Chapter

RNAi-Mutants of *Sorghum bicolor* (L.) Moench with Improved Digestibility of Seed Storage Proteins

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Abstract

Modification of the composition of grain storage proteins is an intensively developing area of plant biotechnology, which is of particular importance for sorghum – high-yielding drought tolerant crop. Compared to other cereals, the majority of sorghum cultivars and hybrids are characterized by reduced nutritional value that is caused by a low content of essential amino acids in the seed storage proteins (kafirins), and resistance of kafirins to protease digestion. RNA interference (RNAi) by suppressing synthesis of individual kafirin subclasses may be an effective approach to solve this problem. In this chapter, we review published reports on RNAi silencing of the kafirin-encoding genes. In addition, we present new experimental data on phenotypic effects of RNAi-silencing of *γ-KAFIRIN-1* gene in sorghum cv. Avans. To obtain RNAi mutants with *γ-KAFIRIN-1* gene silencing we used *Agrobacterium*-mediated genetic transformation. Transgenic kernels had modified endosperm type with reduced vitreous layer and significantly improved *in vitro* protein digestibility (93% vs. 57%, according to the densitometry of SDS-PAGE patterns). SDS-PAGE of transgenic kernels showed lowered level of kafirins and appearance of globulin proteins, which were not observed in the original cultivar. For the first time, the cases of instability of inserted genetic construct were identified: elimination of *ubi1*-intron that is a constituent part of the genetic construct for RNAi silencing, or *nos*-promotor governing expression of the marker gene (*bar*) (in the RNAi mutants of cv. Zheltozernoe 10). The research findings presented in this chapter provide strong evidence that RNA interference can be used for improvement of the nutritional properties of sorghum grain.

Keywords: kafirins, *in vitro* protein digestibility, RNAi-mutants, endosperm, *Sorghum bicolor* (L.) Moench

1. Introduction

Grain sorghum is one of the most promising and relatively poorly studied agricultural crops. With its high drought tolerance, sorghum is capable of producing high grain yields in conditions of minimal moisture supply. This crop is of special

importance in the regions regularly exposed to drought, where the stable production of traditional cereals – wheat, maize, barley – is challenging. Moreover, due to the global warming of climate the importance of this crop will steadily increase. Sorghum is already one of the five most important cereal crops cultivated on the Earth. In addition, sorghum grain is gluten-free and can serve as a source of protein for people with celiac disease who are forced to follow a gluten-free diet.

At the same time, compared with other cereals, sorghum grain has a number of significant disadvantages: its storage proteins (kafirins), the content of which reaches 14–16% in some lines and varieties, are poorly digestible by proteases (pepsin, trypsin) [1–4]. The resistance of kafirins to proteolytic digestion reduces the digestibility of starch, which accumulates in significant amounts in sorghum grain (up to 70–75%) since undigested proteins reduce the availability of amylolytic enzymes to starch grains [3, 5, 6]. In addition, the kafirins have low content of indispensible amino acids – lysine, threonine, and tryptophan – and therefore are characterized by low nutritional value [7, 8]. In this regard, increasing the functionality of proteins in sorghum grain, improving their nutritional value is a very urgent problem that has both applied and fundamental importance.

The resistance of kafirins to proteolytic digestion is caused by several factors [9, 10]. Among them are the chemical composition of kafirins, some of which (γ- and β-kafirins) are abundant with sulfur-containing amino acids capable of forming intra- and intermolecular disulfide bonds, hardening protein molecules, and promoting the formation of oligo- and polymers resistant to protease digestion; interaction of kafirins with non-kafirin proteins and non-protein components, in particular, with tannins, which reduce the proteases activity, and with polysaccharides of starch grains; spatial organization of different kafirins in protein bodies of endosperm cells. It was hypothesized that γ-kafirin, which occupies the outer layer of protein bodies and which is the most resistant to proteolytic digestion, prevents the digestion of the α-kafirins – main storage proteins, located inside the protein bodies [11].

An important argument in favor of this hypothesis was the data obtained in the study of the P721Q mutant, induced by chemical mutagenesis and characterized by increased digestibility of kafirins, and the lines derived from this mutant [12, 13]. In this mutant, the protein bodies of endosperm cells have an irregular shape with invaginations. Moreover, γ-kafirin was located only at the bottom of such invaginations, without forming a continuous layer that impedes the access of proteases to α-kafirins [11, 13]. This mutation leads to the formation of kernels with a floury type of endosperm and an increased lysine content, and therefore was denoted with the symbol *hdhl* (*high digestibility high lysine*). Subsequent studies, however, revealed that the P721Q mutant has a point mutation in the signal sequence of one of the 10 copies of the gene encoding the 22 kDa α-kafirin [14]. This sequence is responsible for the packaging of α-kafirin inside the protein body. It was hypothesized that this mutation decreases the accumulation of α-kafirin in protein bodies that leads to a change in their ultrastructure and increases their sensitivity to the action of proteases [14].

To solve the problem of poor digestibility of kafirins various genetic and biotechnological approaches may be used: experimental induction of mutants with impaired synthesis or altered amino acid composition of kafirins [15]; identification of naturally occurring allelic variants of kafirins [16–19]; obtaining transgenic plants with the genetic constructs that induce silencing of γ- and/or α-kafirin genes [20–23]; editing the nucleotide sequences of kafirin genes in order to obtain lines with complete or partial knockout of these genes [24].

RNA interference (RNAi) technology is an effective genetic tool for gene silencing that was used to obtain metabolically engineered plants with improved virus

resistance, starch and oil content, and health benefits in different agriculturally important crops [25–28]. The proposed RNA silencing mechanism starts with the production of 20 to 25 bp small interfering RNAs (siRNAs), which are produced from genetic constructs encoding hairpin RNAs (hpRNA). A typical hpRNA construct is comprised of a sense and an antisense sequence of a portion of target gene mRNA as inverted repeats, and these inverted repeats are separated by a non-complementary spacer region. In most genetic constructs, a spliceable intron is used as spacer because it significantly improves RNA silencing efficiency in plants [29]. The sense and antisense sequences in the transcribed RNA are complementary to each other and form a hpRNA, which is processed by Dicer-like proteins (DCL). The DCL proteins generate siRNAs from a hpRNA precursor. One strand of the siRNA duplex is incorporated into an Argonaute (AGO) protein forming an RNA-induced silencing complex (RISC). The siRNA molecule guides the RISC to the complementary region of single-stranded RNA, and the AGO protein then cleaves the target mRNA.

