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Epigenetic regulation of Cytoplasmic Male Sterility in Sorghum

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ABSTRACT

Cytoplasmic male sterility (CMS) is a maternally inherited dysfunction of male generative sphere caused by degeneration of pollen grains and disturbed anther dehiscence. CMS arises as a result of remote hybridization that disturbs correct nuclear-mitochondrial interactions. In this review, we summarize experimental data on expression and inheritance of male fertility restoration in sorghum CMS types having large non-dehiscent anthers (9E, A_3, A_4, M35-1A). We demonstrate that additional irrigation at booting and flowering stages, high air relative humidity, and low air vapor pressure deficit are the main factors that affect male fertility restoration in F_1 hybrids in these cytoplasms, perhaps, by up-regulating expression of fertility-restoring genes. "Induced" fertility stably inherited by self-pollination for 12-15 generations and manifested in non-inductive conditions (in the dryland plots); it was transmitted through pollen in testcrosses with CMS lines, although expression of male fertility in newly obtained hybrid genome again required a high level of plant water availability. These data point on epigenetic mechanism, which up-regulates the fertility restorer genes in F_1 hybrids in these cytoplasms. MSAP (*M*ethylation *S*ensitive *A*mplification *P*olymorphism) analysis of F_1 hybrids in the 9E cytoplasm revealed differences in the methylation pattern of the *MYB46* gene in fertile and sterile plants. In addition, polymorphism correlating with restoration of male fertility was found in MSAP spectra obtained with primers to Tos17 RNA-transposon pointing on its possible involvement in fertility restoration of CMS 9E. Apparently, in conditions of drought, the genes that regulate the anther dehiscence and pollen development are repressed by methylation. In conditions of high moisture, this repression is removed, and pollen development and anther dehiscence are restored. These data demonstrate that epigenetic changes in nuclear genes involved in regulation of pollen development and anther dehiscence may be one of the mechanisms of CMS.

Keywords : Cytoplasmic male sterility; fertility-restoring genes; epigenetics; drought stress; DNA methylation; MSAP analysis

Formation of plant tissues and organs, as well as of the whole plant, is determined by the interaction of its genotype with environment. Development of male generative structures is the most sensitive to environmental stresses stage of plant ontogenesis (Dolferus *et al.* 2013). One of the reasons for sensitivity of this ontogenesis stage to environmental stress is high energy intensity inherent in meiosis and microsporogenesis. The energy requirements of a plant are known to be provided by the mitochondrion of the plant cell. At the same time, mitochondria are the primary targets of environmental stresses, which disturb mitochondrial functioning and cross-talk between the mitochondrial and nuclear genomes (Atkin and Macherel 2009, Jacoby *et al*. 2012, Liberator *et al*. 2016). This is especially important for plants with cytoplasmic male sterility (CMS). CMS arises as a result of mutations in the mitochondrial genome, or after remote hybridization that also disturbs nuclear-mitochondrial cross-talk established during co-evolution of nuclear and mitochondrial genomes.

In majority of well-studied CMS types, it is caused by expression of specific mitochondrial CMS-inducing genes. The structure and function of these genes are described in detail in a number of excellent reviews (Hanson and Bentolila 2004, Budar *et al.* 2006, Horn *et al*. 2014). In brief, CMS-inducing genes arise as a result of the recombination processes inherent to the mitochondrial genome. They have chimeric nature and consist from the fragments of other mitochondrial, chloroplast or nuclear genes. They encode abnormal transcripts or specific CMS-associated proteins, which modify synthesis or structure of mitochondrial enzymes or disrupt the normal functioning of mitochondria, in most cases, in tapetum cells or in developing pollen grains (PG). However, such expression occurs only in the absence of specific fertility-restoring genes (*Rf*) in the nuclear genome. Most of these genes encode specific PPR (pentatricopeptide repeat) proteins involved in processing of mitochondrial mRNA.

Another mechanism of CMS is based on phenomenon of retrograde signaling, i.e. regulation of nuclear gene expression by molecular signals from mitochondria and chloroplasts (Fujii and Toriyama 2008, Yang *et al*. 2008). This mechanism has been described for CW-type CMS of rice. This type of CMS may exist in other plant species, since correct nuclear-cytoplasmic interactions are violated in alloplasmic lines, which in fact, in many plant species, are CMS lines. Such a mechanism is characterized by high sensitivity to environmental stresses, in particular, to the effect of abiotic

stress, which violates the expression of the mitochondrial and chloroplast genomes and their interaction with nuclear genes (Li *et al*. 2013, Ng *et al.* 2014). It should also be noted that the alien cytoplasm can cause rearrangements in the nuclear genome, which may affect the genes involved in genetic control of pollen development. In our experiments, significant differences in the amplification spectra of a number of DNA and RNA transposons were noted between the fertile sorghum line Zheltozernoe-10 (Zh10) and its sterile counterpart in the 9E CMS-inducing cytoplasm (Fig. 1).

