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CURRENT STATE OF FOREIGN INVESTIGATION ON GENETICS AND BREEDING OF SUGAR BEET

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S u m m a r y

The authors discussed the results of investigations of foreign scientists during last decade on widening of genetic basis of sugar beet, introgression genes from wild related species for an increase of resistance of varieties and hybrids to plant pests and diseases and also the data on study of genetic variability of varieties of the *Beta* genus with the use of molecular methods.

Keywords: modern foreign investigations, sugar beet, genetics, molecular markers, breeding, wild beet potential, resistance.

Sugar beet is one of 15 most consumed crops, as it has been reported by FAO PNAS USA (1). Main direction of foreign investigation on sugar beet genetics and breeding is increasing its genetic pool by introgression of resistance genes from relative wild species (2). Sugar beet is a "younger" crop with narrower genetic basis than wheat, rice and barley. Successful breeding work on sugar beet necessitates screening of available genetic resources (wild and cultivated) and studying their genetic variability by different methods: conventional (morphological, anatomical, physiological, cytological) and biochemical (protein markers and DNA molecular markers as the most numerous and informative ones) aimed at identification and mapping of genes responsible for economically important traits, as well as detection of carriers of these genes used as a valuable source material for hybridization. Today, all identified Mendelian genes for economically valuable traits are commercialized (2). Some of them (such as genes for rhizomania resistance) are expected to be involved in further genetic engineering work for obtaining a commercial product (3).

Today, foreign investigations on breeding sugar beet is focused on molecular level of its genetic basis. Its prerequisite were the earlier findings about the genome of this crop. Back in the early 1990ies, the species *Beta vulgaris* (sugar beet) was found to have a relatively small genome of 758,000,000 bp. The first molecular markers of DNA mapped in sugar beet were anonymous RFLP-markers (restriction fragment length polymorphism), which helped to build maps of linkage for major genes for resistance to rhizomania, monogerm seed and color of hypocotyl in sugar beets (4). Then, using PCR and molecular markers RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeat) along with some isozyme and morphological ones allowed to obtain genetic maps for all the 9 groups of linked genes in sugar beet.

Even more opportunities for genetic mapping appeared after the development of SNP (single nucleotide polymorphism) – the most numerous class of codominant markers whose identification can be easily automatized and subjected to multiparallel analysis (5). Identification and isolation of genes allowed to apply GISH and FISH techniques of molecular cytogenetics (genome- and fluorescent in situ hybridization) to detect of desired genes in the genome; this is especially important, for example, in interspecific hybrids – candidate carriers of resistance genes transferred from wild species (6).

Studies of DNA polymorphism and SNP-markers were the basis for building the map of expressed genes of sugar beet (7). The authors selected candidate genes according to a common strategy for identification of genes affecting traits (8). These were the evolutionarily preserved genes for carbohydrate metabolism, which were mapped upon the analysis of quantitative trait loci (QTLs) affecting quality of sugar and related to yield (9). Genetic markers were obtained similarly, using resistance gene analogues as candidate genes (10). Firstly, DNA fragments of candidate genes were amplified with degenerative primers designed on the basis of linearly arranged heterologous sequences of Arabidopsis. Today, availability of EST (expressed sequence tag) for sugar beet collections facilitates selection of a pattern. Seven various genotyping techniques were applied including SNP identification by laser desorption and ionization of macromolecules mediated by the matrix (MALDI - matrix assisted laser desorption ionization), mass spectrometry, pyrosequencing and fluorescent screening of labeled nucleotides; during this work, 712 segregation analyses were performed on 538 markers detected in three F_2 populations of sugar beet (7). Anchored SNP- and RFLP-markers were used for mapping the genes for resistance to powdery mildew (11). Three initial resistant forms were crossed with sugar beet to create a splitting population. Plants' resistance was assessed against the artificial background of field tests and in controlled conditions. Two initial forms manifested the level of resistance significantly exceeding currently available cultivars of sugar beet.

AFLP-analysis was combined with the method of splitting sample (bulked segregant) to design markers associated with resistant phenotype in each population. Five major resistance genes were identified and named as the symbols from *Pm 2* to *Pm 6*, and *Pm 3* was found to control total resistance. The partial resistance trait was introduced into the breeding line C39 and transferred to commercial hybrids. In cultivars with low susceptibility, the beginning of powdery mildew epidemic was delayed for 2 weeks at 20-60% lower infectivity of the pathogen. When growing in the controlled conditions (greenhouse), two samples of *B. vulgaris* subsp. *maritima* (WB 242 and WB 97) revealed highly resistant plants, which were involved in reciprocal crosses and transferred this trait to breeding lines. Field trial showed the resistance controlled by one major gene *Pm*. Although *Pm* provides high resistance degree, the disease symptoms were observed on mature leaves at the end of growing season. The resistance gene of WB 242 was mapped in the segment of 6,4 cM between anonymous AFLP-flanking markers on chromosome II.