RNA interference technology has been intensively used to suppress the synthesis of seed storage proteins in different crops including wheat, rice and maize (for review see: [30]). These experiments contributed to obtaining new information on the mechanisms of protein body formation, as well as the role of various classes of prolamins and glutenins in the development of endosperm and the technological properties of flour and dough.

The purpose of our investigations was to obtain the grain sorghum lines with improved digestibility of kafirins using RNA interference technology by

Name	Structure of genetic construction	Reference
pABS032	Maize 19-kDa α-zein promoter; inverted repeats of gene fragments encoding α-A1 (25kDa), α-B1 (19kDa), α-B2 (22kDa), γ1 (27 kDa), γ2 (50 kDa) and δ2 (15 kDa) kafirins, and lysine α-ketoglutarate reductase, separated by the intron of the *ADH1* gene	[20, 34, 35]
pABS166	Maize 19-kDa α-zein promoter; inverted repeats of gene fragments encoding α1 (25 kDa) and γ1 (27 kDa), separated by an intron of the *ADH1* gene	[20, 34, 35]
pABS149	Maize 19-kDa α-zein promoter; inverted repeats of gene fragments encoding γ1 (27 kDa), γ2 (50 kDa), and δ2 (15 kDa) kafirins, lysine α-ketoglutarate reductase, separated by an intron of the *ADH1* gene	[20, 34, 35]
pPTN915	γ-kafirin promoter; complete sequence of the γ-kafirin-1 gene (GeneBank acc. no. X62480), the sequence of the ribozyme gene of the tobacco mosaic virus as a terminator	[21]
pPTN1017	α-kafirin gene promoter; inverted repeats of the α-kafirin (29 kDa) gene fragment, separated by the intron of the Arabidopsis gene encoding the spliceosome D1 protein	[21]
pABS042	Maize 19-kDa α-zein promoter; inverted repeats of δ-kafirin 2 (18 kDa), γ-kafirin 1 (25 kDa), γ-kafirin 2 (50 kDa), and lysine α-ketoglutarate reductase gene fragments, separated by an intron of the alcohol dehydrogenase gene (*ADH1*)	[22]
pABS044	Maize 19-kDa α-zein promoter; inverted repeats of δ-kafirin 2 (18 kDa), γ-kafirin 1 (25 kDa), γ-kafirin 2 (50 kDa), α-kafirin-A1, and lysine α-ketoglutarate reductase gene fragments, separated by an intron of the alcohol dehydrogenase gene (*ADH1*)	[22, 35]
pNRKAF	*35S* promoter; inverted repeats of the γ-kafirin 1 gene fragment (GeneBank accession no. M73688), separated by the maize *ubi1*-intron	[23, 35]

Table 1.
Genetic constructs specially designed to induce RNA silencing of kafirin genes. The molecular masses of kafirins are given in accordance with the author's description.

introducing a genetic construct capable to induce *γ-KAFIRIN-1* silencing. For silencing the *γ-KAFIRIN-1* gene we used the construct pNRKAF [23] that consisted of segment of its nucleotide sequence ([31], GeneBank accession no. M73688) in forward and inverted orientation, which was separated by the sequence of the maize *ubi1*-intron. This construct was driven by the *35S* promoter. Such a construct should suppress the expression of the *γ-KAFIRIN-1* gene using RNA interference. A decrease in the level of γ-kafirin should have "stripped" the protein bodies in transgenic plants and facilitated the digestion of α-kafirins.

In this chapter, we describe phenotypic effects of RNAi-silencing of kafirin genes in two sorghum cultivars – Zheltozernoe-10 (Zh10) and Avans, which contain pNRKAF genetic construct introduced by agrobacterial transformation, as well as characteristic features of other sorghum lines carrying similar genetic constructs for silencing kafirin genes created by other research groups (**Table 1**).

2. Decreased content of kafirins

The primary effect of the functioning of genetic constructs for RNA silencing of kafirin genes is a decreased level of transcripts of these genes. Such an effect was shown for the pPTN915 genetic construct, designed to suppress the expression of

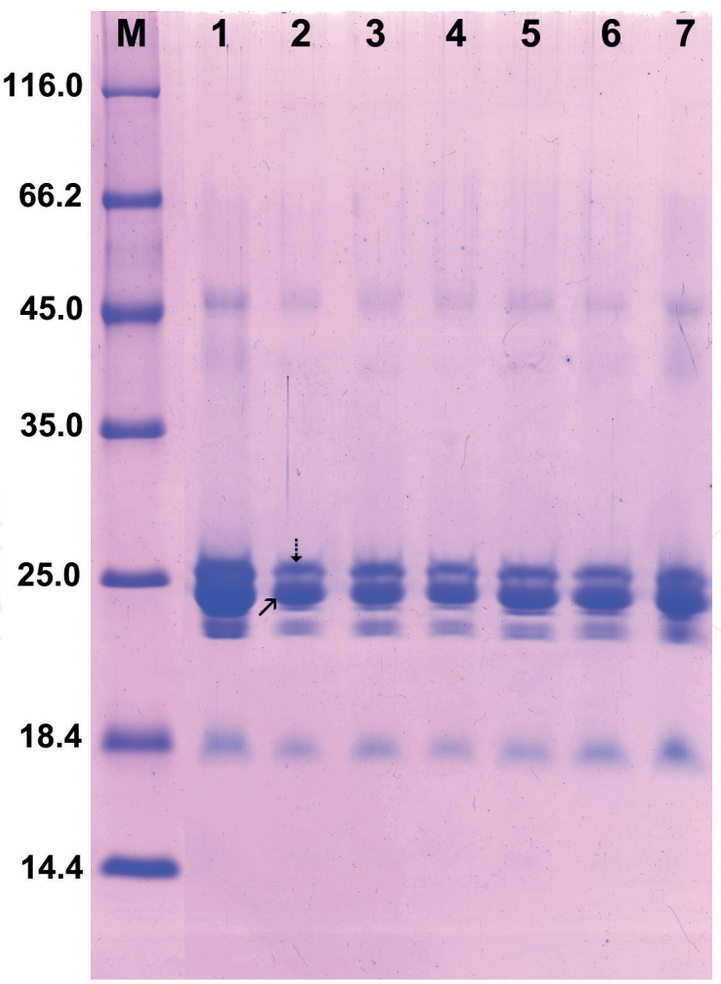

Figure 1.
SDS-PAGE of kafirins from kernels of transgenic plants from the T_1 generation of the RNAi mutant, cv. Avans, isolated under reducing conditions (with the addition of 2-mercaptoethanol). 1 – Original non-transgenic cv. Avans; 2–7 – Individual plants from the T_1 family: 2–6 – Plants with a floury endosperm, containing ubi1-intron; 7 – Plant with a vitreous endosperm, not containing ubi1-intron; M – Molecular mass markers. Kafirins were extracted according to [20]. The arrow marks α-kafirin; the dotted arrow marks γ-kafirin.

