**), one of which turned out to be PCR-positive in the experiment with primers to the *bar* gene (**Figure 3A**).

Experiment	Number of embryos	Number of EC resistant to 2.5 mg/l GA	Number of cultures with regenerants	Number of T$_0$ plants (PCR-positive)[1]	Number of plants in T$_1$ generation		Number of plants in T$_2$ generation[2]	
					Total	Resistant to 2.5 mg/l GA (PCR-positive)	Total	Resistant to 2.5 mg/l GA (PCR-positive)
#1	49	21	11	3 (1)	40	10 (6 out of 6 studied)	141	103 (10 out of 19 studied)
#2	31	14	2	1 (0)	–	–	–	–

Notes: EC = embryogenic cultures; GA = glufosinate ammonium.
[1]PCR with primers to *bar* gene.
[2]Combined progeny from PCR-positive plants from T$_1$ generation.

Table 1. Selection of transgenic plants by cocultivation of immature sorghum embryos of *Zheltozernoe 10* with the *A. tumefaciens* GV3101/pNRKAFSIL.
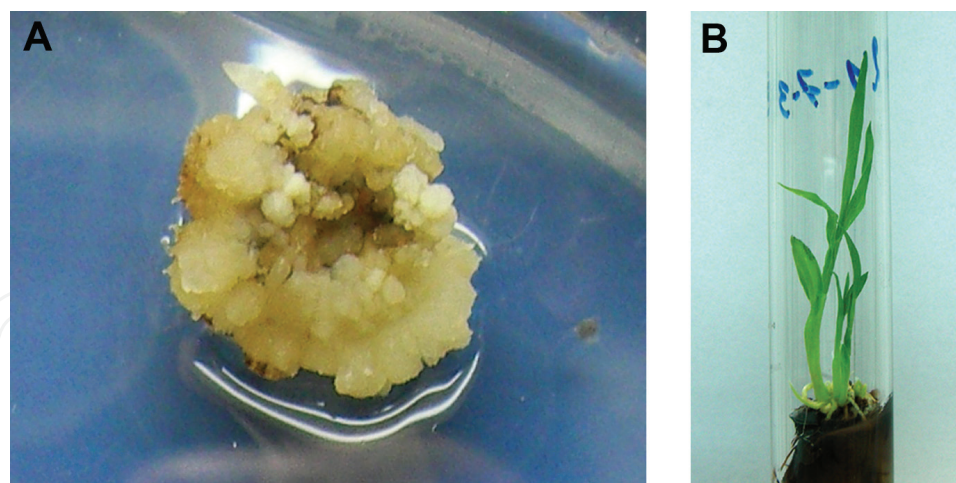
Figure 2. Embryogenic callus developing on M11 medium with 2.5 mg/l glufosinate ammonium (A) and regenerated plants (B) obtained in experiment on *Agrobacterium*-mediated genetic transformation of immature sorghum embryos with *A. tumefaciens* strain GV3101/pNRKAFSIL.

Self-pollinated progeny (T_1) of this plant (#94) was tested for herbicide tolerance by germination on a medium containing 2.5 mg/l of the selective agent (**Figure 4**). This concentration causes browning and death of sensitive non-transgenic plants. Herbicide-tolerant plants were found, and the sensitive plants predominated over tolerant ones (**Table 1**). Some of herbicide-tolerant plants that were tested with the primers to the *bar* gene were proved to be PCR-positive (**Figure 3B**). In the progeny of PCR-positive T_1 plants (i.e., in the T_2 generation) that were grown on the medium with 2.5 mg/l GA, the frequency of herbicide tolerant plants was significantly higher (**Table 1**) and some of these plants were also PCR-positive (data not shown).

These data testify that the progeny of plant #94 inherited the transgenic construct. A low frequency of tolerant plants in the T_1 generation might be explained by silencing of the *bar* gene driven by *nos*-promotor because silencing of transgene is a common phenomenon in sorghum
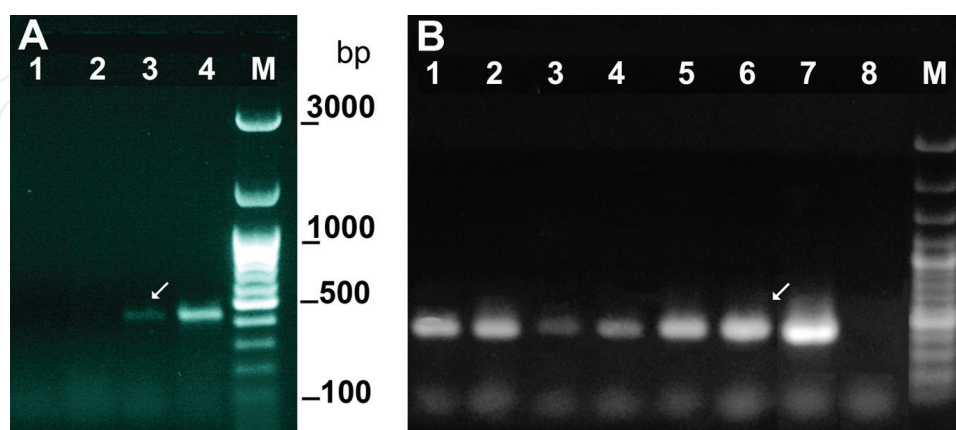

Figure 3. PCR analysis of genomic DNA of plants from T_0 (A) and T_1 (B) generations obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL with primers to *bar* gene. (A) 1—original non-transgenic line, Zheltozernoe 10; 2—negative control without template DNA; 3—T_0 plant (#94); 4—pNRKAFSIL; M—100-bp ladder. (B) 1–6—individual plants from T_1 generation; 7—pNRKAFSIL; 8—negative control without template DNA; M—100-bp ladder. Amplified fragment of the *bar* gene (444 bp) is marked by arrow (published with the permission of the publishing house "Nauka").
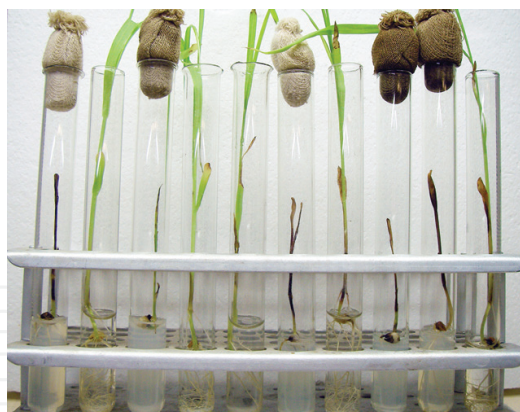
Figure 4. Segregation for tolerance to 2.5 mg/l glufosinate ammonium in the progeny of PCR-positive plant #94 obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL. Note green tolerant plants and necrosis in sensitive plants (tolerant plants survived selection, have been transferred from the agar medium to tap water to improve their survival in soil).