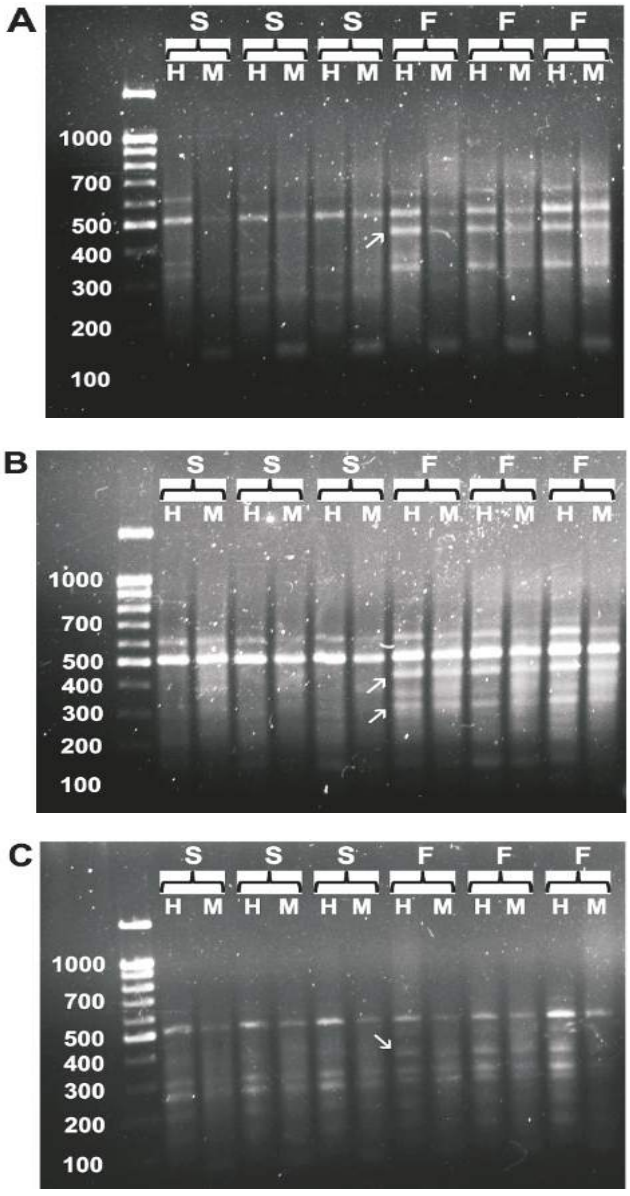

Fig. 1 – MSAP-analysis of plants of fertile line (F) Zheltozernoe-10 (A_1 cytoplasm) and its male-sterile counterpart (S) in the 9E cytoplasm with primers to RNA transposon Cin4 (A), and DNA transposons BoST (B) and Isaak (C). DNA was digested by HpaII-restrictase (H) and MSPI-restrictase (M). Arrows point to DNA fragments of fertile line absent in sterile counterpart.

Sorghum is a convenient model for studying the mechanisms of CMS regulation, since it refers to the most actively evolving plant species. There are a large number of races and subspecies that diverged in the process of evolution and differ in a large number of biological traits, but capable of interbreeding giving rise to progeny that combines nuclear and cytoplasmic genomes of genetically remote parents. To date, a large number of genetically different types of CMS have been identified or experimentally obtained in sorghum (see reviews: Pring *et al.* 1995, Reddy *et al.* 2005). These types of CMS differ in the phenotype of sterile anthers and defective pollen grains, reactions to lines fertility-restorers, mitochondrial and chloroplast genomes, the mechanism of pollen degeneration and the action of fertility-restoring genes, etc. Various types of CMS have been proposed to be designated with the letter "A": A_1, A_2, A_3, etc., or by the name of the source line (9E, M35-1A).

According to Kaul (1988), A_1 and A_2 CMS of sorghum belong to sporogenous type, i.e. occur due to the degeneration processes in microsporogenesis. As a result, anthers of male-sterile plants have small size and contain empty pollen grains (PGs). The fertility-restoring genes *Rf1* and *Rf2* for CMS A_1 (Klein *et al.* 2005, Jordan *et al.* 2010), and *Rf5* (Jordan *et al.* 2011) and *Rf6* (Praveen *et al*. 2015) for CMS A_2 encode PPR proteins. These genes are sporophytic fertility restorers. As a result, in F_1 hybrids with restored male fertility fertile PGs contain either dominant or recessive alleles of fertility restorer genes and after selfing (in the F_2 generation) or backcrossing to CMS-line male sterile plants appear. Molecular mechanisms involved in genetic control of the A_1 and other sporogenous sorghum CMS are poorly studied. A 65-kDa protein was reported to be synthesized in mitochondria of sterile lines with the A_1 cytoplasm, whereas its production in fertile lines was two orders of magnitude lower (Dixon and Leaver 1982). Later it was reported that sterile lines and hybrids with restored male fertility differ in kinetic properties of the Fl component of ATPase from isolated mitochondria, which implicates the *atpA* gene in controlling A_1 type CMS in sorghum (Sane *et al*. 1997).