the γ-kafirin gene [21]. Many studies using SDS-PAGE have also clearly demonstrated a decrease in the content of monomers and polymers of kafirins [20, 22, 23, 32]. In our experiments, SDS-PAGE of proteins extracted from kernels of transgenic plants of Zh10 in non-reducing conditions (without the addition of 2-mercaptoethanol, which breaks the S-S bonds and, thereby, destroys the polymers of the kafirins), showed a decreased content of γ-kafirin monomer (28 kDa), as well as 47 and 66 kDa oligomers, which are supposed to arise as a result of γ-kafirin polymerization [33]. SDS-PAGE of kafirins extracted under reducing conditions from the kernels of transgenic plants of the Avans cultivar (T_1 generation) carrying the same genetic construct revealed also a noticeable decrease in the content of γ- and α-kafirins (**Figure 1**).

3. Improvement of *in vitro* protein digestibility

The main goal of experiments on silencing of kafirin genes is to improve seed storage protein digestibility. Herewith, depending on the structure of the genetic construct, suppression of certain subclasses of kafirins, and the cultivars used in experiments, the level of digestibility varied significantly.

For example, transgenic plants of cv. Tx430 carrying the ABS166 genetic construct containing inverted repeats of several kafirin genes (α, γ, δ) separated by the intron sequence of the alcohol dehydrogenase gene (*ADH1*) and controlled by the 19-kDa α-zein promoter from maize were characterized by improved *in vitro* protein digestibility. Pepsin treatment of the raw flour and flour that underwent the cooking procedure resulted in 78% and 61% digestibility, respectively, while in the non-transgenic control these indicators varied within 40–50% and 34–40%, respectively [34, 35]. The genetic construct for the silencing of δ- and γ-kafirins (ABS149) also improved the digestibility of raw flour, but did not affect the digestibility of the cooked flour.

Subsequently, new transgenic plants were obtained in the sorghum public line P898012 using other genetic constructs ABS042 and ABS044, created during the ABS (Africa Biofortified Sorghum) project [22]. In these plants, an improvement in the digestibility of flour subjected to the cooking procedure was recorded: from 28% in the control to 39% (for the ABS042 construct for silencing γ- and δ-kafirins), and up to 59% (for the ABS044 construct for silencing α-, γ- and δ-kafirins).

Analysis of ultrastructure of protein bodied showed that in transgenic lines with α-kafirin silencing protein bodies were irregular in shape and had invaginations similar to P721Q mutant [34, 35]. In transgenic lines with γ-kafirin silencing, a diameter of protein bodies was reduced in comparison with original non-transgenic line [36]. In addition, in one of the studied lines, 42–1, protein bodies were highly irregular in shape, with deep invaginations present at the periphery, while in the line 42–2, the protein bodies had small peripheral indentations that gave the boundary region a cracked appearance.

In the experiments of T. Kumar et al. [20] the genetic constructs pPTN915 and pPTN1017 designed for the induction of silencing γ- or α-kafirin, respectively, were also introduced into the genome of the Tx430 line through agrobacterial transformation. *In vitro* digestibility of proteins extracted from the flour of transgenic kernels with silencing of γ-kafirin, subjected to cooking procedure, did not differ from the non-transgenic control, while the silencing of α-kafirin by pPTN1017 improved the *in vitro* protein digestibility of flour subjected to cooking.

Transgenic plants of cv. Zh10 obtained in our experiments carrying the genetic construct pNRKAF for silencing *γ-KAFIRIN-1* gene, also had a significantly

improved *in vitro* digestibility of flour proteins [22]. Comparison of electrophoretic spectra before and after pepsin digestion showed that in the transgenic plants the amount of undigested monomers of α-kafirin and total undigested protein was significantly less (1.7–1.9 times) than in the original non-transgenic line. The digestibility level reached 85.4%, while in the original line this value was about 60%. It is noteworthy that in the kernels of transgenic plant No. 94–3-08 (T_2) with a thick vitreous endosperm, the differences in the digestion of kafirins were more pronounced: the amount of undigested monomers was 17.5 times less, and the amount of total undigested protein was 4.7 times less than in the original line, while the level of digestibility reached 92%.

Plants from the T_3 generation inherited the improved digestibility of kafirins. In these plants, kernels had either a modified type of endosperm with reduced vitreous endosperm, or an endosperm with a well-defined vitreous layer. The level of digestibility of endosperm proteins in these plants was 83–90%, significantly higher than that of the original non-transgenic line (**Figure 2**). Apparently, a decrease in the level of γ-kafirin increases the digestibility of α-kafirins. This increase may be due to chemical reasons (decrease in the amount of polymers) and/or physical reasons (changes in the spatial arrangement of α-kafirins in protein bodies, which increase their availability for cleavage by pepsin). The effect of increased digestibility of kafirins was also observed in plants from the T_4 generation; however, in some cases it disappeared, possibly due to the instability of the introduced genetic construct, or due to its silencing (see below).