genetic transformation [34]. In the T_2 generation, segregation of GA-tolerant vs. GA-sensitive plants corresponds to a monogenic ratio 3:1 ($\chi^2 = 0.286$; $0.50 < P < 0.75$) (**Table 1**).

The inheritance of T-DNA in subsequent generations, including T_4, was confirmed by PCR analysis using primers to the marker gene *bar*, with each of the three T_2 families studied contained PCR-positive plants (**Figure 5A**).

To verify the presence of the genetic construct for RNA silencing of the γ-kafirin gene in the transgenic plants, we performed a PCR analysis of a number of plants from T_3 and T_4 generations for the presence of ubiquitin intron. In the studied plants, amplification of a fragment of this gene was observed, which confirmed the presence of a genetic construction for γ-kafirin silencing in the genome of the obtained transgenic plants (**Figure 5B**).
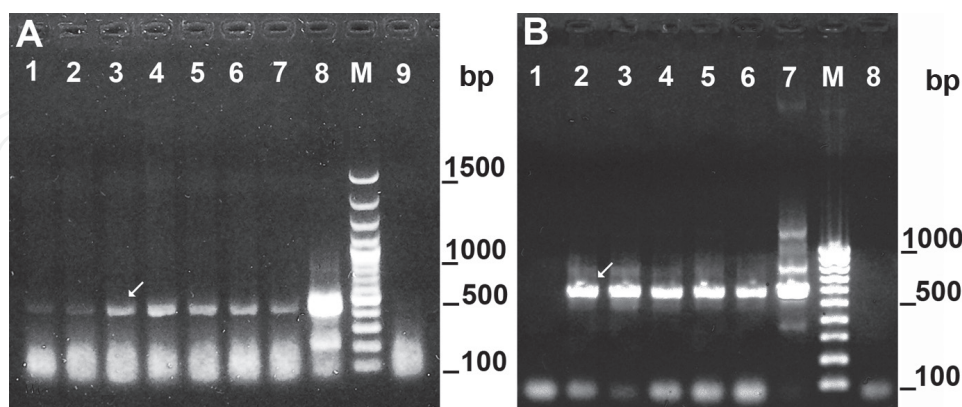

Figure 5. PCR analysis of genomic DNA of sorghum plants from T_3 and T_4 generations obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL with primers to *bar* gene (A) and *Ubi*-intron (B). (A) 1—T_3 94-2-04-1; 2—T_3 94-2-04-3; 3—T_4 94-2-11-2-4; 4—T_3 94-3-04-3; 5—T_4 94-3-08-2-1; 6—T_4 94-3-08-2-3; 7—T_4 94-2-11-2-1; 8—pNRKAFSIL; M—100-bp ladder and 9—negative control (without DNA template). Amplified fragment of the *bar* gene (444 bp) is marked by arrow. (B) 1—Zh10, original non-transgenic line; 2—T_3 94-2-04-1; 3—T_4 94-2-11-2-1; 4—T_0 Ogonek; 5—T_3 94-2-04-2; 6—T_4 94-3-08-3-3; 7—pNRKAFSIL; M—100-bp ladder and 8—negative control (without DNA template). Amplified fragment of *Ubi*-intron (584 bp) is marked by arrow.

3. Analysis of electrophoretic spectra of endosperm proteins in plants with genetic construct for RNA silencing of the γ-kafirin gene

To identify the expression of the introduced genetic construct, the experiments on SDS-PAGE of endosperm proteins were performed. The samples (20 mg of flour) were incubated with a sample buffer (0.0625 M Tris·HCl, pH 6.8) under reducing conditions (2% SDS, 5% β-mercaptoethanol, destroying the S–S bonds of kafirin polymers) or in native, non-reducing conditions (without β-mercaptoethanol) at 100°C for 90 s. The samples were centrifuged, and supernatant was used for SDS-PAGE in 13.0% (w/v) polyacrylamide gel (PAG) according to modified Laemmli method [35]. The gels were stained with Coomassie Brilliant Blue R-250. The electrophoretic spectra were carefully studied, and particular attention was paid to the γ-kafirin content, the suppression of which was to be expected, and to content of kafirin oligomers (≈47 and ≈66 kDa), which consist from α- and γ-kafirins [14, 17, 18].

It was found that in kernels of original non-transgenic line Zh10 content of polypeptides with Mr ≈47 and ≈66 kDa was markedly higher than in transgenic plants. These differences were observed both in SDS-PAGE performed in non-reducing conditions (**Figure 6**) and in reducing conditions (see Section 5).

