ISSN 2079-0597, Russian Journal of Genetics: Applied Research, 2017, Vol. 7, No. 2, pp. 145–152. © Pleiades Publishing, Ltd., 2017.
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Obtaining Interspecific Hybrids for Introgressive Pea Breeding

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Received March 13, 2015; in final form, August 12, 2015

Abstract—Crosses between cultural pea varieties and a wide range of accessions of wild *P. fulvum* species were performed. All seeds from the combination *P. sativum* × *P. fulvum* were developed and hybrid. Crosses *P. fulvum* × *P. sativum* resulted in the formation of seeds with unfilled embryos. The hybrid nature of *P. fulvum* × *P. sativum* plants was confirmed using biochemical and morphological markers. The regenerated plants were obtained by in vitro culturing eight-day-old immature embryos of *P. fulvum* (I592589) ×*P. sativum* (Aist cultivar). Some accessions of wild *P. fulvum* species were found to have the unique protein band 7, which was absent in the electrophoretic spectra of the cultural pea. Hybridological analysis was performed and the inheritance of protein band 7 was determined as a monogenic and dominant.

Keywords: pea, *Pisum fulvum*, interspecific hybridization, seed proteins, introgression

DOI: 10.1134/S2079059717020046

INTRODUCTION

The pea (*Pisum sativum* L.) is an important leguminous crop of a temperate climate. Pea breeding based on a high and stable yield, resistance to biotic and abiotic stresses, a high content and quality of storage proteins is the main condition for increasing the competitiveness of the crop (Ali et al., 1994; Bobkov and Uvarova, 2010).

Molecular genetic studies have revealed a paradoxically narrow genetic base of modern pea cultivars (Baranger et al., 2004). The involvement of new alleles for economically valuable traits in the breeding process will increase the genetic diversity of new pea varieties. There are two ways to transfer genes (alleles) of interest into the elite breeding lines of pea: genetic engineering and introgression from their wild relatives (Ellis, 2011; Smykal et al., 2011). Genetic engineering is expensive and insufficiently developed method for pea. Interspecific hybridization leads to the introgression of both useful and unwanted genes. Therefore, measures should be taken during the introgression of the genes of interest to block the transfer of unwanted genetic material. Using backcrosses with cultivars and elite breeding lines promotes the selective introgression of valuable genes. These strategies for transferring genetic material are not exclusive and there are good prospects for their combined use.

Among wild peas, *P. fulvum* is an important source of new alleles for economically valuable traits. The genome size of *P. fulvum* is 108.9% of *P. sativum* (Baranyi et al., 1996). The species is of interest for use in pea breeding for resistance to diseases, pests, and abiotic stress factors (drought and extreme temperatures) (Ochatt et al., 2004; Byrne et al., 2008; Clement et al., 2009). The *P. fulvum* roots penetrate the soil at a high rate to a greater depth (Ali et al., 1994).

It is known that interspecific hybridization of pea faces the problem of reproductive incompatibility (Ben-Ze'ev and Zohary, 1973; Bogdanova and Kosterin, 2007) and is characterized by a low efficiency of crosses (Ochatt et al., 2004).

A considerable phylogenetic divergence between *P. sativum* and *P. fulvum* is manifested in sufficiently strong reproductive isolation (Ben-Ze'ev and Zohary, 1973; Ellis, 2011; Smykal et al., 2011; Zaytseva et al., 2012). It is possible to obtain hybrids from the crosses *P. sativum* × *P. fulvum* (Ochatt et al., 2004; Byrne et al., 2008; Fondevilla et al., 2010; Bobkov and Lazareva, 2012); however, reverse hybridization is facing severe incompatibility. It is reported on receiving nonviable seeds (Ben-Ze'ev and Zohary, 1973) and one hybrid plant derived from 100 crosses (Bogdanova and Kosterin, 2007).

Despite some advances in the introgression of genes for resistance from *P. fulvum* into the genome of the cultural species (Fondevilla et al., 2010), reproductive isolation restricts the use of interspecific hybrids in pea breeding. Overcoming interspecific incompatibility barriers is a paramount and important task (Ochatt et al., 2004).

