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Isolation of Promoters and Fragments of Genes Controlling Endosperm Development Without Fertilization in Arabidopsis and Engineering of the Antisense Constructions*

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Abstract

Apomixis is asexual seed reproduction without both meiosis and fertilization based on the complex developmental processes such as apomeiosis, parthenogenesis and specific endosperm development. This investigation is aimed at engineering of apomixis in *Arabidopsis thaliana* with sexual seed reproduction. The fragments of known genes of endosperm formation MEA, FIE, FIS2 and gene of apomeiosis DYAD (as control) were isolated using Q5 high fidelity DNA polymerase. These gene fragments of interest at the antisense orientation were fused with isolated constitutive and meiosis specific promoters of Arabidopsis at NcoI sites. The fused promoter-gene fragment modules were cloned in pCambia1301 at SalI cites. The engineered constructions will be used for the floral dip transformation of Arabidopsis and down regulation of these genes at engineering of apomixis.

Keywords: *Arabidopsis thaliana*, apomixis, endosperm formation, *MEA, FIE, FIS2*.

Introduction

Apomixis (from Greek apo, without + mixis, mixture) is asexual seed reproduction of floral plants at which germs arise without both meiosis and fertilization [1 – 4]. In fact apomixis is a natural system of maternal genome cloning by seeds. Applied usage of apomixis promises the economic and social benefits exceeding a prize from the "green revolution" which solved a hunger problem in many developing countries. So, only the profit on world production of apomictic rice is estimated more than at $2.0 billion in a year [4].

Already the first apomictic experiments of G. Mendel showed that there are objects for which general principles of inheritance don't work (see experiments with Hieracium plants). The molecular nature of an apomixis still remains unclear [5 – 7]. As it is established so far, it occurs in

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the case of specific abnormal alteration of normal course of meiosis. That is why apomixis may be result of the sophisticated deregulation of classical sexual seed reproduction. The first attempt of engineering of apomixis de novo in Arabidopsis plants with sexual reproduction proved to have promising prospects [8].

Probably the construction of transgene plants with differently expressed genes of apomictic triad (sporogenesis, gametogenesis and an endosperm formation) under the control the cell-specific promoters will allow to construct prototype of apomixis at many agricultural forms with sexual reproduction [7]. **This approach could allow understanding** how genome works upon transition from amphimixis to apomixis in floral plants. The goal of work is the creation of genetically engineered constructions with genes involving in the endosperm formation in Arabidopsis plants.

Materials and methods

1. Biological material

The selection of model objects is very important at the investigations of reproductive systems of floral plants in general and the apomixis in particular [9]. Extensive molecular and genetic resources (completely sequenced genome, mutants ets) are available for Arabidopsis thaliana that facilitates search of the genes participating in the control of apomixis. there The plants of Arabidopsis thaliana of the ecotypes Ler2n, Col 0 (2n) and Wass were in the focus of researches.

2. Methods of molecular biology

Total DNA was extracted from sprouts and leaves of plants by salt extraction method [10]. The screening of perspective promoter and gene candidates was performed from literary data [4, 11]. The analysis of nucleotide sequences and selection of primers carried out with usage of Genbank data and other Resources of PubMed (http://www.ncbi.nlm.nih.gov**).** Promoters and fragments of genes were isolated by means of HiFi Q5 polymerase (NEB). 1% agarose gel-electrophoresis of DNA fragments were carried out in 1x TAE buffer. The subsequent cleaning of DNA preparations was carried out using special kits for cleaning of DNA (Cytokin, Russia) and Quantum PrepTM Freeze'N Squeeze DNA Gel Extraction Spin Columns (BioRad). Restrictase FastDigestTM NcoI (Fermentas) was used to create NcoI sticky ends at promoters and antisense fragments of genes. The fusion of promoters and antisense fragments of genes at NcoI ends was carried out using T4 DNA ligase (Fermentas) or (NEB). The alloyed constructions were amplified by HiFi Q5 polymerase (NEB) and fractioned in agarose gel slab for the subsequent cleaning. Resrictase FastDigestTM SalI (Fermentas) was used for the creation of sticky ends and following cloning of the constructions in pCambia1301 plasmid. Further selection of plasmids and control of inserts were carried out. Nucleotide sequence analysis of DNA preparations was carried out at Beckman Coultier or AppliedBiosystems 3500 analyzers. Thus, in our work we used the following promoters: constitutive (as positive control) – *pMEF1* and *pUBQ* promoters [12], meiosis specific – *pAtDMC1* and *pMS5* promoters [13], ovule specific – *pAGL11* promoter [14], and also gene fragments in the antisense orientation – *DYAD* [15], *MEA* [16], *FIE* [17] and *FIS2* [18].