Notably, electrophoresis in non-reducing conditions revealed that the level of polypeptide corresponding to γ-kafirin (28 kDa, marked by an arrow) in transgenic plants was significantly lowered compared to the original non-transgenic line, which was to be expected with the silencing of the γ-kafirin gene. In addition, as we found previously in experiments on SDS-PAGE in reducing conditions, content of α-kafirin monomers (25 and 23 kDa) was also
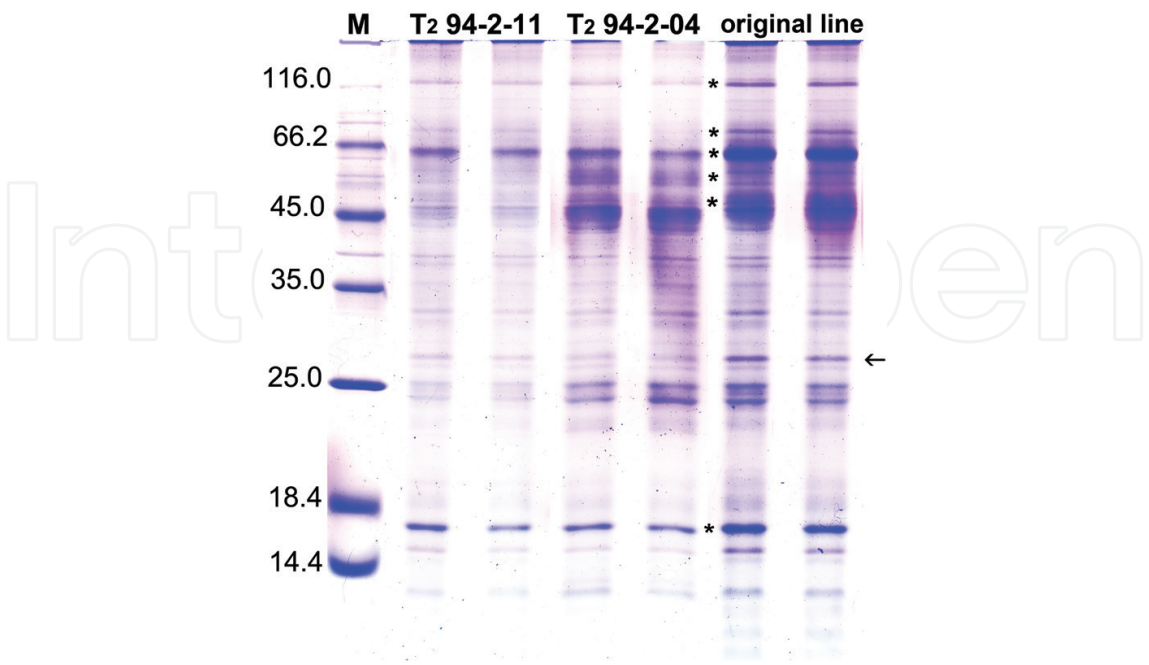

Figure 6. SDS-PAGE of endosperm proteins of transgenic sorghum plants with genetic construct for silencing of the γ-kafirin gene in non-reducing conditions. γ-kafirin is marked by arrow; the proteins, the amount of which varies in the original line and in transgenic plants, are marked by asterisks.

reduced in transgenic plants ([28], see Section 5). Perhaps the suppression of the synthesis of γ-kafirin caused also effect on synthesis or accumulation of α-kafirins.

Noteworthy, lowered amount of protein in the lanes of transgenic plants (**Figure 6**) is not an artifact, since in each sample the same amount of flour was taken in the study; all samples were subjected to the same treatment, and the same amount of extract was taken when carrying out SDS-PAGE. Therefore, such reduced protein content is due to the genetic characteristics of the samples. A similar decrease in protein content was observed in transgenic maize plants carrying constructs for RNA silencing of γ-zein [36].

4. Endosperm texture in plants with genetic construct for RNA silencing of the γ-kafirin gene

It is known that one of the consequences of silencing of γ-prolamins in maize and sorghum is a disruption of the formation of the vitreous layer of the endosperm. In previously obtained transgenic sorghum lines with genetic constructs for γ-kafirin silencing [24, 26, 27], as well as in the mutant with high digestibility [37], the kernels had a floury endosperm type. In transgenic maize plants, silencing of γ-zein also resulted in reduction of the vitreous layer and the formation of floury endosperm that suggests its role in interaction with starch granules and in the formation of the vitreous endosperm [36]. In this connection, we paid special attention to the endosperm texture in the kernels of our transgenic plants.

Careful examination of the kernels developed on panicles of T_1 plants obtained in our experiments revealed three plants, #94-3, #94-4 and #94-6, in which the kernels with almost floury endosperm were found (**Figure 7A**) [28]. Such kernels clearly differed from those of the original non-transgenic line, which have a thick vitreous layer (**Figure 7B**). The amount of such kernels varied in different panicles of one and the same T_1 plants. For example, in T_1 plant, #94-2, all kernels developed on its first panicle did not express floury phenotype, although kernels on its second panicle had either almost floury or modified structure of endosperm. In such kernels, the vitreous layer was significantly reduced and developed as sectors or blurs surrounded by floury endosperm (**Figure 7C–E**). Remarkably, these kernels resemble the kernels of recombinant sorghum lines obtained by hybridization of highly digestible mutant with floury endosperm (*hdhl*) with ordinary sorghum lines with low protein digestibility and vitreous endosperm [38]. Formation of this endosperm type in our transgenic plants apparently reflects peculiarities of expression of inserted genetic construct during kernel development.

Modified endosperm type of plant #94-2 inherited for three generations and was observed in T_2 and T_3 families (94-2-04; 94-2-05 and 94-2-11) characterized by high *in vitro* protein digestibility (see Section 5), although kernels with thin or irregularly developed vitreous endosperm (**Figure 7F–H**) also formed in panicles of plants from these families. The plants from T_2 and T_3 families from the progeny #94-3 (94-3-04; 94-3-08) had both modified, irregularly developed and normal vitreous endosperm types.