Based on the fact that various genes of interest may be in different accessions of *P. fulvum* (Byrne et al.,

Table 1. Interspecific hybridization of the pea (2012 and 2013)

Index no.	*P. fulvum* accessions	*P. sativum* × *P. fulvum*			*P. fulvum* × *P. sativum*		
		Stabil	Aist	Sum	Stabil	Aist	Sum
1	K2523	2*	–**	2	9	–	9
2	K6070	5	–	5	5	–	5
3	I582583	–	–	–	2	–	2
4	I592573	1	–	1	3	–	3
5	I592575	–	–	–	1	–	1
6	I592577	–	–	–	1	–	1
7	I592579	–	3	3	–	2	2
8	I592589	2	3	5	–	4	4
9	I582593	–	2	2	–	2	2
10	I592595	1	–	1	–	2	2
11	I592597	–	–	–	1	–	1
12	I582598	–	1	1	2	4	6
13	I592602	–	–	–	1	3	4
14	I592603	1	1	2	2	4	6
15	I592608	1	–	1	–	–	–
16	I592609	–	–	–	–	1	1
17	I592612	–	–	–	1	–	1
18	I592615	–	1	1	–	1	1
19	I592619	–	–	–	2	–	2
20	I592626	–	–	–	1	–	1
21	I592881	1	–	1	–	–	–
22	I609881	–	–	–	6	–	6
23	I609884	–	–	–	–	1	1
TOTAL		14	11	25	37	24	61

*Number of pollinated flowers; **crosses were not performed.

2008; Fondevilla et al., 2010), studying interspecific incompatibility should be carried out taking into account the genetic diversity of wild species. This work can be combined with the searching for and identification of accessions that are the sources of economically valuable traits.

The study was aimed at investigating the characteristics of interspecific pea hybridization using a wide range of wild species accessions to create *P. sativum* introgression lines with inclusions of new genes and alleles of economically valuable traits from the *P. fulvum* genome. Hybrids and unique *P. fulvum* genetic material were identified using the electrophoretic spectra of seed storage proteins.

MATERIALS AND METHODS

The following cultivars of *P. sativum* ssp. *sativum* were used in the experiments: Stabil (*af*, semi-leafless morphotype), Aist (leafy morphotype), and line VI 9402 (*tl*, acacia like morphotype), as well as the accessions of *P. fulvum* K2523, K6070, I592573, I582583, I592575, I592577, I592579, I592589, I582593, I592595, I592597, I592598, I592602, I592603, I592608, I592609, I592612, I592615, I592619, I592626, I592881, I609881, and I609884 from the international VIR collection (Table 1). Interspecific hybridization of the pea was carried out in June and July 2012 and 2013 in a greenhouse based on a reciprocal scheme. The direction *P. sativum* × *P. fulvum* and *P. fulvum* × *P. sativum* included 61 and 25 crosses, respectively. The correlation coefficient between the number of *P. fulvum* accessions, used in different directions of crosses, was 0.45 ($p < 0.05$).

Additional crosses were conducted in the direction *P. fulvum* × *P. sativum* for the in vitro cultivation of isolated ovules and embryos. One cross was carried out in each combination I592595 × Stabil, I592583 × Stabil, I592602 × Stabil, I592612 × Stabil, I592589 × Aist, and K2523 × Stabil. Three crosses were carried out in combinations K6070 × Stabil and K2523 × Stabil, and six crosses in combination K2523 × Aist. One cross was

Table 2. Efficiency of interspecific pea hybridization

Direction of crosses	Number of flowers	Pods		Seeds		
		Number	Number per cross	Number	Number per cross	Number of seeds in one pod
P. sativum × *P. fulvum*	25	9	0.36 ± 0.1	20	0.8 ± 0.08	2.22
P. fulvum × *P. sativum*	61	4	0.07 ± 0.03**	13	0.21 ± 0.05***	3.25

**Differences are significant at $p = 0.005$; **differences are significant at $p = 0.001$.

carried out in reverse combinations Stabil × K6070 and Stabil × K2523.

Isolated ovules and embryos derived from interspecific crosses were planted on agarized nutrient media for the initiation of morphogenic calli, the prolonged subcultivation of the regenerating calli, and obtaining regenerated plants (Bobkov, 2014). Ovules were isolated and placed on nutrient media at the age of four to six days. In cross combinations K2523 × Aist and I592589 × Aist, immature embryos were isolated at the age of eight days.

Comparing the effectiveness of the crosses in the direction *P. fulvum* × *P. sativum* and *P. sativum* × *P. fulvum*, the number of pods and seeds (the absolute number and the calculated number per crossing), as well as the average number of seeds per pod have be taken into account (Kosterin and Bogdanova, 2014).