Results

At first the selection of the most perspective candidate genes of autonomous endosperm development was carried out as it discussed in the articles [4, 11]. Summary of these genes are in Tab.1. Our work is devoted to creation of recombinant plasmids on the basis of the binary vector pCambia 1301 for genetic transformation of plants. Genes *DYAD, SERK* and fragments of genes of interest (*DYAD, FIE, MEA, FIS2*) under constitutive and ovule specific promoters were cloned in the T-DNK field of pCambia 1301. The scheme of vector constructions is submitted in Fig. 1.

Table 1: Genes candidates used in work

##	**Elements of apomictic triad**	**Genes**	**Gene product / function**
I	Apomeiosis	*DYAD (SWI1)*	Meiosis specific chromatin associated protein (phospholipase C), participates in chromosome non-disjunction
II	Parthenogenesis	*SERK1* (Somatic embryogenesis receptor kinase)	Serin-treonin protein kinase, initiates a somatic embryogenesis
III	Autonomous endosperm development	1. *FIE* (Fertilization-independent endosperm) 2. *MEA/ MEDEA* (Maternal embryogenesis control protein) 3. *FIS2* (Fertilization-independent seed 2)	Proteins of Polycomb system, repress development of the central cell in ovule

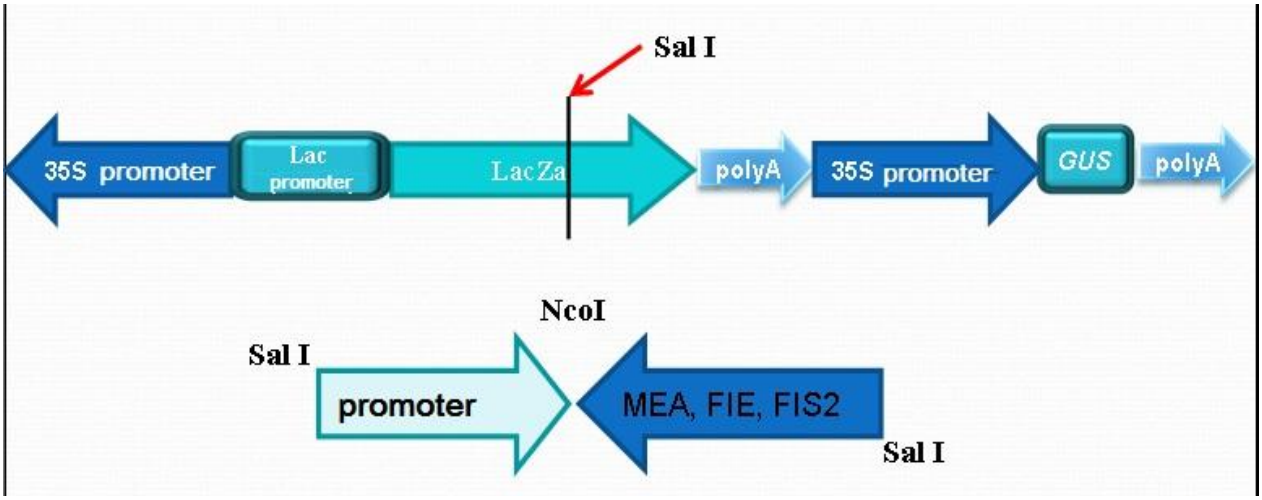

Fig. 1. The scheme of isolation and cloning of promoters and fragments of genes in the antisense orientation