After experiments with the model cv. Zh10, we set the task of obtaining RNAi mutants with improved digestibility of kafirins in the new commercial cultivar Avans, which is characterized by a number of agronomically valuable traits. The analysis of the *in vitro* digestibility of proteins from kernels that set on one of the transgenic plants (#1–1) obtained by *Agrobacterium*-mediated genetic transformation with the strain carrying pNRKAF genetic construct showed a significantly higher level of digestibility compared to the original non-transgenic cultivar (**Figure 3**) (93% vs. 57%, according

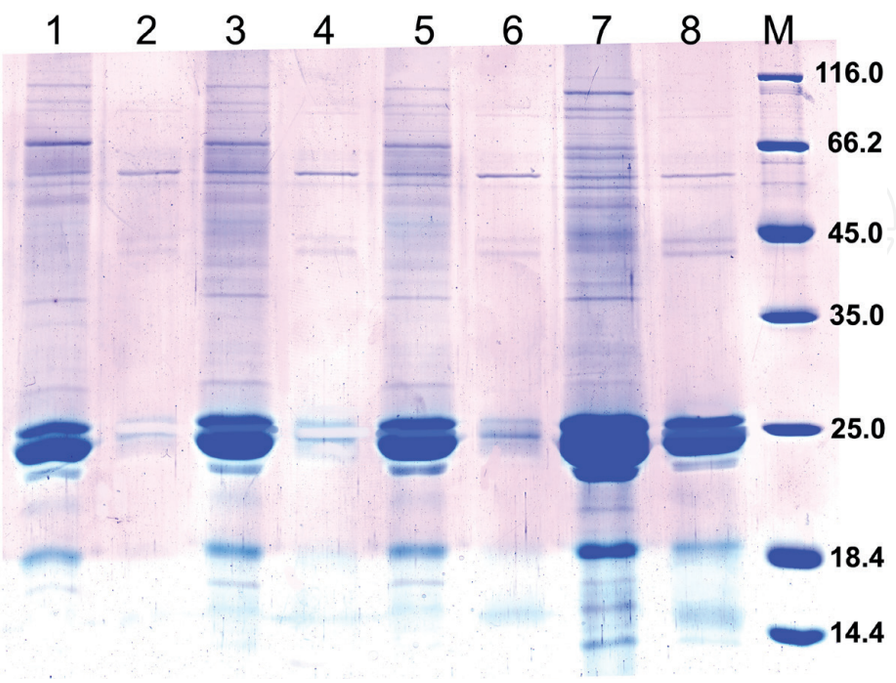

Figure 2.
Electrophoretic spectra of proteins from the flour of transgenic plants from T_3 family #94–3-08 with normal vitreous endosperm. 1–6 – Individual plants from T_3 generation; 7, 8 – Original non-transgenic line Zh10. 1, 3, 5, 7 – Before, 2, 4, 6, 8 – After pepsin digestion. M - molecular mass markers (kDa). [23].

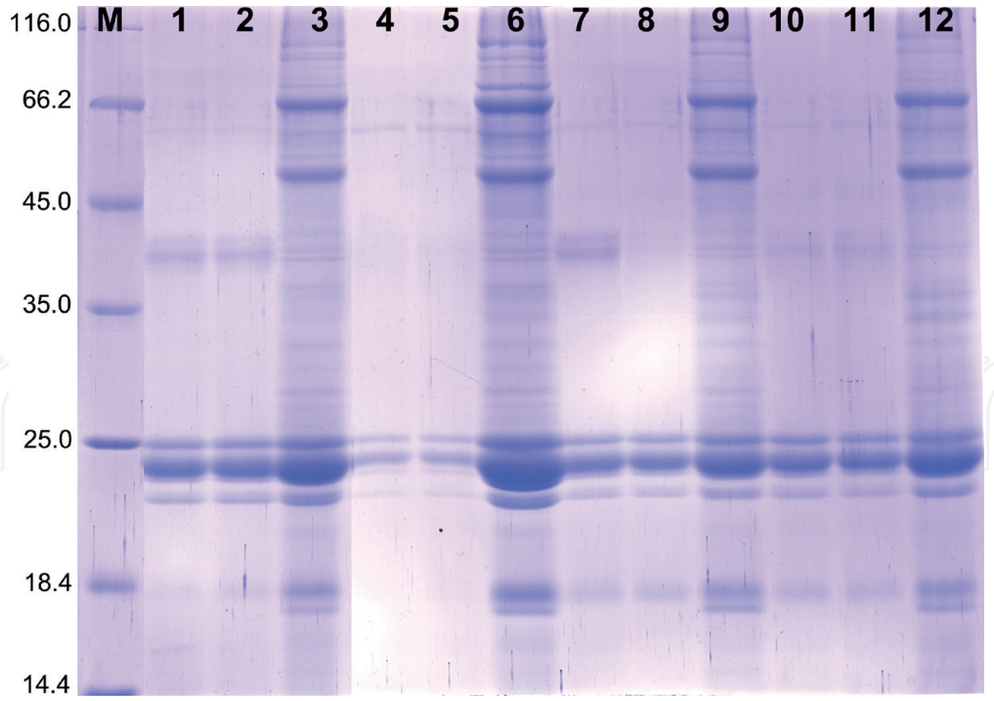

Figure 3.
Electrophoretic spectra of proteins from the flour of sorghum cv. Avans (1–3), transgenic plant #1–1 (4–6) and non-transgenic plants #5–1 (7–9) and #6–4 (10–12). M - molecular mass markers (kDa). 3, 6, 9, 12 – Before, 1, 2, 4, 5, 7, 8, 10, 11 – After pepsin digestion.

to the densitometry of SDS-PAGE patterns). A high level of kafirin digestibility was observed also in the next generation, T_1.

4. Modification of endosperm texture

An important consequence of the functioning of genetic constructs for silencing kafirin genes is a change in the texture of endosperm: in transgenic plants, in most cases, there is complete or partial loss of the vitreous layer, as a result of which the kernels contain only floury endosperm [21, 22, 34, 35]. In our experiments, the