No variation of endosperm type was observed in the kernels developed in other PCR-positive T_1 plants, #94-1 and #94-5.
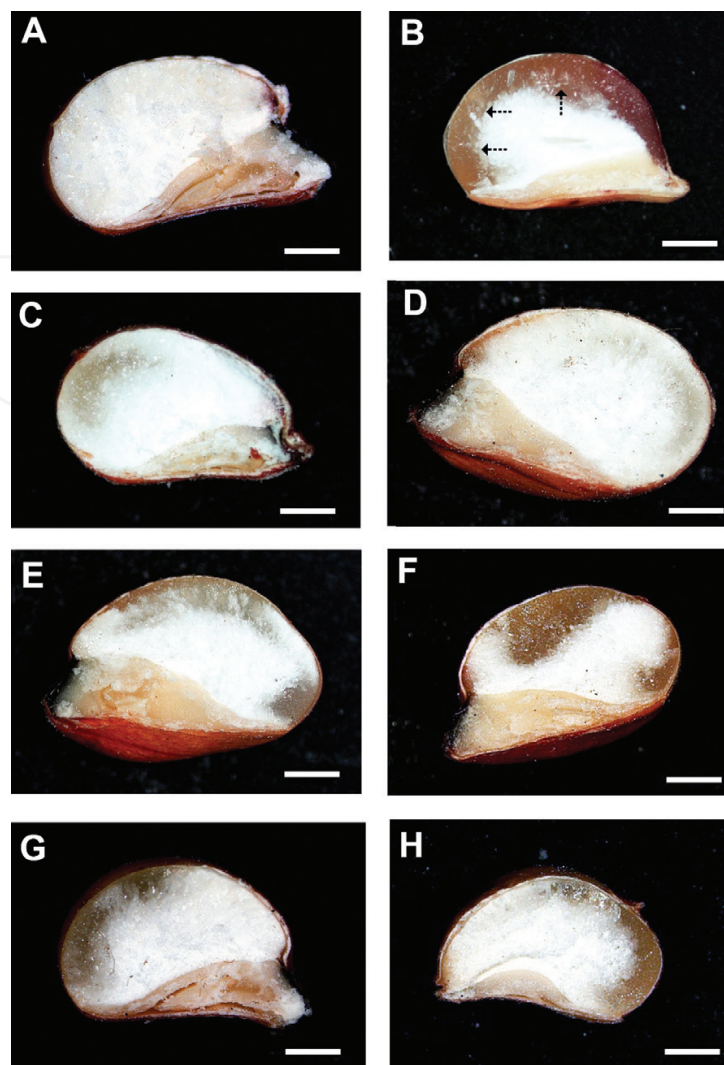
Figure 7. Cross sections of kernels with different types of endosperm of transgenic sorghum plants with genetic construct for silencing of the γ-kafirin gene. (A) Kernel with floury endosperm (T$_3$ 94-2-05-1); (B) kernel of original non-transgenic line Zheltozernoe 10 with thick vitreous endosperm (marked by arrows); (C–E) modified endosperm type with blurs and sectors of vitreous endosperm (T$_2$ 94-2-05, T$_2$ 94-2-04, T$_1$ 94-6, respectively); (F–H) irregularly developed vitreous endosperm (T$_2$ 94-3-08; T$_3$ 94-2-05-2; T$_3$ 94-2-11-2, respectively). Bar = 1 mm.

5. *In vitro* digestibility of endosperm proteins

To study *in vitro* protein digestibility, the method of whole-grain flour pepsin treatment, widely practiced in the past few years, was used [37–42]. The flour (20 mg) of transgenic samples (kernels of transgenic plants from T$_1$–T$_3$ generations) and of original non-transgenic line Zh10 was treated with 5 ml of 0.15% pepsin solution (Sigma-Aldrich, activity: 806 units/mg of protein) in a 0.1 M potassium phosphate buffer (pH 2.0) for 120 min at 37°C with repeated shaking. The control samples were incubated in potassium phosphate buffer without pepsin addition under the same conditions. For quantitative estimation of protein digestibility, the digested and control samples were centrifuged and the pellet was incubated with a sample buffer (0.0625 M Tris·HCl, pH 6.8) under reducing conditions (see above). The samples were subjected to SDS-PAGE (see above). After electrophoresis, the gels were scanned. The amount

of protein, expressed as volume (intensity × area) of kafirin bands or of total protein bands in the lane, was quantified with the Scangel program (Dr. A.F. Ravich, Agricultural Research Institute of the South-East Region, Saratov, Russian Federation) [41]. The digestibility value was counted as the percent ratio of the difference between protein volume in the control sample and in digested sample to the protein volume in the control sample. All experiments were performed in two replications.

It was found that transgenic plants obtained in our experiments significantly differed in digestibility of endosperm storage proteins from the original non-transgenic line Zh10 [28]. Comparison of electrophoretic spectra before and after pepsin digestion of proteins of T_1 plant #94-2 (almost floury endosperm; **Figure 8A**, lanes 1, 2) with Zh-10 kernels (**Figure 8A**, lanes 5, 6) revealed that in transgenic plant the amount of undigested α-kafirin monomers and total undigested protein was significantly fewer (in 1.7–1.9 times) than in original non-transgenic line (**Table 2**). The digestibility value reached 85.4%, whereas in original line this value was about 60%, usual index for sorghum flour (**Table 3**). Remarkably, in kernels of transgenic plant #94-3-08 (T_2 generation) with thick irregularly developed vitreous endosperm (**Figure 8A**, lanes 3, 4), the differences in kafirin digestion, in comparison with original line Zh-10 (**Figure 8A**, lanes 5, 6), were more pronounced: the amount of undigested monomers was 17.5 times fewer, and the amount of total undigested protein was 4.7 times fewer than in original line (**Table 2**). The digestibility value reached 92% (**Table 3**).

One should note considerable differences in content of kafirin oligomers between original non-transgenic line Zh10 and transgenic plants (**Figure 8**). Decreased content of kafirin oligomers, which apparently was caused by reduction of γ-kafirin synthesis, might be the reason of higher protein digestibility in transgenic plants.

Another examples of significantly improved kafirin digestibility in transgenic plants obtained in our experiments are presented in **Figure 8B**, where almost complete disappearance of kafirin monomers after pepsin digestion was observed in plants from T_2 generation with both floury (#94-2-11, lanes 5, 6) and modified endosperm (#94-2-04, lanes 1, 2, and #94-2-05, lanes 3, 4). Total protein digestibility indices in 94-2-05 and 94-2-11 plants reached 74.1% and 90.7%, respectively, that significantly differed from original non-transgenic line (**Table 3**). Remarkably, in electrophoretic spectra of digested samples of transgenic plants, one should note the polypeptides with molecular weights approx. 40 and 42 kDa. Previously, we found that these polypeptides were more prominent in electrophoretic spectra of more digestible lines than in spectra of poorly digestible ones [41]. In this study, appearance of these polypeptides in transgenic samples coincides with almost complete digestion of kafirin monomers and slightly reduces total protein digestibility values (**Table 3**).