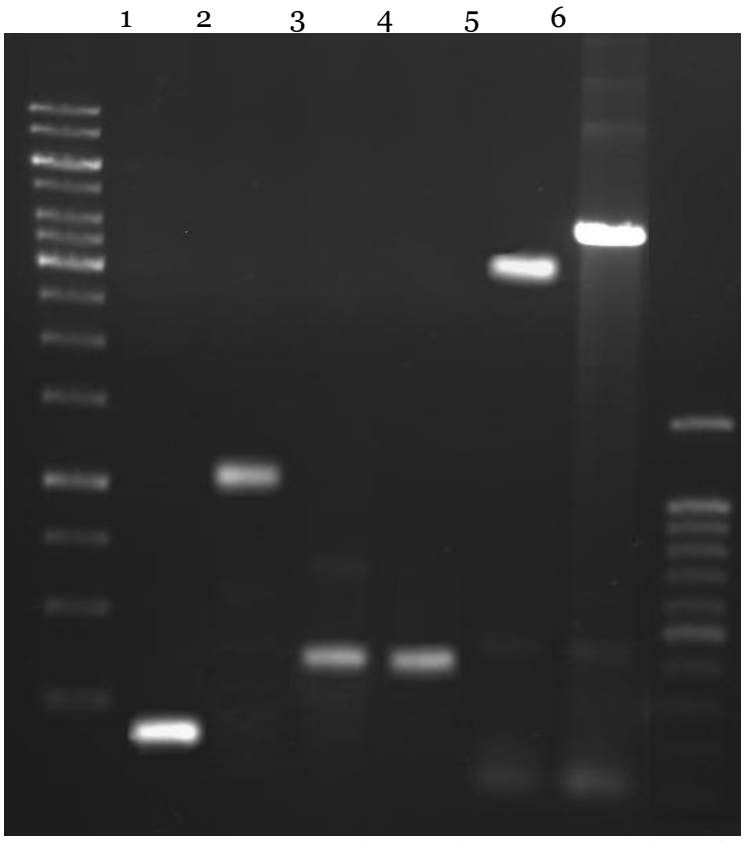

Fig. 2. The electrophoresis separation of isolated fragments of genes.
Fragments of genes are in the antisense orientation:
1 - *DYAD* (0.2 kb); 2 - *MEA* (1.0 kb), 3 - *FIE* (0.4 kb); 4 - *FIS2* (0.4 kb). Full size gene in sense orientation: 5 – *DYAD* (3.0 kb); 6 - *SERK* (4.0 kb)