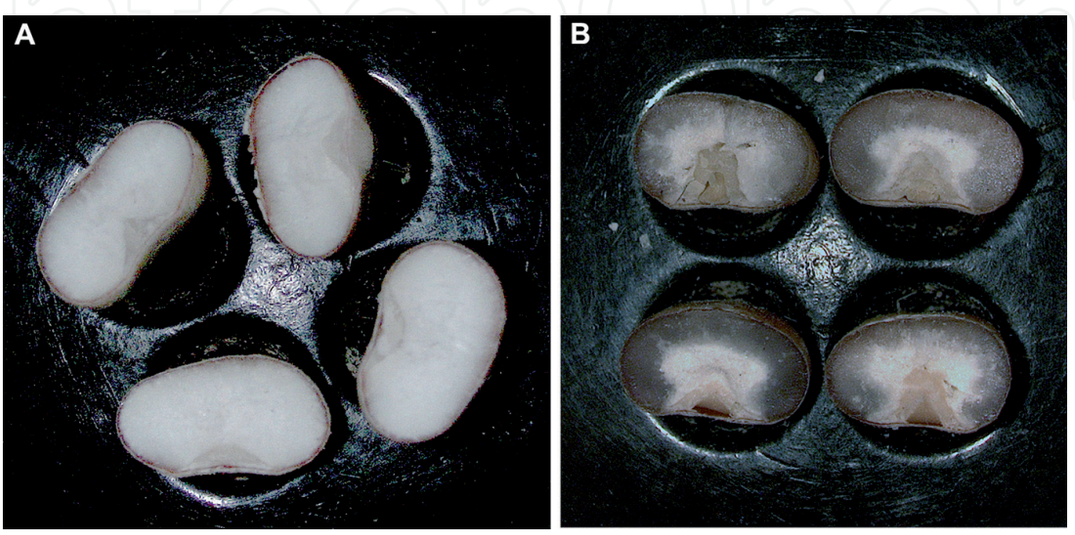

Figure 4.
Cross sections of the kernels of the RNAi mutant #1-1 (A) carrying genetic construct pNRKAF for RNAi silencing, and original cv. Avans (B).

RNAi mutant #1–1 of cv. Avans, had also a floury type of endosperm (**Figure 4**). It should be noted that, in similar experiments in maize, silencing of different zein genes also resulted in reduction of the vitreous endosperm and formation of kernels with floury endosperm [37–39]. It was shown that γ-zein gene plays an important role in the formation of the floury endosperm, and silencing of this gene modified the structure of protein bodies and their connection with starch grains that result in formation of floury endosperm [38].

Unfortunately, the presence of floury endosperm is a significant disadvantage of the obtained lines, since the absence of a vitreous layer increases the fragility of the kernels and reduces its resistance to fungal diseases. It should be noted that the floury (opaque) type of endosperm is characteristic of the P721Q mutant and many

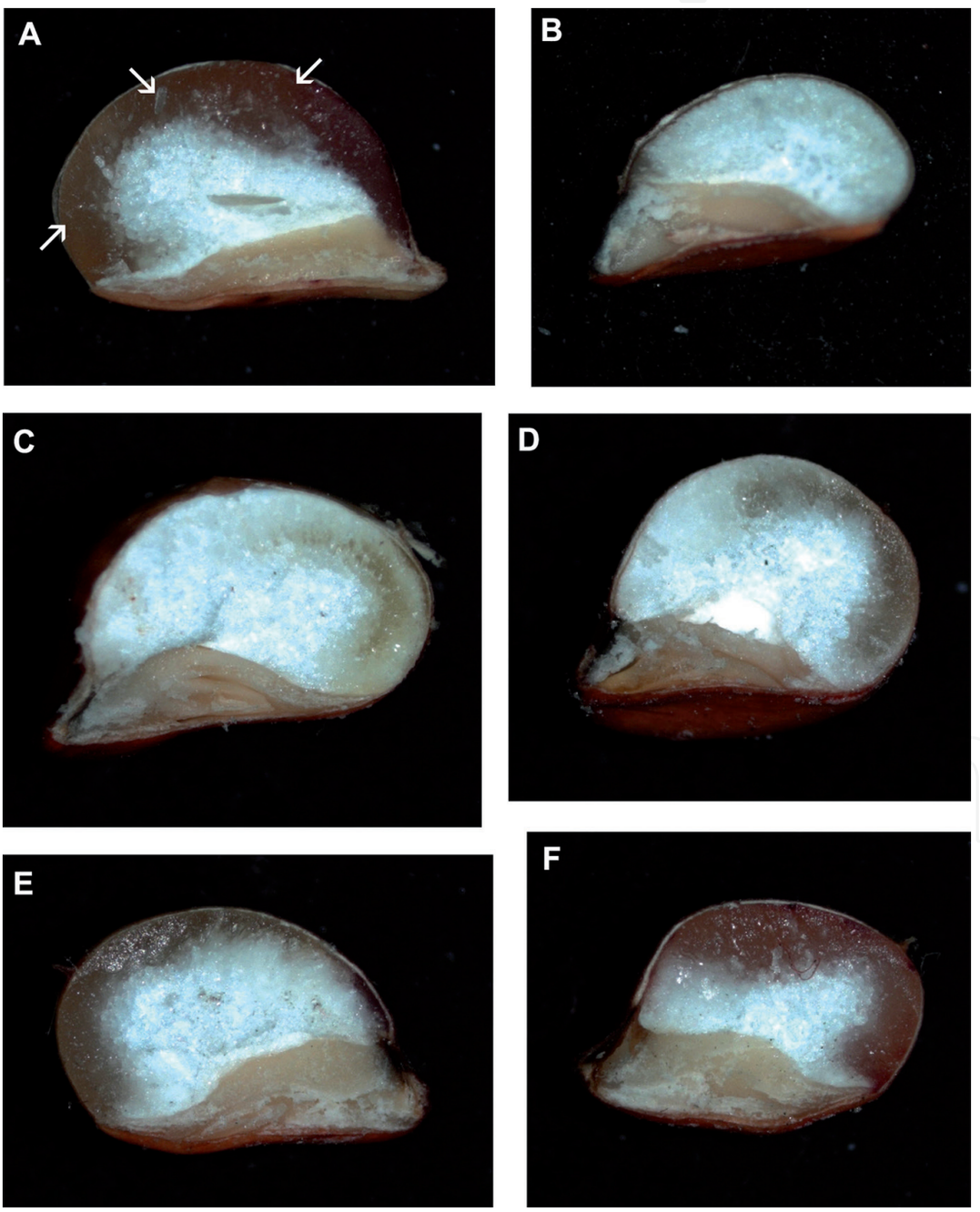

Figure 5.
Longitudinal sections of kernels of the original non-transgenic line Zheltozernoe-10 (A) and transgenic plants carrying pNRKAF genetic construct for RNA silencing of the γ-KAFIRIN-1 gene (B-F), differing in the degree of development of the vitreous endosperm. Vitreous endosperm is marked with white arrows.

breeding lines with improved digestibility of kafirins derived from it. Overcoming this correlation is an extremely difficult and urgent task [8].