In the A_3 CMS type, pollen degeneration occurs at the late development stages; the fertility-restoring genes (*Rf3* and *Rf4*) function as gametophytic restorers (Pring *et al.* 1999), as a result, in the F_1 hybrids with restored male fertility develop both fertile PGs carrying fertility restorer genes and sterile PGs, not capable of fertilization. There was also report on sporophytic fertility restoration system in A_3 CMS derived from sudan-grass accession (Tang *et al.* 2007). The A_3 CMS to date is the only sorghum CMS system with identified mitochondrial CMS-inducing gene (*orf107*) (Pring *et al.* 1999; Tang *et al.* 1999). This gene is chimeric and consists of fragments of two sequences: one homologous to the *atp9* gene and the other homologous to the *orf79* gene associated with *boro* CMS of rice. *orf107* encodes specific 12kDa CMS-

Table 1 – Examples of inheritance of male fertility restoration in the 9E CMS-inducing cytoplasm (from Elkonin *et al.* 2005)

Self-pollination				Testcrosses to CMS-lines			
Generation	Number of plants			Hybrid combination	Number of plants		
	f	ps	s		f	ps	s
[9E] P614 Persp.-1							
F_1	9	9	7				
F_2	14	-	-				
F_3	18	-	-	[9E] P614 F_2	-	-	8
F_4	7	10	2	[9E] Zh10 F_3	-	-	35
[9E] P614 IS12603							
F_1	2	3	-				
F_2	4	2	-				
F_3	31	-	-	[9E] Zh10 F_2	-	6	16
[9E] T 398 Persp.-1							
F_1	19	-	-				
F_2	11	18	9	[9E] P614 F_1	-	3	32

f – fertile (>50% seed set, usually 80-100%), ps – partially sterile (<50% seed set, usually 10-20%), s – sterile plants (0% seed set or few seeds); P614 = Pishchevoe-614; Zh10 = Zheltozernoe-10.

associated mitochondrial protein (Tang *et al.* 1996). Restoration of male fertility by gametophytic fertility-restoring gene *Rf3* correlated with enhanced transcript processing of *orf107*. Fertility restoration by sporophytic fertility restoration system did not involve enhanced transcript processing of *orf107* (Tang *et al.* 2007).

Male sterility in other sorghum CMS types (9E, A_4, and M35-1A) is caused by disturbance of anther dehiscence; most anthers usually have large size and contain some portion of stainable PGs. This phenomenon is illustrated in Figure 2, in which cross-sections of anthers of male-sterile and male-fertile plants of the F_1 hybrids in the 9E CMS are shown. The anthers of male-sterile plant contain both degenerated and stainable PGs and clearly differ from the anthers of fertile plant by strongly developed epidermis that may prevent their dehiscence. In addition, in fertile anthers, lignin accumulated predominantly in endotecium, whereas in sterile anthers strong lignification was observed both in endotecium and in epidermal cell walls that may impede their dehiscence (Fig. 2).

It was shown that fertility restoration in these CMS types is unstable and vary in different seasons; with male-fertile revertants often appear in advanced backcross generations (Elkonin *et al*. 1998; 2005). In the self-pollinated progeny of fertile F_1 hybrids, the fertility-restoring genes function stably as dominant genes, but they do not function or are weakly expressed in the testcrosses of these hybrids to CMS-lines with the same type of cytoplasm (Table 1). Segregation in the progeny of restored F_1 hybrids in the 9E and A_4 cytoplasms corresponded to di-genic mode of fertility restoration (15:1) suggesting involvement of two fertility-restoring genes, Rf^{9E}_1 and Rf^{9E}_2, although in drought seasons monogenic segregation ratio (3:1) was observed, which testified to high sensitivity of one of fertility-restoring gene to drought (Elkonin and Tsvetova 2012).

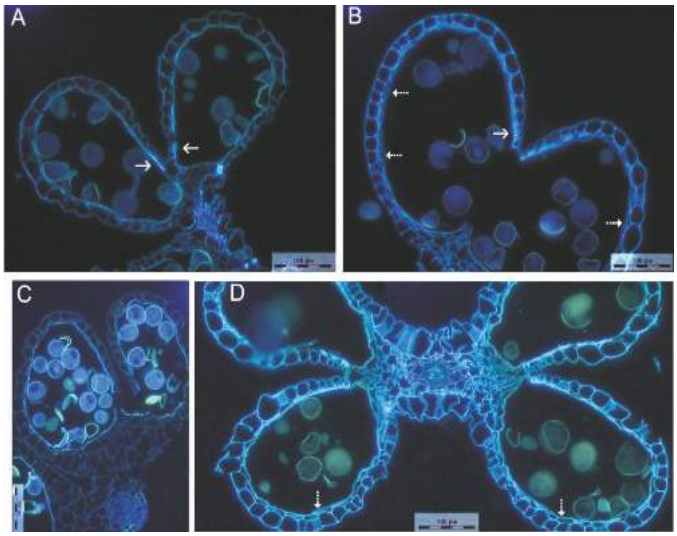

Fig. 2 – A, C-Cross-sections of anthers of fertile, B,D- Cross-sections of male-sterile anthers of plants from the F_1 hybrids 9E Rannee-7 / KVV-263 (A, B) and 9E Zh10 / rev.-188 (C, D). Endotecium is marked by arrows; the remnants of the tapetum in the anthers of sterile plants are marked by the dotted arrows. UV fluorescence, ×20.