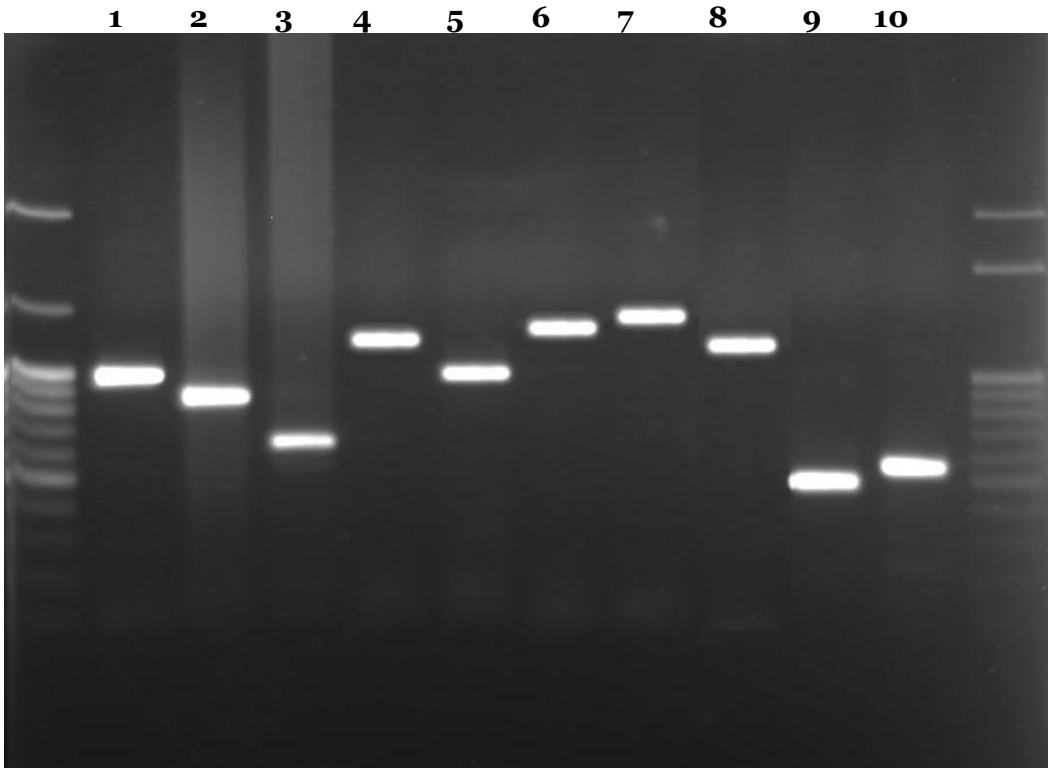

Fig. 3. The electrophoresis separation of isolated promoters
Promoters: 1 - p1 (*pEF1a*), 2 - p2 (*pUBQ*), 3 - p4 (*pMS5*), 4 - p11 (*pRKD1*), 5 - p10 (*pDD45*), 6 - p7 (*pAt5g40260*), 7 - p8 (*pAt5g40260*), 8 - p11 (*pRKD1*), 9 - p9 (*pEC1*), 10 - p13 (*pRKD2p*)

Table 2: The scheme of fused constructions including promoters and fragments of genes in the antisense orientation

NN, names, and sizes	**Genes**			
Promoters	**1) *DYAD*, 0.2 kb**	**2) *MEA*, 1.0 kb**	**3) *FIE*, 0.4 kb**	**4) *FIS2*, 0.4kb**
1) *pEF1a*, 1.0 kb	1.2 kb	2.0 kb	1.4 kb	1.4 kb
2) *pUBQ*, 0.9 kb	1.1 kb	1.9 kb	1.3 kb	1.3 kb
3) *pDMC1*, 3.0 kb	3.2 kb			
4) *pMS5*, 0.6 kb	**0.8 kb**	**1.6 kb**	**1.0 kb**	**1.0 kb**

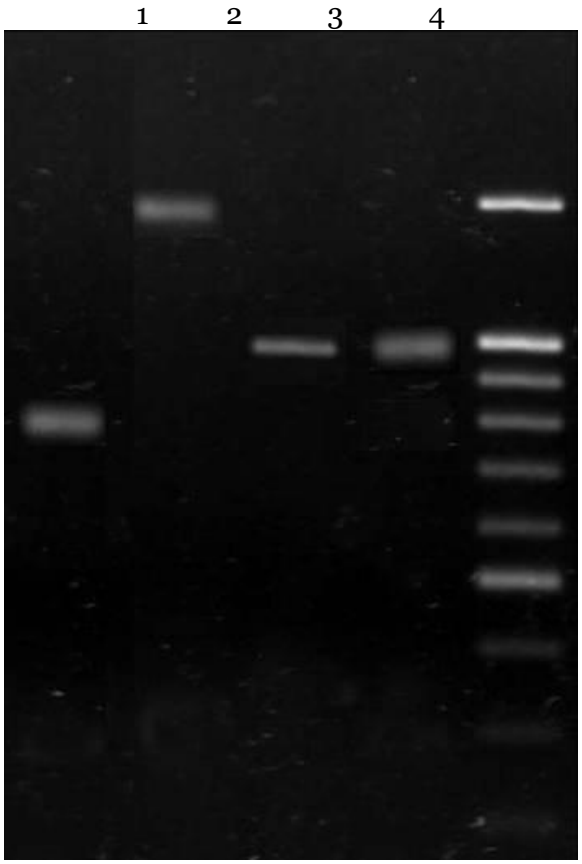

Fig. 4. The electrophoresis separation of constructions containing fragments of genes in the antisense orientation under pMS5 promoter

The created constructions with the fragments of genes in the antisense orientation:
1 – *pMS5_DYAD* (0.2 + 0.6 = 0.8 kb); 2 - *pMS5_MEA* (1.0 + 0.6 = 1.6 kb) ; 3 - *pMS5 _FIE* (0.4 + 0.6 = 1.0 kb); 4 - *pMS5 _FIS2* (0.4 + 0.6 = 1.6 kb).