Plants from T_3 generation inherited improved digestibility of kafirins. Comparison of electrophoretic spectra of proteins obtained from plants #94-2-11-2 and # 94-2-11-3 (**Figure 9A**, lanes 1–4), which were characterized by almost floury or modified endosperm, with the spectrum of the original line (**Figure 9A**, lanes 5, 6) before and after pepsin digestion showed that in transgenic plants, the amount of undigested α-kafirin monomers was significantly fewer (3.4–6.0 times, respectively) (**Table 2**). Likewise, the total sum of undigested proteins was also reduced (2.9–3.2 times). The digestibility value reached 85.5–87.8%, whereas in the original line this value was 59.3%, the usual index for sorghum flour (**Table 3**).
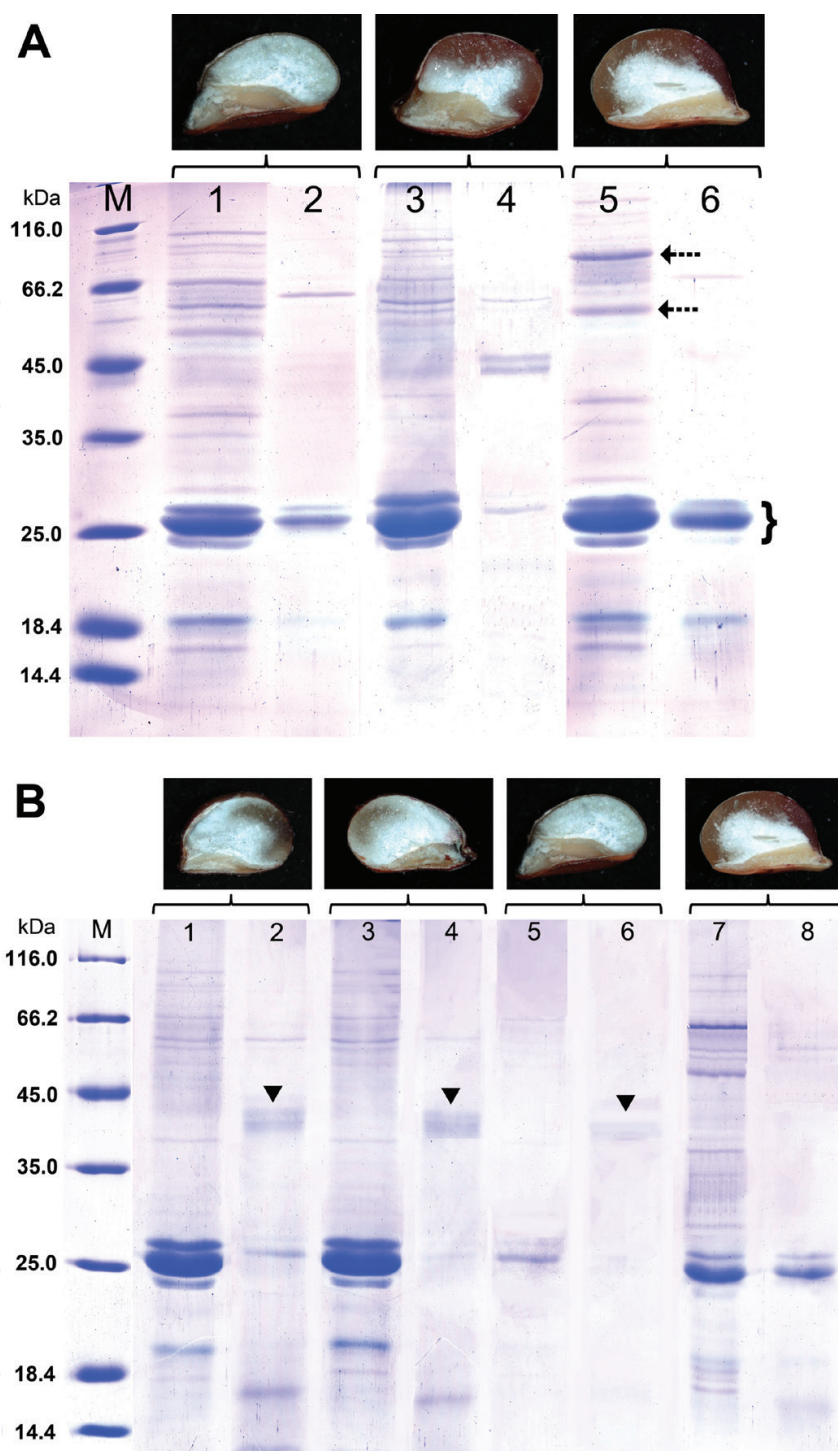
Figure 8. SDS-PAGE of endosperm proteins of kernels developed on transgenic sorghum plants with genetic construct for silencing of the γ-kafirin gene in reducing conditions. (A) 1, 2—#94-2 (T_1 generation) with almost floury endosperm; 3, 4—#94-3-8 (T_2 generation) with thick vitreous endosperm; 5, 6—original non-transgenic line Zheltozernoe 10 (Zh10) with normal vitreous endosperm; M—molecular weight markers (kDa; Thermo Scientific). 1, 3, 5—before, and 2, 4, 6—after pepsin digestion. Dashed arrows indicate probable kafirin oligomers. α-kafirin monomers are indicated by brace. (B) 1, 2—#94-2-04; 3, 4—#94-2-05, both with modified endosperm, in which vitreous layer is covered by thin floury layer (**Figure 4C**); 5, 6—#94-2-11 with floury endosperm; 7, 8—original non-transgenic line Zh10. 40 and 42 kDa appeared in digested samples are marked by arrows. 1, 3, 5, 7—before and 2, 4, 6, 8—after pepsin digestion (**Figure 8A** is published with the permission of the publishing house "Nauka").