Interspecific hybrids were identified by the electrophoretic spectra of individual seeds and morphological markers of vegetating plants.

Seed proteins were isolated and separated in polyacrylamide gel according to (Konarev, 2000). Proteins were extracted from flour during 20 h at 3–4°C in an electrode buffer (TRIS, glycine, sodium dodecyl sulfate), pH 8.3. After centrifugation, 10 μL of the extract was transferred into well of microplate and mixed with an equal volume of the sample buffer (sodium dodecyl sulphate, Tris-HCl, glycerol, β-mercaptoethanol, bromophenol blue). Electrophoresis was performed in a polyacrylamide gel using a vertical electrophoretic VE-4 unit (Helicon, Russia). The concentrations of separating and stacking gels were 12.5 and 5%, respectively.

Markers with a molecular weight of 6.5 to 200 kDa (SIGMA-ALDRICH, United States) were used to identify the unique protein bands of *P. fulvum* (Shand et al., 2007). The inheritance of the protein band 7 was analyzed using the electrophoretic spectra of 19 F_2 hybrid seeds.

The significance of differences was assessed using Student's t and chi-square tests (Dospekhov, 1985).

RESULTS

Effectiveness of the Crosses

The cross direction *P. sativum* × *P. fulvum* included 25 pollinated flowers, where nine pods containing a total of 20 seeds were formed (Table 2). The number of formed pods and seeds calculated per cross equaled 0.36 and 0.80, respectively. Sixty-one crosses in the direction *P. fulvum* × *P. sativum* resulted in four pods and 13 seeds. The number of pods and seeds calculated per cross equaled 0.07 and 0.21, respectively. Using *P. fulvum* as female parent in our experiments resulted in low hybridization efficiency. The differences in the number of formed pods and seeds between these directions of crosses were statistically significant ($p = 0.005–0.001$). The average number of seeds per pod in the crosses *P. fulvum* × *P. sativum* and *P. sativum* × *P. fulvum* was 3.25 and 2.22, respectively.

Productive combinations of crosses were identified. In the direction *P. sativum* × *P. fulvum*, seeds were obtained in Stabil × K2523, Stabil × K6070, Stabil × I592589, Stabil × I592595, Stabil × I592603, Aist × I592579, and Aist × I582598 cross combinations. In the direction *P. fulvum* × *P. sativum*, seeds were obtained in I592577 × Stabil, I692603 × Stabil, I582593 × Aist, and I592602 × Aist crosses (Table 3). Productive crosses in this direction led to the formation of pods with undeveloped seeds. Cross I592577 × Stabil gave one filled seed that proved to be viable. The vegetating I592577 × Stabil F_1 plant was well developed (Fig. 1).

The morphogenic calli, regenerating callus tissues, and the regenerated plants were derived from the in vitro culture of isolated eight-day-old embryos from the I592589 × Aist cross.

Identification of Pea Interspecific F_1 Hybrids According to the Protein Band Composition of Electrophoretic Spectra

Individual pea seeds obtained from interspecific crosses in combinations Stabil × I592603, Stabil × I592595, I592603 × Stabil, I582593 × Aist, and I592602 × Aist were taken for the electrophoretic analysis (Table 3). The resulting protein spectra were compared with the spectra of the parents' seeds.

Table 3. Effective cross combinations and identification of hybrids

Index no.	Combination	Number of pollinated flowers	Number of pods	Number of seeds in a pod	Identification of hybrids
			P. sativum × *P. fulvum*		
1	Stabil × K2523	2	1	3	Analysis was not performed
2	Stabil × K6070	5	3	2 + 1 + 1 = 4	Analysis was not performed
3	Stabil × I592589	2	1	1	Analysis was not performed
4	Stabil × I592595	1	1	3	SDS-PAGE: all are hybrids
5	Stabil × I592603	1	1	7	SDS-PAGE: all are hybrids
6	Aist × I592579	3	1	1	Analysis was not performed
7	Aist × I582598	1	1	1	Analysis was not performed
			P. fulvum × *P. sativum*		
1	I592577 × Stabil	1	1	1	A vegetative plant was obtained and identified as a hybrid because of its red flowers and stipules with serrated edges
2	I692603 × Stabil	1	1	4	SDS-PAGE: no spectra
3	I582593 × Aist	2	1	2	SDS-PAGE: bands of the spectra are poorly expressed
4	I592602 × Aist	3	1	6	SDS-PAGE protein analysis of one seed: hybrid