Hitherto only monogenic form of resistance to powdery mildew was known in sugar beet, which gene was mapped using molecular markers. Marker-assisted analysis of the sample PI 504236 *B. vulgaris* subsp. *maritima* detected the presence of two quantitative traits (QTLs) closely associated with resistance (13). Over 600 samples were tested in field and in greenhouse conditions to analyze their resistance to powdery mildew, which was found to be significant variable within the section *Beta* ($P < 0,05$) (14). Resistant samples were crossed with susceptible breeding lines with the purpose of creation splitting populations and their labeling.

Genetical basics of resistance to cyst nematode *Heterodera schachtii* Schm. is an extremely important subject of study owing to high severity of this pest in sugar beet. Monogenic resistance to the nematode was introduced into sugar beet from wild species (*B.*

procumbens, *B. patellaris*, *B. webbiana*). There are two groups of resistant material: monosomic additional lines (*2n* = 19) carrying the chromosome (a whole one of the wild type or a fragment) providing resistance and diploid forms (*2n* = 18) containing the chromosome translocated from wild beets. The latter are widely used as introduction lines for breeding work.

However, low productivity of these lines and low rate of transfer of nematode resistance genes (owing to meiosis disturbances) lead to new attempts to isolate the resistance gene and transfer it to susceptible lines of high breeding value. This work includes the use of many techniques: selection of mono-copy and repeated samples (probes) closely linked with resistance genes in a fragment of the chromosome in additional or translocation lines of *B. procumbens* and B. *patellaris*; formation of representative libraries of their clones, preferably with yeast artificial chromosomes (YAC) based on corresponding resistant parental material; cloning of continuous linked fragments of DNA (contigs) using YAC-vectors; identification of resistance gene by genetic complementation during the transformation of *Agrobacterium rhizogenes*, which causes the formation of "hairy roots" indicating the transfer of nematode resistance gene encloned in *Agrobacterium*.

YAC-technology is suitable for analysis of eukaryotic genomes as it allows to isolate relatively large DNA fragments from 100 to 1000 kbp. When a high-density linkage map contains STS as a marker site or gene-tester for screening of such libraries and the distribution of individual YACs in the contig of overlapping fragments, long fragments of DNA can be cloned using YAC as a vector and desired genes can be selected. Sugar beet has certain advantages for studying the cloned gene for nematode resistance. This crop has the established map of RFLP linkage, a relatively small genome and it can be easily transformed using *A. rhizogenes* or *A. tumefaciens*. For sugar beet, there is a physical map of translocation carrying the nematode resistance gene - $Hs1^{pro-1}$ from the I chromosome of *B. procumbens* and the representative library of YAC with DNA of resistant translocation line of sugar beet, as well as the isolated YAC-clones covering more than 70% translocations. The material of research we two diploid (2*n* = 18) introduction lines carrying in the I chromosome translocated genes of *B. procumbens*. A splitting population was obtained by backcrosses – sensitive breeding line was pollinated with pollen from the population heterozygous on nematode resistance. The control were sensitive plants from the splitting population - carriers of monosomic fragment of the genome from additional line with the I chromosome of *B. procumdens*. The two-year translocation line was subjected to 4-fold reciprocal crosses with early-blooming (annual) sensitive line. The resulting annual line BC_4 was hemizygous for translocation and homozygous for dominant allele providing early bolting (BB). This line was used to create the library of bacterial artificial chromosomes (BAC) aimed at formation of the complete physical map of gene translocation from the wild beet (*B. procumdens*) into the genome of sugar beet (15).

Translocations are fairly rare events. In sugar beet, a few translocations from the wild beet were mapped using RFLP-markers on the end of chromosome IX, which region is considered as location of translocation “hot spot”. Genetic mapping data were confirmed by hybridization in situ with specific translocation samples of DNA showing signals in the end of chromosome IX. Translocations were inherited according to Mendelian pattern, but their carriers demonstrated meiotic instability manifested as chromosome breakage and loss of translocations leading to reduced productivity.