Plant	Lane[1]	Estimated protein quantity[2]		Percent of undigested protein[3]	
		α-kafirin monomers	total	α-kafirin monomers	total
Figure 8A					
T_1 94-2	1 (c)	$4.887 \cdot 10^6$	$15.083 \cdot 10^6$	23.8	15.15
	2 (p)	$1.163 \cdot 10^6$	$2.285 \cdot 10^6$		
T_2 94-3-08	3 (c)	$8.166 \cdot 10^6$	$19.077 \cdot 10^6$	2.6	5.45
	4 (p)	$0.340 \cdot 10^6$	$1.925 \cdot 10^6$		
Zheltozernoe 10 (original line)	5 (c)	$5.124 \cdot 10^6$	$11.899 \cdot 10^6$	45.4	25.9
	6 (p)	$2.328 \cdot 10^6$	$3.079 \cdot 10^6$		
Figure 8B					
T_2 94-2-04	1 (c)	6.782	10.658	9.3	28.9
	2 (p)	0.633	3.085		
T_2 94-2-05	3 (c)	$6.667 \cdot 10^6$	$12.917 \cdot 10^6$	6.7	23.0
	4 (p)	$0.448 \cdot 10^6$	$1.949 \cdot 10^6$		
T_2 94-2-11	5 (c)	$1.277 \cdot 10^6$	$4.495 \cdot 10^6$	6.1	9.4
	6 (p)	$0.078 \cdot 10^6$	$0.421 \cdot 10^6$		
Zheltozernoe 10 (original line)	7 (c)	$3.802 \cdot 10^6$	$12.034 \cdot 10^6$	48.7	37.2
	8 (p)	$1.853 \cdot 10^6$	$4.481 \cdot 10^6$		
Figure 9A					
T_3 94-2-11-2	1 (c)	$4.601 \cdot 10^6$	$7.055 \cdot 10^6$	13.9	11.6
	2 (p)	$0.638 \cdot 10^6$	$0.816 \cdot 10^6$		
T_2 94-2-11-3	3 (c)	$4.249 \cdot 10^6$	$6.829 \cdot 10^6$	7.9	10.4
	4 (p)	$0.336 \cdot 10^6$	$0.710 \cdot 10^6$		
Zheltozernoe 10 (original line)	5 (c)	$7.248 \cdot 10^6$	$21.939 \cdot 10^6$	47.8	33.4
	6 (p)	$3.464 \cdot 10^6$	$7.329 \cdot 10^6$		
Figure 9B					
T_3 94-3-08-2	1 (c)	$4.900 \cdot 10^6$	$8.845 \cdot 10^6$	6.8	13.9
	2 (p)	$0.331 \cdot 10^6$	$1.191 \cdot 10^6$		
T_3 94-3-08-3	3 (c)	$5.630 \cdot 10^6$	$10.256 \cdot 10^6$	4.3	8.7
	4 (p)	$0.243 \cdot 10^6$	$0.896 \cdot 10^6$		
T_3 94-3-08-1	5 (c)	$5.793 \cdot 10^6$	$8.656 \cdot 10^6$	13.5	12.6
	6 (p)	$0.782 \cdot 10^6$	$1.091 \cdot 10^6$		

Plant	Lane[1]	Estimated protein quantity[2]		Percent of undigested protein[3]	
		α-kafirin monomers	total	α-kafirin monomers	total
Zheltozernoe 10 (original line)	7 (c)	$10.090 \cdot 10^6$	$19.495 \cdot 10^6$	56.4	38.8
	8 (p)	$5.692 \cdot 10^6$	$7.570 \cdot 10^6$		

[1]c—control sample; p—pepsin treatment.

[2]Values are expressed as amount of dots (intensity × mm²).

[3]Percentage from estimated protein quantity in undigested sample.

Table 2. Quantitative analysis of SDS-PAGE of total flour proteins from kernels of transgenic sorghum plants obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL.

Plant	Endosperm type	Protein digestibility (%)
Plants from T_1 and T_2 families		
T_1 94-2	Floury	85.4 c
T_1 94-6	Floury	85.2 c
T_2 94-2-05	Modified	74.1 b
T_2 94-2-11	Floury	90.7 cd
T_2 94-3-08	Vitreous, irregular	92.0 d
Zheltozernoe 10 (original non-transgenic line)	Vitreous	60.4 a
F		71.52**
Plants from T_3 families		
T_3 94-2-11-2	Modified	87.8 b
T_3 94-2-11-3	Modified	85.5 b
T_3 94-2-04-2	Modified	85.2 b
T_3 94-3-04-1	Floury	83.1 b
T_3 94-3-04-1	Modified	90.3 c
T_3 94-3-08-2	Vitreous, irregular	86.2 b
T_3 94-3-08-3	Vitreous, irregular	88.3 b
Zheltozernoe 10 (original non-transgenic line)	Vitreous	59.3 a
F		68.311**

Notes: Each value is a mean from two replications. Data followed by the same letter did not differ significantly ($P < 0.05$) from plant from the same group of families according to Duncan Multiple Range Test. Protein digestibility was calculated as percent ratio of difference between total estimated protein quantity in the control and digested sample to total estimated protein quantity in the control sample.