The spectra of proteins derived from the seven individual seeds of the suggested hybrids were analyzed in the cross Stabil × I592603 (Fig. 2). All spectra contained the protein bands of the Stabil cultivar and the male parent–I592603 accession of the wild species *P. fulvum*. The presence of protein bands in the spectra of both parents was an indicator for the hybrid nature of the obtained seeds. The spectra of three individual seeds from the suggested hybrids were analyzed in the Stabil × I592595 cross. All the analyzed spectra had unique bands from the Stabil cultivar and the I592595 accession of *P. fulvum* (Fig. 4). Consequently, all the obtained seeds were hybrid.

The formation of unfilled seeds was observed in the crosses *P. fulvum* × *P. sativum*. The cross I692603 × Stabil resulted in four unfilled seeds having practically no embryos. The electrophoretic analysis showed no spectra. Two seeds obtained in the cross combination I582593 × Aist were unfilled. However, the electrophoretic analysis revealed the presence of the random protein bands in the spectra (Fig. 3). A small amount of protein did not allow us to carry out a comparative analysis of the polymorphic bands needed for the identification of hybrids.

The I592602 × Aist cross combination resulted in six seeds (Table 3). One seed was almost filled, and the remaining five did not contain any rigorous embryos.

The electrophoretic protein analysis of the filled seed revealed the presence of five unique protein bands from the Aist cultivar (Fig. 4). The spectrum also contained the bands of the *P. fulvum* accession I592602, which allowed us to identify the obtained seed as a F_1 hybrid.

The vegetating plant obtained from the cross I592577 × Stabil was characterized by red flowers and stipules with serrated edges, indicating its hybrid nature (Fig. 1). We note that the petals of the flower buds originally had a light yellow color; however, by the time of the disclosure they acquired a red pigmentation.

Identification and Introgression of Genes Encoding the Unique Proteins from P. fulvum

Identification of unique bands in the accessions of the wild *P. fulvum* species, which are absent in the spectra of *P. sativum* cultivars and lines, is very important at performing the comparative analysis of the electrophoretic spectra of seed proteins. The unique bands in the spectra of *P. fulvum* may contain the proteins that play an important role in plant resistance to pests and diseases and may serve as a marker of genes for economically valuable traits.

Earlier, the protein band 7 (numbered according to the defined bands of the soybean seed protein spectra), which was absent in pea cultivars and lines and was unique to the *P. fulvum* accession I609881, was identified. It was shown that the band was inherited in

F_1 hybrid combinations 109b × I609881 and Stabil × I609881 (Bobkov, Lazareva, 2012). Later, band 7 was detected in the electrophoretic spectra of the *P. fulvum* accessions K2523 and K6070 and in the K296 accession of *P. sativum* ssp. *transcaucasicum*.

The hybridization of the VI 9402 line of the acacia-like pea morphotype and I609881 accession was carried out to determine the inheritance of protein band 7. The cross resulted in F_1 and F_2 hybrids. Electrophoretic analysis was performed for proteins from the individual seeds of F_1 and F_2 hybrids. Band 7 was present in the spectra of all F_1 hybrid seeds. The spectra of 19 seeds of F_2 hybrids showed the genetic segregation for this band (Fig. 5). Phenotypes with and without band 7 were distributed in the ratio of 13 : 6. In accordance with the hypothesis of monogenic dominant inheritance, the theoretical segregation corresponds to a ratio of 3 : 1. The actual segregation ratio was 2.2 : 1. The results were compared by the chi-square test, which revealed the coincidence of the actual and theoretical segregations ($\chi^2_{act} = 0.44$; $\chi^2_{0.5} = 3.84$). Therefore, the inheritance of protein band 7 was monogenic and dominant.

DISCUSSION

There are various methods for transferring the genetic material into new breeding lines: genetic engineering and interspecific hybridization. Genetic engineering is expensive and insufficiently developed for pea (Ellis, 2011). Interspecific hybridization leads to the creation of the material that is barely suitable for use in breeding due to the parallel transfer of economically valuable and unwanted genes (alleles). Methods of marker-assisted breeding, using cultural pea lines as the recurrent parents for backcrosses, are capable of creating introgressive pea lines without any unwanted alleles.