In this regard, the transgenic plants of cv. Zh10 with the genetic construction pNRKAF for *γ-KAFIRIN-1* gene silencing are of special interest, since in most cases they had sectors or of the vitreous endosperm in the kernels, or the vitreous endosperm formed a continuous thin layer along the periphery of the kernels (**Figure 5**). It is noteworthy that the formation of the vitreous endosperm in such kernels did not reduce the digestibility of kafirins. Moreover, a plant (94–3-08) was found in T_2, in whose progeny (T_3, kernels is shown in **Figure 5F**) a high level of kafirin digestibility (88–90%) was combined with normal vitreous endosperm [23]. The fact of obtaining such plants shows that an increase in the digestibility of sorghum kafirins may not be associated with the reduction of the vitreous layer and formation of floury endosperm. Further investigation of these plants is needed to understand the role of the γ-kafirin in development of hard endosperm in sorghum.

Previously, transgenic plants with inclusions of vitreous endosperm surrounded by a floury endosperm were also observed in the transgenic plants of cv. Tx430, which contains a genetic construct for silencing α- and γ-kafirins [35]. At the same time, co-suppression of the δ-kafirin and γ-kafirin subclasses did not change the endosperm type in this cultivar. Apparently, the formation of different types of endosperm is due to the peculiarities of the expression of genetic constructs in the genome of the recipient line.

In this regard, it should be noted that the nucleotide sequence that we used in the genetic construct pNRKAF was homologous not only to the γ-*KAFIRIN-1* gene located in the chromosome 2 of the sorghum genome but also to the locus of the chromosome 9 encoding bi-functional protease inhibitor protein (Pfam: PF00234) belonging to the LTP-family (lipid transfer proteins) [23]. It is possible that a higher kafirin digestibility in plant 94–3-08 and its progeny could be due mainly to the suppression of the synthesis of the protease inhibitor, which did not entail a change in the texture of the endosperm.

These data indicate a possible effect of protease inhibitors on the digestibility of proteins in sorghum flour, which remains poorly understood. Purposeful designing of genetic constructs for RNA-silencing of protease inhibitors and their introduction into sorghum genome can help to obtain lines with improved digestibility of kafirins, in which the endosperm could be of the usual vitreous type.

5. Increased synthesis of other proteins

An important consequence of silencing of the prolamine genes in cereals is an increase in the synthesis of other proteins, including those with a higher content of essential amino acids. For example, in transgenic maize plants with α-zein silencing, a double content of tryptophan and lysine was observed [40]. In rice, it was shown that silencing of 13 kDa prolamine increases the total lysine content up to 56% as a result of a compensatory increase in the synthesis of lysine-rich glutelin, globulins, and chaperones [41]. A significant increase in the lysine content (up to 3.3 g / 100 g of protein, compared to 2.1 g/100 g of protein in the non-transgenic control) was found in transgenic sorghum plants carrying complex genetic constructs for RNA silencing of kafirins (ABS032, ABS149) [35]. However, these genetic constructs carried, along with the fragments of the kafirin genes, the fragments of the lysine ketoglutarate reductase gene, which controls the catabolism of free lysine. This fact does not allow drawing a conclusion on the effect of kafirin silencing on the increase in the lysine content in sorghum.

In the transgenic plants obtained in our experiments with a high *in vitro* kafirin digestibility, the total amino acid content in the kernels of plants of the T_2 generation decreased by 22.8–40.2% as compared with the original, non-transgenic line [23]. At the same time, the relative content of the two main essential amino acids, lysine and threonine, has increased significantly. The proportion of lysine increased 1.6–1.7 times: from 1.54% of the total amino acid content in the flour of the original non-transgenic line to 2.41–2.63% in transgenic plants. This increase, combined with a significant decrease in the total level of amino acids, was apparently caused by a decrease in the content of α-kafirins, which are poor in lysine and threonine, while the synthesis of other proteins was not impaired. Accordingly, the relative proportions of lysine and threonine increased. It is possible that suppression of the synthesis of γ-kafirin prevents the accumulation of α-kafirins but does not affect the synthesis of other proteins richer in lysine and threonine. The appearance of new proteins in transgenic sorghum plants carrying a genetic construct for silencing α-kafirin gene was described by T. Kumar et al. [21].

It is noteworthy that in the transgenic plants of the cv. Avans with a construct for silencing γ-*KAFIRIN-1* (RNAi mutant #1–1), along with a decrease in the content of γ- and α-kafirins (**Figure 1**), an increase in the content of a number of globulins occurs, possibly resulting from the re-balancing of the proteome of the kernels (**Figure 6**).

Protein rebalancing in the endosperm is a frequent phenomenon in transgenic plants with genetic constructs for RNA silencing of seed storage proteins. In maize, it was suggested that a compensatory mechanism, which is sensitive to the protein