Influence of plant water-availability conditions on the restoration of male fertility in the 9E and related CMS types of sorghum—Careful analysis of the data on fertility restoration in the 9E cytoplasm revealed that level of fertility-restorer gene expression in the newly obtained F_1 hybrids is regulated by water availability to plants before anthesis (Elkonin *et al.* 2005). The most pronounced example of this was observed in the hybrid combination 9E T398/KVV-112 (Fig. 3). The F_1 grown in the greenhouse was fertile. In the F_2 generation, which was grown in the field, both fertile and

sterile plants were found; their ratio suggested segregation of two dominant fertility-restorer genes. After selection of fertile plants in the F_2 and F_3 generations, a fertile homozygous line in the 9E cytoplasm (KVV-263) was obtained. Nevertheless, this line produced only sterile and partially sterile F_1 hybrids in the testcrosses to CMS lines 9E T398 and 9E Zh10, which had been obtained by backcrossing Zh10 to 9E T398. However, during wet seasons, there were no significant deviations from the regular pattern of expression of fertility-restorer genes: fertile and partially fertile plants were observed in test crosses with CMS lines. Remarkably, the line KVV-263 was completely fertile in both drought and in wet conditions at anthesis. Consequently, the dominant fertility-restorer genes functioned efficiently, but their up-regulation in the newly obtained F_1 hybrids was suppressed in conditions of water stress during pollen formation.

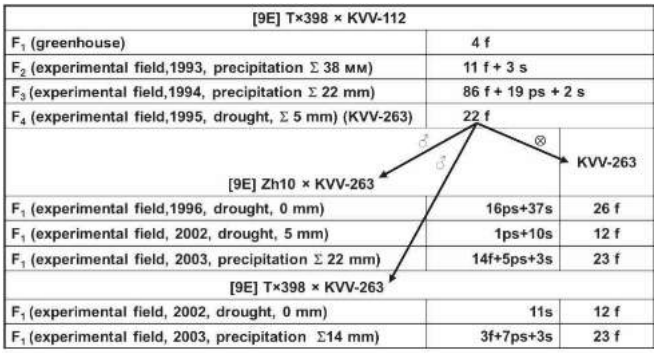

Fig. 3 – Inheritance of male fertility restoration in the hybrid combination 9E T×398 / KVV-112 (from Elkonin *et al.* 2005).

Detailed analysis of fertility restoration in the hybrid combination F_1 [9E] Zh10/ KVV-263 revealed a high positive correlation (r=0.99; P>0.99) between the level of male fertility and total precipitation 7 days before anthesis. A similar dependence of male fertility on plant water availability during the development of the panicle and flowering was observed in F_1 hybrids in the M35-1A cytoplasm (Elkonin *et al*. 2005). It was shown that additional irrigation at the booting and flowering stages significantly increased the proportion of fertile plants in the 9E, A4 and M35-1A cytoplasms (Elkonin *et al.* 2005).

Results of experiments on parallel growing of hybrid populations in the dryland plot and in the irrigated plot clearly demonstrate that the level of male fertility of the F_1 and BC_1 hybrids with the 9E cytoplasm was significantly higher in conditions of additional irrigation (Table 2). These data prove that the conditions of plant water availability during the development of the male generative sphere regulate the expression of the fertility-restoring genes for the 9E CMS, the high water availability regime is "inductive", i.e. favorable for the functioning of fertility restorer genes.

A very significant example of the induction of expression of fertility restorer genes by the conditions of plants water availability are F_1 hybrids 9E Zh-10/KVV-263, which have almost completely sterile phenotype in the dryland plot. However, in the irrigated plot few semi-sterile plants appeared, and in the self-pollinated progeny of these semi-sterile plants, the fertile and partially fertile plants prevailed (Table 2). It means that fertility restorer genes were up-regulated in F_1 generation by high water availability regime and function in F_2 as dominant genes.

It was also found that along with the irrigated plot, the conditions of growing in a greenhouse cause similar inductive effect: when transferring sterile plants from F_1 progenies grown under dryland plot to a greenhouse, fertile shoots were formed and seed setting was observed. Remarkably, reversions to male fertility were observed not only in the F_1 hybrids, which were heterozygous for fertility-restoring genes but also in the male-sterile plants segregating-out in the F_2 families as recessives, presumably devoid of dominant fertility-restoring genes (Elkonin and Tsvetova 2012).

Table 2 – Effect of plant water availability at panicle development stage on level of male fertility of sorghum hybrids in the 9E CMS-inducing cytoplasm.

Hybrid combination, generation		Number of plants in					
		Irrigated plots, >25 mm			dryland plots, 0 mm		
		f	ps	s	f	p	s
[9E] P614 / IS12603	F_1	17**	8	1	3	16	2
[9E] Rannee-7 / KVV-263	F_1	9***	18	-	-	7	15
	F_2	26	3	-	17	5	2
[9E]T×398 / KVV-263	F_1	16**	-	-	7	5	-
	F_2	15	5	5	24	2	2
[9E] Zh10 / KVV-263	F_1	-	**3***	56	-	-	60
	F_2	24	6	7	9	4	4
[9E] Zh10 / Persp.-1	F_1	22	1	-	19	4	2
	F_2	25	6	2	25	5	3
[9E] Zh10 / ([9E] Zh10 / Persp.-1)	BC_1	13*	13	9	3	2	9
[9E] Rannee-7 / Persp.-1	F_1	27**	-	-	9	3	-
[9E] Volzhskoe-615 / KVV-263	F_1	-	**4****	10	-	-	25

*, **, *** P<0.05, P <0.01, and P <0.001, respectively, in comparison with the dryland plot, according to F-criterion

Male fertility, "induced" in the irrigated plot or in greenhouse conditions, was inherited and manifested in the progeny of fertile revertants grown in non-inductive conditions (in the dryland plot, in the field) (Fig. 5). In the progeny of such revertants, fertile plants often predominated suggesting that revertants had dominant fertility-restoring genes.