At the beginning it is necessary to confirm the establishment of interspecific hybrids during the interspecific crosses. Previously, interspecific hybrids were identified by marker genes for their morphological traits, the flower color of vegetating F_1 plants (Byrne et al., 2008; Bobkov and Lazareva, 2012), the electrophoretic spectra of the isoenzymes, and DNA-markers (Ochatt et al., 2004). In our experiments, we used a method based on comparing the seed protein spectra of the parents and products of hybridization. It allows us to identify hybrids with no need to obtain vegetating plants and in case of nonviable seeds. Additionally, the morphological markers were used to identify the hybrids.

It is known that the *Pisum* genus is characterized by the reproductive isolation of the taxa. For example, disorders of the meiosis due to the nuclear-cytoplasmic incompatibility were described in hybrids derived from crosses between the accessions of the wild subspecies *elatius* and the cultural subspecies *sativum* of *P. sativum* L. (Bogdanova and Galiev, 2009). The hybridization of pea (*P. sativum*) with wild species (*P. fulvum*) faces a more severe reproductive incompatibility (Ben-Ze'ev and Zohary, 1973). It is known that interspecific hybrids were obtained only in

Fig. 1. Vegetating plant of the interspecific F_1 hybrid I592577 × Stabil having stipules with serrated edges.

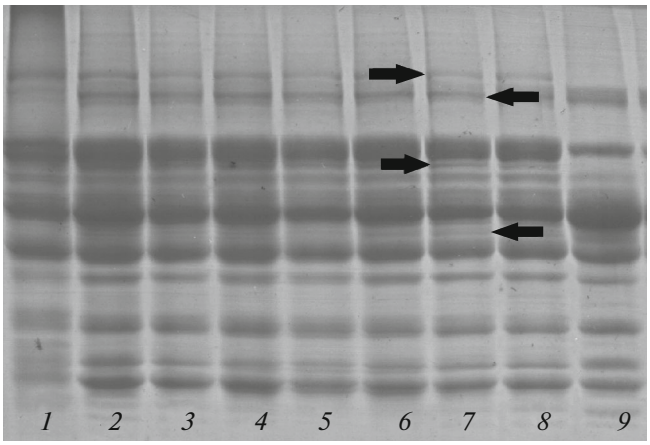

Fig. 2. Electrophoretic protein analysis of individual seeds from parents and F_1 hybrids in the Stabil × I592603 cross. Electrophoretic spectra: *1*—*P. fulvum* accession I592603; *2*–*8*—F_1 hybrids, *9*—cultivar Stabil. The right arrows denote protein components of the accession I592603, the left arrows denote the protein bands of the cultivar Stabil.

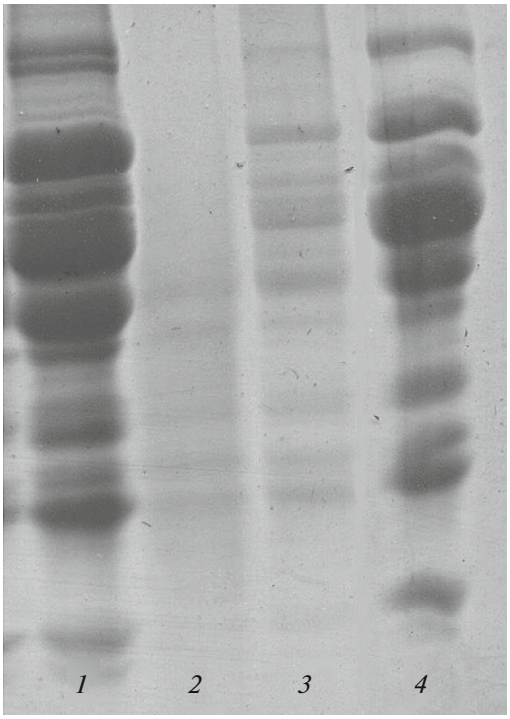

Fig. 3. Electrophoretic protein analysis of individual seeds from the parents and offspring obtained from the I582593 × Aist cross. Electrophoretic spectra: *1*—*P. fulvum* accession I582593; *2*, *3*—seeds obtained from the I582593 × Aist hybridization; *4*—cultivar Aist. Unfilled seeds contained embryos with an insufficient amount of proteins to perform the electrophoretic analysis (spectra *2* and *3*).