**Significant at $P < 0.01$.

Table 3. *In vitro* protein digestibility of sorghum flour from kernels of transgenic plants obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL.
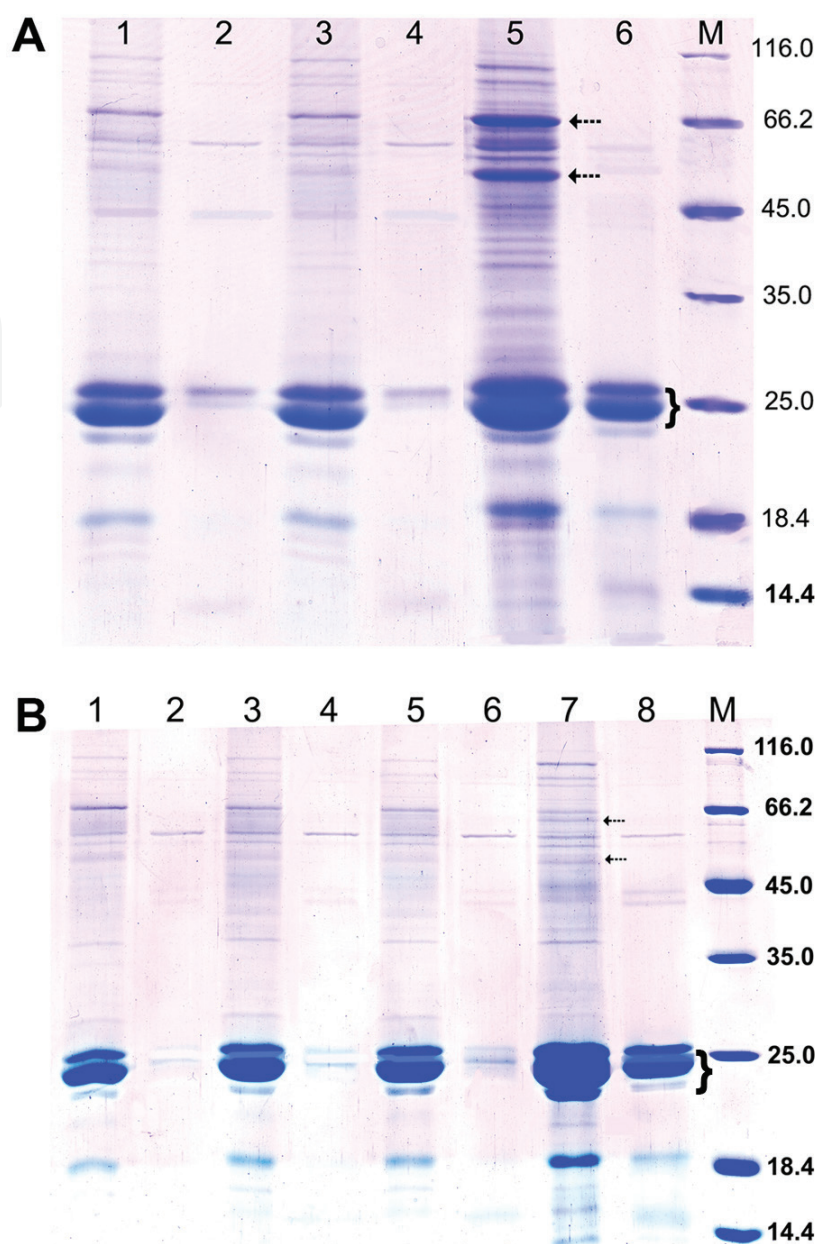
Figure 9. SDS-PAGE of endosperm proteins of kernels of transgenic sorghum plants from T$_3$ families #94-2-11 (with modified endosperm) and (with irregular vitreous endosperm) in reducing conditions. (A) 1, 2—#94-2-11-2; 3, 4—#94-2-11-3; 5, 6—original non-transgenic line Zh10; M—molecular weight markers (kDa). Dashed arrows indicate fraction of kafirin oligomers; brace—α-kafirin monomers. 1, 3, 5—control samples; 2, 4, 6—samples after pepsin digestion. (B) 1–6—Three individual plants from #94-3-08 family; 7, 8—original non-transgenic line Zh10. 1, 3, 5, 7—before and 2, 4, 6, 8—after pepsin digestion (published with the permission of the publishing house "Nauka").

Improved *in vitro* protein digestibility was observed also in plants from other T$_3$ families: #94-2-04, #94-3-04 and #94-3-08 (**Table 3**). In these plants, kernels had either floury or modified endosperm (#94-2-04-2; #94-3-04-1) or endosperm with irregularly developed vitreous layer (#94-3-08). Quantitative analysis showed that the level of digestibility of endosperm proteins in these plants was 83–90%, significantly differing from the digestibility of proteins in the original non-transgenic line.

Thus, the comparison of electrophoretic spectra of endosperm proteins before and after pepsin treatment showed a high level of kafirin digestibility in transgenic sorghum plants, harboring genetic construct for silencing of the γ-kafirin gene. Such electrophoretic spectra of digested endosperm proteins are not characteristic of ordinary sorghum cultivars obtained by classical breeding [40–42] except highly digestible sorghum mutant (*hdhl*) and its hybrids [37–39]. Apparently, a decrease in the level of γ-kafirin increases the digestibility of α-kafirins. This increase may be due to chemical reasons (reduction of polymerization) and/or physical reasons (change in the spatial arrangement of α-kafirins in the protein bodies that increase their availability to pepsin digestion).

Earlier it was reported on obtaining of transgenic sorghum plants carrying genetic constructs for silencing of γ- and α-kafirins, which were characterized by increased *in vitro* protein digestibility [25–27]. However, electrophoretic spectra of endosperm proteins after pepsin treatment were not shown in these studies. It should be noted also that in these studies improvement of kafirin digestibility was induced by complex genetic constructs that contained inverted repeats of several kafirin genes ($\delta 2$, $\gamma 1$, $\gamma 2$; or $\alpha 1$, $\delta 2$, $\gamma 1$, $\gamma 2$). These repeats were separated by the sequence of ADH1 intron, and the constructs were driven by the maize 19-kDa α-zein promoter [24–26]. In another work [27], the genetic construct included the complete sequence of the γ-kafirin gene, which was terminated by a nucleotide sequence of the self-cleaving ribozyme of tobacco ringspot virus that should destroy γ-kafirin mRNA. In our study [28], the effect was achieved by using a simpler genetic construct, containing inverted repeats of a short segment of the gene γ-kafirin (307 bp) separated by *ubi1*-intron gene, under the control of the constitutive *35S*-promoter, which allowed us to reach apparently rather high level of silencing of a target gene.

6. High-pressure liquid chromatography (HPLC) analysis of total amino acid content

An important feature of transgenic plants of sorghum and maize with silencing of prolamin genes is an increased proportion of essential amino acids in kernels, in particular the lysine proportion. Previously, this effect was observed in the silencing of genes of the main prolamin fractions: α-zein [22, 23] and α-kafirin [24, 27]. It was assumed that the suppression of the synthesis of these proteins, characterized by a low content of lysine, results in upregulating non-storage protein genes and appearance of lysine-rich proteins [23, 27].