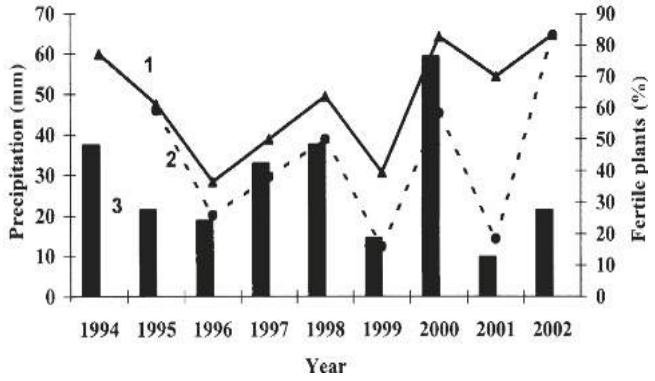

Fig. 4 – Variability in fertility in the self-pollinated progeny of selections in the M35-1A CMS-inducing cytoplasm (1) and in testcrosses of fertile plants to CMS line [M35-1A] P614 (2) and total precipitation 3 weeks prior to anthesis (3) from 1994 to 2002 (from Elkonin *et al.* 2005)

With using this approach, we have obtained a large number of fertile lines in the 9E cytoplasm, which stably inherit fertility up to 12-15 generations and express it under drought. In a number of cases "induced" fertility was transmitted through pollen to F_1 hybrids when crossing the revertants to CMS lines with 9E cytoplasm (Fig. 5) that indicated the presence of two nuclear fertility-restoring genes in genome of the revertants (Elkonin *et al*. 2015). However, in majority of cases, the fertility-restoring genes of these fertile lines did not function in testcrosses, in newly obtained hybrid genome. Evidently, the fertility-restoring genes were suppressed by genome of CMS lines.

Nevertheless, such suppression can be overcome if testcross hybrids were grown in the plot with additional irrigation. It means that the fertility-restoring genes of fertile revertants (for example, No. 23 from the family 9E Zh10 / KVV-263, Fig. 5) require a high level of plant water availability for expression in the hybrid genome. This conclusion is supported by data on the simultaneous growth of F_1 hybrids and their paternal parents – revertant lines – under conditions of dryland plot and irrigated plot (Table 3), which clearly demonstrate that F_1 hybrids carrying fertility-restoring genes of revertants need a high level of moisture supply for manifestation of male fertility, although in the revertants themselves, the fertility restorer genes function in both humid conditions and under conditions of drought.

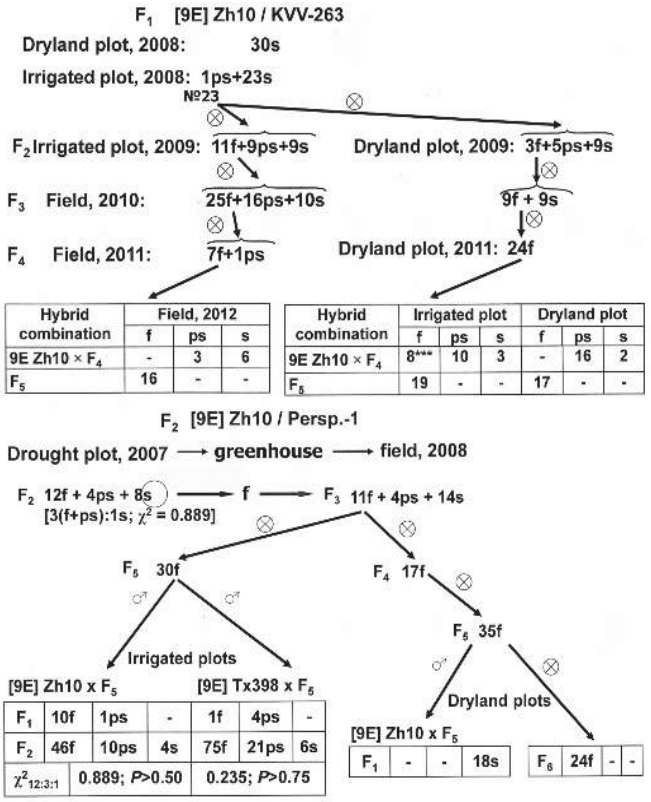

Fig. 5 — Examples of inheritance of reversions to male fertility induced in irrigated plots in the F_1 9E Zh10 / KVV263 and in male-sterile plant from F_2 9E Zh10 / Perspektivnyi-1 transferred to the greenhouse (from Elkonin *et al.* 2015, with modification).

Table 3 – Manifestation of male fertility in testcrosses of revertants obtained from sterile sorghum hybrids with the 9E type CMS grown under drought conditions (D) and additional artificial irrigation (Ir) (from Elkonin *et al.* 2015)

Hybrid combination	Growing conditions	Number of plants, %		
		f	ps	s
9E P614 / rev.170-11	Ir	64.3**	28.6	7.1
	D	31.6	57.9	10.5
9E Zh10 / rev.170-11	Ir	20.0	66.7	13.3**
	D	16.7	8.3	75.0
Rev.170-11	Ir	100	-	-
	D	100	-	-
9E Zh10 / rev.188-12	Ir	77.8**	22.2	0
	D	4.5	45.5	50.0
Rev.188-12	Ir	100	-	-
	D	100	-	-

** $P < 0.01$, in accordance with the *F*-test, in comparison with the family grown in drought conditions.

It was also revealed that an important factor regulating the fertility of testcrosses of revertants is the level of relative air humidity during the flowering period (Table 4). The high

Table 4 – Dependence of the level of male fertility of testcrosses of revertants on the relative air humidity during the flowering period.