crosses, where *P. fulvum* was used as the male parent (Ochatt et al., 2004; Byrne et al., 2008; Bobkov and Lazareva, 2012). These crosses did not characterized by high efficiency (Ochatt et al., 2004). Using *P. fulvum* as a female parent led to appearance of nonviable seedlings (Ben-Ze'ev and Zohary, 1973). One hybrid seed and vegetative plant was obtained as a result of 100 crosses in the direction *P. fulvum* × *P. sativum* (Bogdanova and Kosterin, 2007). The use of DNA markers showed that the obtained four F_2 hybrid plants contained plastids from the male parent (*P. sativum*).

In our experiments, the average efficiency of the pod formation in the direction *P. fulvum* × *P. sativum* was 7%, which was significantly ($p = 0.005$) lower compared to the crosses, where *P. fulvum* was used as the male parent (36%). Significant ($p = 0.001$) differences were observed also for the number of seeds produced, calculated per cross in the *P. fulvum* × *P. sativum* (0.21) and *P. sativum* × *P. fulvum* (0.8) directions. The number of seeds in one pod in the direction *P. fulvum* × *P. sativum* was equal to 3.25; it was higher than that in the reverse combination (2.22).

In the *P. sativum* × *P. fulvum* crosses, all the obtained seeds were well developed and hybrid; however, the reciprocal combinations resulted in the formation of unfilled seeds. Four seeds derived from the I592603 × Stabil cross had almost no embryos (arrested development in the early stages). Two seeds obtained in the I582593 × Aist cross combination were unfilled. The electrophoretic analysis revealed the presence of the spectra; however, the insufficient amounts of protein (the polymorphic bands were absent) did not allow us to identify the hybridization products. However, the fact of the presence and separation of storage proteins in polyacrylamide gel (Fig. 3) indicated the formation and growth of the embryos.

Among the six seeds formed in the I592602 × Aist cross combination, only one seed was characterized by the presence of a filled embryo (Table 3). The electrophoretic analysis of proteins confirmed hybrid nature

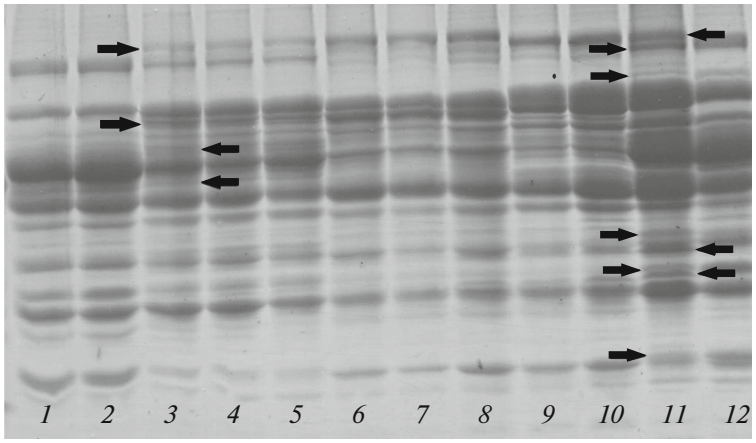

Fig. 4. Electrophoretic protein analysis of individual seeds from parents and F_1 hybrids in the Stabil × I592595 and I592602 × Aist crosses. Electrophoretic spectra: *1*, *2*—Stabil cultivar; *3*–*5*—F_1 Stabil × I592595 hybrids; *6*–*8*—*P. fulvum* accession I592595; *9*, *10*—*P. fulvum* accession I592602; *11*—F_1 hybrid I592602 × Aist; *12*—Aist cultivar. The right arrows in spectrum 3 denote the protein bands of the accession I592595, the left arrows denote the protein bands of the cultivar Stabil. The right arrows in spectrum *11* denote the protein bands of the cultivar Aist, the left arrows denote the protein bands of the *P. fulvum* accession I592602.