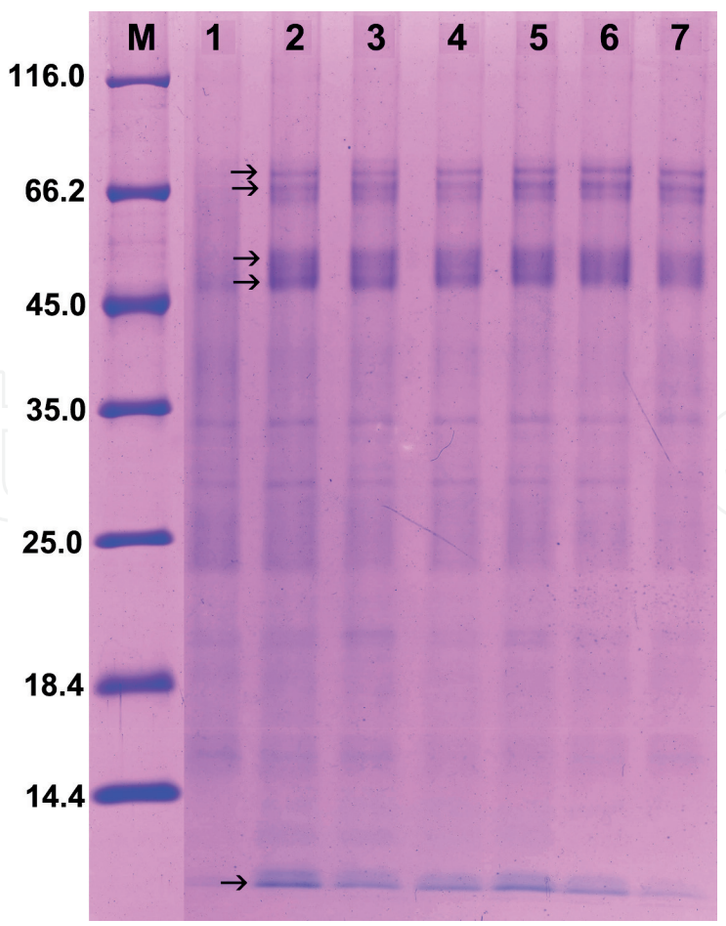

Figure 6.
Electrophoretic spectra of globulins from the kernels of transgenic plants from T_1 generation of RNAi mutant #1–1. 1 – Original non-transgenic cv. Avans; 2–7 individual T_1 plants; M – Molecular mass markers (kDa). Globulins were extracted according to [42].

content exists in the kernels; and a violation of zein synthesis in developing kernels enhances the translation of other mRNAs [43]. It is noteworthy that in transgenic soybean plants with suppressed synthesis of the main storage proteins, the seeds retained an almost identical level of total protein characteristic of untransformed soybean varieties [44]. These data suggest that restoration of proteome balance may be quite common phenomenon, providing a constant supply of nitrogen during seed maturation.

6. Instability of the genetic construct for RNA silencing

In our experiments, we found that the offspring of transgenic plants with a high *in vitro* digestibility of endosperm proteins sometimes lose this trait. Even different panicles of the same plant had different digestibility values. Such instability is an interesting phenomenon, which may be caused by silencing of introduced genetic construct possibly by RNA-dependent DNA methylation that is characteristic to hairpin genetic constructs [45], or by environmental factors, such as temperature, soil moisture, air humidity, etc. It has been reported that temperature causes a significant impact on RNAi-silencing [46]. It was also shown that mRNA degradation induced by microRNA and translation inhibition, depends on the temperature of plant growth [47]. Consequently, the efficiency of inhibition of kafirin synthesis by RNAi-silencing may be sensitive to plant growing conditions, and this was really shown in our experiments [48].

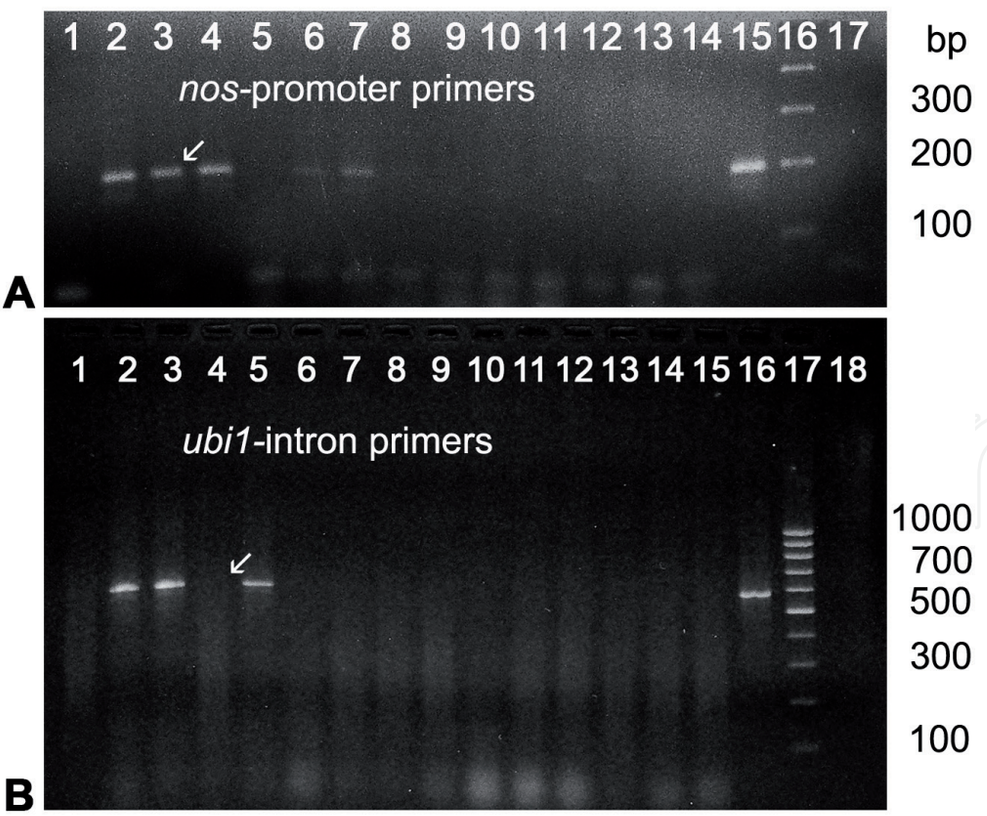

Figure 7.
PCR analysis of plants from the offspring of the RNAi mutant (#1–1, cv. Avans) carrying the genetic construct for silencing γ-KAFIRIN-1, with primers to the nos-promoter (A) and ubi1-intron (B). 1 – Original non-transgenic cv. Avans; 2-4 (A) and 2-5 (B) – individual T_1 plants (A: #2, #3, #4; B: #1, #2, #3, #4, respectively); 5–14(A) and 6–15 (B) – Plants from another experiment; 15 (A), 16 (B) – A. tumefaciens GV3101/pNRKAF; 16 (A), 17 (B) – DNA markers; 17 (A), 18 (B) – Negative control (no DNA). The nos-specific primers amplified the 202 bp fragment (A). The ubi1-intron specific primers amplified the 588 bp fragment (B). The arrows mark the products of DNA amplification in plant #3.