Hybrid combination	Plant fertility	Number of plants	Relative humidity, %	*r*
9E T398 / rev.170-11	f	10	73.6	0.82±0.14**
	pf	6	68.4	
	ps	1	58.6	
	s	1	52.4	
9E Zh10 / rev.164-11	f	4	70.0	0.63±0.27*
	ps	5	57.6	
	s	1	50.2	
9E P614 / rev.170-11	ps	1	61.8	0.0
	s	10	62.0	
9E P614 / rev.164-11	ps	7	56.4	0.0
	s	6	56.4	

***P*>0.99; **P*>0.95; s – sterile plants (seed set 0%); ps – partially sterile plants (1-30%); pf – partially fertile plants (40-70%); f – fertile plants (75-100%)

relative humidity of the air (68-70%) significantly increased the fertility of the panicles of the F_1 hybrids with CMS lines 9E T×398 and 9E Zh10, with a correlation coefficient of 0.82 and 0.63, respectively.

Consequently, the restoration of male fertility under the influence of the environment is not due to changes in the structure of genes involved in the control of 9E CMS, but with a change in their regulation in the genome of the original hybrid plants under the moisture supply regime. Apparently, there is an epigenetic mechanism, controlled by environmental conditions, which "up-regulates" the fertility-restorer genes in the genome of F_1 hybrids under the influence of a high level of moisture supply of plants.

MSAP-analysis of male-sterile and fertile F_1 hybrids in the 9E cytoplasm—Assuming that one of these epigenetic mechanisms controlling the fertility-restorer genes of the 9E CMS can be DNA methylation under drought conditions, we performed an MSAP analysis (*M*ethylation *S*ensitive *A*mplification *P*olymorphism) of the gene sequences involved in the control of anther dehiscence and pollen development in male-sterile and male-fertile plants from the F_1 hybrid 9E Zh10/KVV-263 grown, respectively, in the dryland plot and irrigated plot (Elkonin *et al*. 2015).

It was found that MSAP spectra obtained with using the primer to the gene encoding transcriptional regulator MYB46 differed in sterile and fertile plants (Fig. 6). The spectrum of sterile plants obtained with *Hpa*II contains a fragment of H≈700 bp, which is absent in fertile plants. At the same time, the spectra of the fertile plants obtained using *Msp*I contain a fragment of H≈650 bp, whereas sterile plants in the *Msp*I-spectrum show amplification of both the 700 and 650 bp fragments. This result may indicate a difference in the *Myb46* gene methylation in the genome of fertile and sterile plants, which may reduce the level of this transcriptional regulator in sterile plants. MYB46 is a transcription factor regulating the biosynthetic pathways of the components of the secondary cell wall – cellulose, xylan, and lignin (Zhong *et al*. 2007). Correct deposition of lignin in anther tissues is known to be an important factor of anther dehiscence (Wilson *et al.* 2011). Perhaps, changes in methylation pattern of *MYB46* gene may cause male sterility in the 9E cytoplasm. Differences in lignification of cell walls between sterile and fertile plants in the 9E cytoplasm (Fig. 2) confirm this hypothesis.

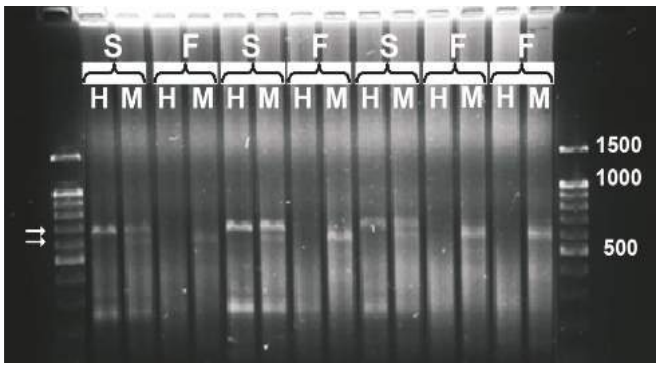

Fig. 6 – MSAP-profiles of sterile (S) and fertile (F) plants from hybrid combination F_1 9E Zh10 / rev. № 188 obtained with using restriction enzymes *Hpa*II (H) and *Msp*I (M) and primer to*Myb46*. Arrows indicate fragments differentially amplified in sterile and fertile plants (from Elkonin *et al.* 2015).

Comparative MSAP-analysis of sterile F_1 hybrids 9E Zh10 / KVV-263 and male-fertile revertants derived from them in the growth chamber (under 70% air relative humidity) also showed differences in the experiment using the primer to the *Myb46* gene (Fig. 7). These data indicate that a change in methylation pattern in the *Myb46* gene may be involved in reversion to male fertility. It is noteworthy that the sterile and fertile counterparts of the line Zh10 (maternal line for this hybrid) likewise differ in the methylation of the *Myb46* gene (data not shown here).