In our experiments, the total amino acid content in the kernels of three transgenic plants from the T_2 generation with high *in vitro* protein digestibility: #94-2-11, #94-2-04 (both with modified endosperm) and #94-3-08 (with vitreous endosperm) was studied by using HPLC [28]. As can be seen from **Table 4**, content of a number of amino acids (leucine, proline, serine, isoleucine, histidine, tyrosine) and total amino acid content were significantly reduced in transgenic plants #94-2-04 (−40.2%, in comparison with the original non-transgenic line) and #94-3-08 (−22.8%). At the same time, the relative content of two major essential amino acids, lysine and threonine, significantly increased. Lysine proportion is increased by 1.6–1.7 times: from 1.54% of total amino acid content in the flour of original non-transgenic line Zh10 to 2.41–2.63% in transgenic plants #94-3-08 and #94-2-04, respectively, with vitreous and modified endosperms.

Amino acid	Control (Zh10)	T$_2$ 94-2-11	T$_2$ 94-2-04	T$_2$ 94-3-08
Glu	2.84 ± 0.28	2.63 ± 0.15	**1.62 ± 0.07*** (−43.0%)	2.17 ± 0.08
Leu	1.72 ± 0.12	1.54 ± 0.08	**0.97 ± 0.04****(−43.6%)	**1.27 ± 0.03*** (−26.2%)
Ala	1.10 ± 0.11	1.02 ± 0.06	**0.65 ± 0.02*** (−40.9%)	0.85 ± 0,04
Pro	0.98 ± 0.07	0.85 ± 0.02	**0.58 ± 0.02**** (−40.8%)	**0.76 ± 0.02*** (−22.4%)
Asp	0.76 ± 0.03	0.74 ± 0.05	**0.44 ± 0.01**** (−42.1%)	0.57 ± 0.04
Phe	0.73 ± 0.01	0.59 ± 0.01	**0.39 ± 0.01*** (−46.6%)	0.49 ± 0.01
Ser	0.58 ± 0.03	0.52 ± 0.02	**0.35 ± 0.01**** (−39.7%)	**0.45 ± 0.01*** (−22.4%)
Val	0.57 ± 0.05	0.53 ± 0.02	**0.35 ± 0.01*** (−38.6%)	0.45 ± 0.01
Ile	0.48 ± 0.03	0.44 ± 0.02	**0.29 ± 0.01**** (−39.6%)	**0.36 ± 0.02*** (−25.0%)
Thr	0.40 ± 0.03	0.39 ± 0.01	**0.27 ± 0.00*** (−32.5%)	0.31 ± 0.08
Tyr	0.40 ± 0.02	0.38 ± 0.03	**0.24 ± 0.01**** (−40.0%)	**0.30 ± 0.02**** (−25.0%)
Arg	0.38 ± 0.02	0.33 ± 0.02	0.25 ± 0.02	0.32 ± 0,01
Gly	0.35 ± 0.03	0.34 ± 0.01	**0.25 ± 0.00*** (−28.6%)	0.31 ± 0.00
His	0.26 ± 0.01	0.23 ± 0.01	**0.15 ± 0.01**** (−42.3%)	**0.19 ± 0.01**** (−26.9%)
Lys	0.19 ± 0.04	0.23 ± 0.02	0.19 ± 0.00	0.22 ± 0.00
Cys/2	0.04 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.04 ± 0.01
Met	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Total	11.85 ± 0.86 c	10.86 ± 0.50 c	7.09 ± 0.24 a (−40.2%)	9.15 ± 0.23 b (−22.8%)
Lys (%)	1.54 a	2.14 b	2.63 c	2.41 bc
Thr (%)	3.37 a	3.59 a	3.86 b	3.42 a

Notes: Values are mean ± standard error from three replications. Data marked in bold differ significantly from the original non-transgenic (control) line Zh10 at $P < 0.05$ (*) and $P < 0.01$ (**) according to Student's T-test. Percentage of reduction is indicated in parenthesis.

Data for total amount of amino acid content and for percentage of lysine and threonine from the total amino acid content followed by the same letter did not differ significantly ($P < 0.05$) according to Duncan Multiple Range Test.

Table 4. Total amino acid content in kernels of transgenic sorghum plants obtained by genetic transformation with *A. tumefaciens* GV3101/pNRKAFSIL (g/100 g flour) (published with the permission of the publishing house "Nauka").

Such increase of the relative content of lysine and threonine in transgenic sorghum plants, coupled with a significant reduction of the total level of amino acids (**Table 4**), presumably was caused by decrease in the content of α-kafirins poor in lysine and threonine, whereas the synthesis of other proteins remained undisturbed. Accordingly, the relative proportions of lysine and threonine increased. The lower α-kafirins content in transgenic plants relative to the original non-transgenic line is clearly evident in the above electrophoresis photographs (**Figures 8** and **9**). Perhaps the suppression of the synthesis of γ-kafirin disrupts the formation of protein bodies and prevents the accumulation of α-kafirins, but does not affect the synthesis of other proteins richer in lysine and threonine.

7. Conclusion

Summarizing, using *Agrobacterium*-mediated genetic transformation with the strain carrying the genetic construct for silencing of γ-kafirin gene, we have obtained transgenic sorghum plants with significantly improved *in vitro* protein digestibility. The basis of such improved digestibility may be a reduction in the level of γ-kafirin, which causes formation of poorly digestible kafirin oligomers and development of vitreous endosperm. Further studies of these plants, including analysis of the expression of the genetic construct at the molecular level, will contribute to the understanding of regularities of endosperm development and possible use of these plants in sorghum breeding.

Obstacles along this path have both scientific reasons (instability of transgene expression, effects of transgenes on agronomically important traits) and social basis (public opposition to genetically modified plants). In future, to overcome public fears on "danger" of genetically modified organisms, sorghum plants with a modified synthesis of kafirins should be obtained by using marker-free technologies of genetic engineering or technologies of genome editing. With obtaining objective data from biosafety experiments, genetically modified sorghum plants with improved kafirin digestibility will be in demand on the market because they will combine favorable traits of sorghum (high grain productivity, resistance to drought stress) with a high nutritive value.

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Author details

Lev A. Elkonin[1]*, Julia V. Italyanskaya[1], Valery M. Panin[1] and Nikolai Yu. Selivanov[2]

*Address all correspondence to: lelkonin@gmail.com

1 Agricultural Research Institute of the South-East Region, Saratov, Russia

2 Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov, Russia

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