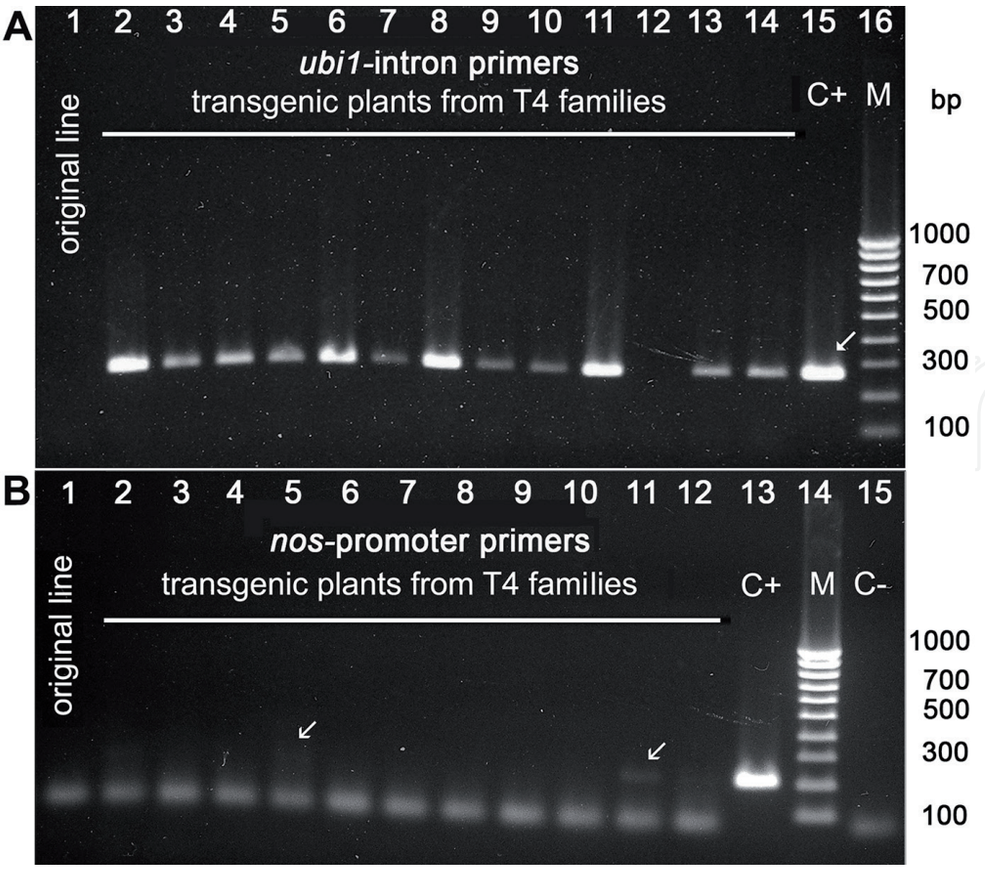

Figure 8.
PCR analysis of transgenic sorghum plants (T_4 generation) carrying a genetic construct pNRKAF [23] with primers to the ubi1-intron (A) and nos-promoter (B). 1 (A, B) – Original non-transgenic line Zh10; 2–-14 (A), 2-12 (B) – DNA of individual transgenic plants from the T_4 families; 15 (A), 14 (B) – A. tumefaciens GV3101/pNRKAF (positive control); 16 (A), 14 (B) – DNA markers; 15 (A) – Negative control (no DNA). The ubi1-intron specific primers amplified the 267 bp fragment (A). The nos-specific primers amplified the 202 bp fragment (B). Amplified gene-specific fragments are marked with arrows [50].

In addition to instability at the epigenetic level, we have found the genetic instability of introduced construct for RNAi-silencing. In this regard, analysis of the progeny of the RNAi mutant #1–1 (cv. Avans), carrying a construct for silencing γ-*KAFIRIN-1*, is indicative. Of the 4 studied T_1 plants grown in the experimental field plot, all plants were transgenic, because carried the *nos*-promoter driving the expression of the marker gene *bar*, located in T-DNA of pNRKAF, along with a genetic construct for the γ-*KAFIRIN-1* gene silencing (**Figure 7A**). At the same time, one of these plants (#3) lacked the *ubi1*-intron, which is a part of the genetic construct for silencing (**Figure 7B**). All kernels developed in the panicle of plant #3 had the vitreous type of endosperm, characteristic to the original cultivar (**Figure 4A**), while in the panicles of other plants, in which the *ubi1*-intron was present, the kernels had a floury type of endosperm (**Figure 4B**), characteristic for transgenic plants with γ-kafirin silencing.

In addition, in Zh10 transgenic plants from the T_4 families with high digestibility of kafirins, probable elimination of the *nos*-promoter, which controls the expression of the marker gene *bar* in the pNRKAF genetic construct [23] was found [49]. **Figure 8** clearly shows that in the plants from the T_4 families, amplification of the *ubi1*-intron fragment was observed, while amplification of the *nos*-promoter located in the construct in front of the marker gene *bar* was absent. Thus, these plants probably turned out to be functionally marker-free transgenic plants. This fact is of significant interest, since the presence of marker genes in the genetic constructs hinders the practical use of transgenic lines in practical plant breeding.

7. Conclusions

The research findings presented in this chapter provide strong evidence that RNA interference can be used for the improvement of the nutritional value of grain sorghum. RNAi mutants are characterized by significantly improved digestibility of kafirins and higher content of essential amino acids, in particular lysine. In some cases, these mutants retain vitreous endosperm that is highly important for grain hardiness and in ensuring the resistance of kernels to fungal diseases.

Nevertheless, in most cases the kernels with suppressed synthesis of γ- or α-kafirins have floury endosperm that strongly reduces their use in sorghum breeding. Such a correlation between the traits of high digestibility of kafirins and the floury type of endosperm, which was originally observed in the P721Q mutant and lines created on its basis is a serious problem (see review [8]). In maize, the correlation between the floury endosperm and the increased lysine content was disrupted using modifier genes that enhanced the accumulation of γ-zein [42, 51, 52]. However, in sorghum, an increase in the synthesis of γ-kafirin may decrease the level of kafirin digestibility due to a high content of sulfur-containing amino acids, which contribute to the polymerization of kafirins. Possibly, one of the ways to solve this problem may be down-regulation of genes that encode protease inhibitors, which can also affect the level of digestion of kafirins by exogenous proteases. In this case, the resulting lines would have a hard endosperm in combination with a high digestibility of kafirins.

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