The control DNA sequencing the cloned elements validated correct assemblies of the constructions.

Discussion

The idea of apomixis engineering de novo due to the deregulation of the genes involved in sexual process at plants (first of all in arabidopsis) was stated in 1993 [19]. It is known that apomictic ways don't function independently from a classical sexual way of seed plants, and, therefore, the genes controlling apomixis can't be entirely new in the function, and, most likely, are subjected to regulatory changes [20]. We need take into account classical model plants, such as arabidopsis and corn, have no apomixis, and however have abundance of forms and lines reminding apomixis ("miming apomixis"). Availability of information about some genes involved in functioning of an apomictic triad – apomeiosis, parthenogenesis and endosperm formation (see Table 1.) create fine prerequisites for the creation of apomictic prototype at arabidopsis.

So, the group of d'Erfurth managed to create in the lab a MiMe genotype of arabidopsis for which meiosis is entirely replaced with a mitosis due to a combination of three various genes involved in meiosis [9]. The simultaneous suppression of *OSD1/TAM, At-Spo11-1* and *Atrec8* genes leads to formation of male and female gametes. Thus artificial mutants of MiMe only demonstrate a sign of apomeiosis (the first elements of an apomictic triad) and aren't capable to a parthenogenesis. Thus, MiMe genotype plants bring forth offspring with the doubled set of chromosomes, and special schemes of crossings are necessary for lowering of the ploidy level. We will note that artificial MiME system of arabidopsis isn't known at natural apomicts. Thus, the engineering of apomixis de novo is an excellent alternative to unsuccessful attempts cloning genes of apomixis.

The strategy of our researches is to place the genes of interest under the transcriptional control of cell-, tissue-, and/or organ- specific promoter, for example, the promoter, specifically expressed in an ovule (ovule-specific promoter). It is known that arabidopsis genes of *FERTILIZATION-INDEPENDENT ENDOSPERM (FIE)* [17], *MEDEA (MEA)* [16] and *FERTILIZATION-INDEPENDENT SEED2 (FIS2)* [18] suppress development of endosperm. Perhaps, the suppression of these genes could initiate the process of the autonomous endosperm development. As a result of this work, we created the genetically engineered constructions containing the key genes in the antisense orientation for down regulation of their expression and following the fertilization-independent seed development (see Fig.1 – 4).

In our lab work we faced with the task to clone genes of *FIE, MEA* and *FIS2* in antisense orientation in binary vector of pCambia 1301 under various promoters. We described the received results in this article. In further work we should introduce vector constructions into arabidopsis plants and to estimate how their work in various lines (especially in mutants with different deviations of developments). The comparative cytogenetic analysis will be used for this purpose. Ploidy levels of control and transgenic plants will be studied too.

The nature of inheritance of transgene constructions and deviation from splitting in progeny of transgene plants will be investigated. Discussions about prospects of use of these or those elements of future constructions for engineering of apomixis is proceeding up to now [4, 6]. However messages except works of d'Erfurth group practically are out [8]. A few references (given in article) show common progress in this field and importance of our results.

Conclusion

All efforts to use of biotechnological of advantages of apomixis assume the transfer of an apomixis in crops. In this direction three key strategy may be useful [21]: (1) a direct introgression of apomixis in agricultural plants by means of traditional schemes of selection; (2) genetic transformation of agricultural plants by means of transfer of the alien genes controlling an expression of an apomixis; and (3) artificial deregulation of own genes controlling sexuality. The recent works performed at Arabidopsis, corn and rice allowed to perform identification of apomixis-like mutants and the corresponding genes [4, 6, 7, 11].

In this investigation we reported about the cloning of *DYAD* gene fragment (as control) and *FIE, MEA* and *FIS2* fragments of genes in the antisense orientation under constitutive and cell- and meiosis specific promoters in binary vectors of pCambia 1301 for the down regulation of the genes of interest controlling autonomous endosperm development. The fused constructions will be used for the production of transgene arabidopsis plants by floral dip method.

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