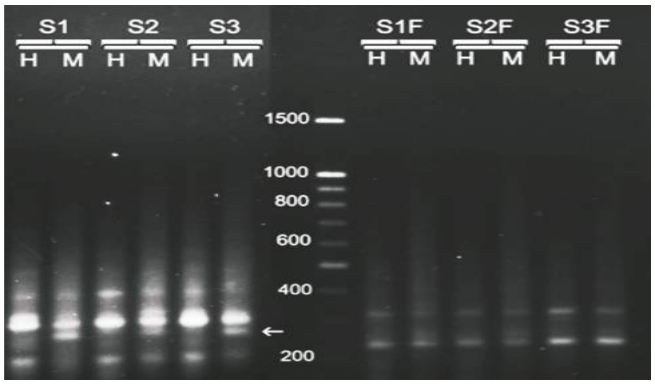

Fig. 7 – MSAP-profiles of sterile (S) plants from the hybrid combination F_1 9E Zh10 / rev. №188 and fertile shoots (F) derived from them in the growth chamber. MSAP-profiles were obtained using restriction enzymes *Hpa*II (H) and *Msp*I (M) and primer to *Myb46*. Arrow indicates fragment differentially amplified in sterile and fertile shoots.

In addition, MSAP analysis with primer to RNA transposon Tos17 performed on DNA of sterile F_1 9E Zh10 / KVV-263 and fertile lines obtained from this hybrid combination in irrigated plots also revealed intriguing polymorphism correlating with the manifestation of CMS. A fragment (H≈500 bp) was detected, the amplification of which was observed in sterile plants in both *Hpa*II and *Msp*I-spectra, but in fertile lines it was absent from the *Msp*I-spectrum (Fig. 8).

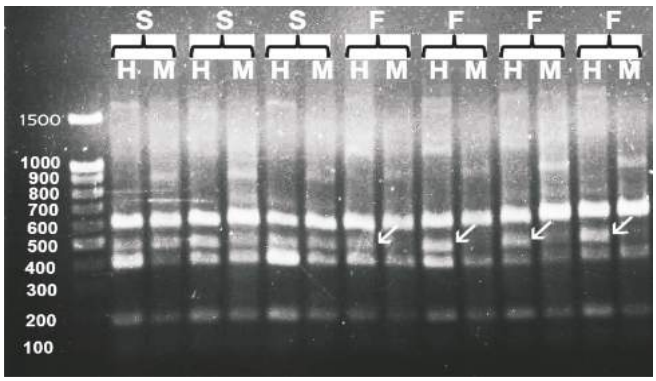

Fig. 8 – MSAP-profiles of sterile (S) plants from the hybrid combination F_1 9E Zh10 / KVV263 and fertile plants of revertant lines (F) obtained from this hybrid combination in irrigated plots. MSAP-profiles were obtained using restriction enzymes *Hpa*II (H) and *Msp*I (M) and primer to RNA transposon Tos17. Arrow indicates the fragment differentially amplified in sterile and fertile plants.

Apparently, in conditions of drought, the genes that regulate the opening of the anthers and pollen development are in a repressed state, as was shown by the MSAP analysis, caused by methylation of their nucleotide sequences. In conditions of high moisture availability, this repression is removed, the functioning of these genes is restored, hybrids normalize the formation of fertile pollen, and fertilization takes place. As a result of self-pollination of heterozygous plants, homozygotes appear that appear to be less sensitive to drought conditions than parental heterozygous plants and exhibit male fertility both under high and low levels of moisture supply. In the testcrosses of the revertants to CMC lines, these genes again appear in the heterozygous state, and since the inherited changes in the genes themselves did not occur in the course of reversion, and the epigenetic labels (in particular, the character of methylation) in F_1 hybrids are erased and are established *de novo*, then, for the functioning of these genes, a high level of moisture supply of plants is again required. The obtained data are consistent with the known facts on the role of the external environment, in particular, of water availability to plants, for the determination of the nature of methylation and the plant epigenotype (Tan 2010, Verhoeven *et al.* 2010, Wang *et al*. 2011, Tricker *et al*. 2012, Yaish 2013).

Vapor pressure deficit as a trigger of fertility restoration in the A_3 cytoplasm—A bright example of epigenetic control of male fertility restoration in CMS is the A_3 cytoplasm of sorghum. This cytoplasm is known as one of the most difficult for fertility restoration because of the rare frequency of restorer genes among sorghum accessions and low seed set in restored F_1 hybrids (Torres-Cardona *et al.* 1990, Dahlberg and Madera-Torres 1997). In this cytoplasm, an unusual phenomenon of poor expression of fertility restoring alleles in backcrosses of fertile hybrids to A_3 CMS lines was found, and was explained by paramutation of the *Rf* genes caused by sterility-maintaining alleles (Tang *et al.* 2007).

In our experiments it was found that the main factor regulating expression of the fertility restorer genes for A_3 CMS is the air vapor pressure deficit (VPD) during the flowering period (Kozhemyakin *et al.* 2017). VPD is an integral indicator that connects air humidity and air temperature. Studying restoration of male fertility in the hybrid combinations A_3 Topaz/(F_3 A_3 Karlik-4/IS1112C) we found strong correlations of fertility of testcrosses and their paternal parents and VPD value: $r = 0.96$ ($P > 0.99$) and $r = 0.99$ ($P > 0.99$), respectively. Noteworthy is that paternal plants tolerated much higher moisture deficit than F_1 hybrids. An identical fertility level of panicles of paternal plants and F_1 hybrids was observed under different values of VPD.