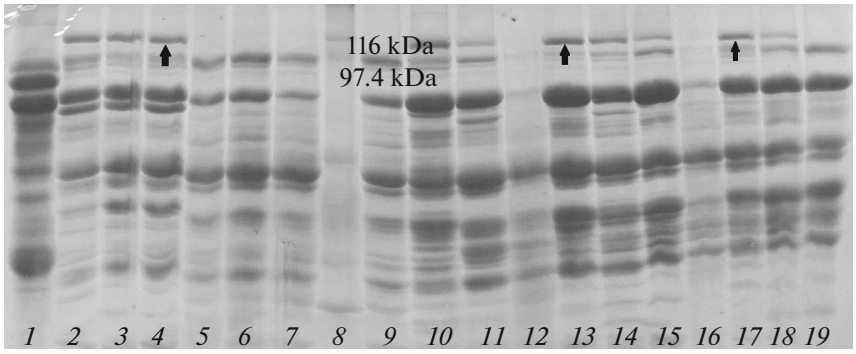

Fig. 5. Electrophoretic spectra of seed proteins from parents and interspecific F_2 hybrids VI 9402 (*P. sativum*) × I609881 (*P. fulvum*): *1*—soybean cultivar Lantsetnaya; *2–4*—accession I609881; *5–7*—line VI 9402; *8*—molecular weight markers (SIGMA-ALDRICH, United States); *9–19*–spectra of F_2 hybrids. The arrows indicate band *7* (~110 kDa).

of the seed in the *P. fulvum* × *P. sativum* cross, where *P. fulvum* was used as the female parent (Fig. 4).

It is known that interspecific pea hybridization is possible when *P. fulvum* is used as the male parent. The main cause of the reproductive incompatibility in the *P. fulvum* × *P. sativum* combination is the lack of germination of *P. sativum* pollen grains on the stigmata of *P. fulvum* (Ochatt et al., 2004). Obtaining hybrid seed can be explained by the fact that the pollen germinates and fertilization takes place in some cases.

Interspecific hybridization in legumes faces postzygotic incompatibility, leading to the death of the embryos in the early stages of development. In vitro embryo culture is used to save them. Cultivation of isolated embryos from interspecific hybrids in nutrient media is considered as an important method for the introgression of the genetic material from chickpea wild species (Clarke et al., 2006). In vitro embryo culture has been successfully used in the interspecific hybridization of lentil (Suvorova, 2014).

Overcoming the postzygotic incompatibility of the pea with embryo culture is especially important for inefficient interspecific *P. fulvum* × *P. sativum* crosses. In our experiments, the in vitro culture of isolated eight-day-old pea embryos from the I592589 × Aist cross led to positive results. Morphogenic callus tissues and regenerated plants were obtained.

Interspecific *P. fulvum* × *P. sativum* pea hybrids should have mitochondria from *P. fulvum*, which are known to be inherited through the maternal line (Bogdanova and Galiev, 2009). Creating introgressive pea lines with the mitochondrial genome from *P. fulvum* opens up new possibilities for genetic and physiological studies.

Some accessions of the *P. fulvum* wild species are characterized by an increased resistance to pests and diseases (Byrne et al., 2008; Fondevilla et al., 2010). Therefore, the question whether the unique protein bands of *P. fulvum* seeds serve as markers for increased resistance to adverse environmental factors is very relevant. It is known that increased resistance to pests and diseases can be kept by unique isoforms of lipoxygenase (Porta and Rocha-Sosa, 2002). Lipoxygenases were detected in mature and immature seeds and vegetative organs of the pea (Domoney et al., 1990).

Accessions characterized by the presence of the unique band 7 with a protein molecular mass of ~110 kDa were found among the representatives of the *P. fulvum* species. This protein band was located in close proximity to two major isoforms of the pea lipoxygenase with molecular weights of ~90 and ~97 kDa (Casey et al., 1985). Using affinity chromatography, a pea lipoxygenase isoform with a molecular mass of ~100 kDa was revealed (Domoney et al., 1990). Manganese lipoxygenases of a pathogenic fungus, *Gaumannomyces graminis*, are localized on electrophoretic spectra in the range 100–140 kDa (Su and Oliw, 1998).

The presence of band 7 near the bands of pea lipoxygenases is not a sufficient reason to consider it as an earlier unknown isoform. Issues on whether the protein band 7 belongs to the lipoxygenases or other proteins and its functional properties require additional research.

The introgression of unique *P. fulvum* genes in the genome of the cultural pea using protein markers has no significant technical constraints for use in pea breeding. Electrophoretic analysis requires small amounts of the desired protein extracted from one cotyledon or a part of it. As a result, the bud of a second cotyledon germinates and a normal plant grows from it.

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Translated by M. Shulskaya