Remarkably, in the F_2 generation of testcrosses, fertile, partially fertile, partially sterile, and completely sterile plants were observed (Table 5). Formally, their ratio corresponded

to the di-genic segregation 9f: 3pf: 3ps: 1s ($\chi^2 = 4.543$; $0.25 > P > 0.10$). However, detailed analysis revealed that plant fertility was strongly correlated with air vapor pressure deficit during flowering. For example, plants that flowered under low VPD (1.1–1.4 kPa) were completely fertile, whereas plants flowering under 2.0–2.3 kPa VPD showed complete or partial male sterility. Correlation of VPD and plant fertility was evaluated by the Chuprov contingency coefficient, confirming this finding (T = 0.446; $P < 0.001$). It is noteworthy that a distribution curve of plants with different levels of fertility in the F_2 [A_3 Topaz/F_3 (A_3 K4/IS1112C)] was the mirror image of a curve showing VPD changes during the growing season (Fig. 9). With VPD at 1.25–1.4 kPa, fertility increased almost to 100%, whereas increases in VPD reduced the level of fertility to 0 (at 2.5 kPa) (Kozhemyakin *et al.* 2017).

Table 5 – Effect of vapor pressure deficit (VPD) during the flowering period on male fertility of F_2 plants of the testcross hybrid A_3Topaz / F_3 (A_3Karlik-4 / IS1112C) (from Kozhemyakin *et al.* 2017).

VPD, kPa	Number of plants with different fertility level			
	f	pf	ps	s
1.1±0.1	6	-	-	-
1.4±0.1	22	5	-	-
1.7±0.1	6	12	-	-
2.0±0.1	-	-	16	4
2.3±0.1	-	-	-	3
Total	32	17	16	7

Average VPD for five days beginning two days prior to the beginning of anthesis of the middle part of the panicle. Correlation was evaluated by Chuprov contingency coefficient: T = 0.446; $P < 0.001$.

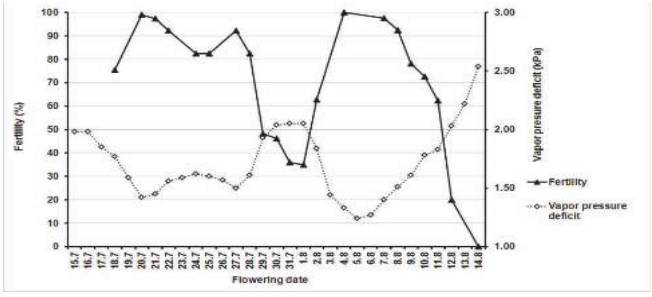

Fig. 9 – Distribution of plants by fertility level in the F_2 generation of the testcross hybrid A_3 Topaz / F_3 (A_3Karlik-4 / IS1112C) and indicators of air vapor pressure deficit (kPa) during the growing season. The indicator of fertility level at each date is the average for all plants in which the middle of the panicle was in bloom on that date (from Kozhemyakin *et al.* 2017).

Therefore, the appearance of sterile plants in F_2 generation was not the result of genetic segregation but was caused by environmental conditions. This may be also true for BC-hybrids. Apparently, expression of the fertility-restoring genes *Rf3* and/or *Rf4* depends upon air humidity. In this connection, restoration of male fertility in the A_3 CMS type, as well as in 9E, appears to be an epigenetically regulated trait.

An interesting observation that is possibly linked with epigenetic control of fertility restoration in A_3 cytoplasm is the difference in restoration ability of plants from F_2 families grown in the dryland and irrigated plots. In testcrosses of plants from the family grown in the irrigated plots, the frequency of fertile and partially fertile individuals was 43.2%, whereas that in testcrosses of plants from the same F_2 family grown in the dryland plots was 17.9% (differences are significant at $P<0.01$). In plants from the F_3 generation, the differences in restoration ability not only persisted but became more extreme, in that plants from families derived from the dryland plots almost completely lost their ability to restore fertility of testcrosses (1.2% fertile hybrids), while families derived from the irrigated plots produced 18.8% fertile testcross hybrids. Such effects are typical for cases of so-called transgenerational inheritance (Daxinger and Whitelaw 2010, Hauser *et al.* 2011). It should be noted that further selection in the progeny of hybrid derived from the irrigated plot resulted in development of stable line fertility restorer for the A_3 cytoplasm.

CONCLUSIONS

The interactions of the genotype and the environment are among the key problems of the biology of plant development. To date, a lot of data on the influence of environmental factors, such as temperature, illumination conditions, water availability, and mineral nutrition on various epigenetic mechanisms regulating the realization of genetic information encoded by a DNA nucleotide sequence have been accumulated in the literature (Kim *et al.* 2010, Fisher and Franklin 2011, Paszkowski and Grossniklaus 2011, Yaish 2013, Yuan *et al.* 2013). The data presented above demonstrate that cytoplasmic male sterility, which arises from the interaction of nuclear and mitochondrial genomes, can be a convenient model for studying these processes. We found that nuclear fertility-restoring genes for a number of CMS-inducing cytoplasms of sorghum may be repressed by genome of CMS-lines under drought conditions, and this repression can be removed by high relative humidity of air and plant irrigation during the booting and flowering stages. We show that such repression in the 9E cytoplasm might be caused by DNA methylation. Therefore, epigenetic changes in nuclear genes, determined by environmental conditions, may be one of the mechanisms of fertility restoration. It is possible that such a mechanism is characteristic not only for sorghum CMS, but also for certain types of CMS in other plant species.

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