Numerous molecular markers localized in translocation from wild beets have been isolated. Some of them were the repetitive elements capable for hybridization only with wild beet DNA, so these are the best probes for fingerprinting analysis of translocation lines and for screening of translocation-specific clones from genomic libraries. Using this method, many YAC-clones covering over 800 000 bp translocation were designed upon the line A 906001. This line was the source of gene $Hs1^{pro-1}$ cloned by positional mapping of specific DNA samples of wild beet. It has been proved that another gene - *Hs1-1* - is localized in translocations of lines A 906001 and Pro 4, because $Hs1^{pro-1}$ alone doesn’t ensure complete resistance to nematode after its transfer into sugar beet. Moreover, the totally resistant line Pro 4 doesn’t carry $Hs1^{pro-1}$ although its translocation partially overlaps the translocation A 906001 of the I chromosome of *B. procumbens*. This fact suggests cloning of the overlapping region with the purpose to identify the gene *Hs1-1* and other genes of wild beets, such as the gene providing decrease in yields. Sequence analysis in the point of translocation discontinuity can help understanding why the chromatin of wild beets was translocated mainly into this region of sugar beet genome.

It has been reported about the complete physical map of translocation of wild beet chromosome fragment into the chromosome of sugar beet, which showed the overlapping region between two translocations in the lines A 906001 and Pro 4 (15). After the long-range restriction mapping of homologous translocations was performed upon these two lines, it has been established the uneven length of their translocations while the overlapped region amounted to about 350 kbp.

It has been found many repeated elements in the translocated fragment and in other regions of sugar beet genome. These elements, particularly microsatellites, can be used for identification of cultivars and lines. Physical and genomic structure of these microsatellites were studied, which data were used at fingerprinting of several samples of sugar beet (16).

Resistance to rhizomania (the most harmful disease of sugar beet) was studied by molecular-genetic methods in two directions - the search for resistance genes in the host plant and the study of genetic nature of this pathogenic virus. Rhizomania now is widespread in the world; for the first time it was described in Italy, then - in Japan. There’s a wide discussion about the nature of rhizomania, ways of combating it and results of breeding resistant sugar beet. Rhizomania is caused by beet necrotic yellow vein virus (BNYVV) classified as benivirus (furovirus), which is transmitted by the soil fungus *Polymyxa betae* Keskin. Severe rhizomania results in yield decrease by more than 50% with the reduce in sugar content up to 16-18 or even 10%. Infected roots contain increased sodium content against the reduced content of amino nitrogen, which disturbs sugar synthesis. BNYVV infects all varieties of sugar beet, fodder beet, red beet, leaf beet, spinach and some other species of the family *Chenopodiaceae*.

Furovirus virions are morphologically shaped as a hard rod structure. Their subdivided genome consists of 2 or more 4 plus-strands of RNA. In root cells of infected sugar beet, all four components of BNYVV genome were found: RNA 1, RNA 2, RNA 3 and RNA 4 – the compact particles of, respectively, 390, 265, 100 and 85 nm length and 20 nm width. RNA 1 encodes viral RNA polymerase, RNA 2 - capsule protein (21 kDa). RNA 3 encodes a product affecting virus expression, reproduction and expansiton in plant roots, RNA 4 controls factors affecting virus transmission by the fungus. In Japan, RNA 5 has been established in virus isolates taken from *B. macrocarpa* Guss. (systemic host of the virus), which caused enhanced pathogenicity. RNA 1 - RNA 4 of European BNYVV and RNA 5 of the Japanese virus are known to be completely sequenced (3). The peculiarity of interaction between the virus and its vector has been shown: high stability of the virus during a dormancy of its vector.

Resistance to BNYVV was described in samples of *B. vulgaris* L. subsp. *vulgaris* and subsp. *maritima* (L.) Arcang. To identify the genes responsible for resistance in splitting populations, BNYVV reproduction and expansion were compared in resistant and susceptible samples during inoculation of the virus in root cells of seedlings.

The emergence of rhizomania in North America caused an intense screening of genetic resources the cultural and wild species aimed at identification of potential sources of resistance and its transfer to elite sugar beet germplasm (17). The dominant

resistance gene - so-called Holly-gene (*Rz 1*) providing high resistance to BNYVV has been established in Italian cultivar Rizor, and this was the first major resistance gene detected in commercial cultivars of sugar beet (3, 17). Transmission of allele *Rz 1* can be easily controlled, and it is widely used in reciprocal crosses and selection programs for improvement the germplasm.

However, resistance determined by the only dominant gene could appear to be unstable, which fact stimulated the search for extra sources. Since the lack of genetic resources in cultivated sugar beet, it has been studied *B. vulgaris* ssp. *maritima* easily crossing to cultural beets. Resistant samples were identified by ELISA (plants collected in the field and grown in greenhouses) and then used in recurrent crosses with sugar beet lines. The sample WB 42 (*B. vulgaris* ssp. *maritima* from Denmark) when testing in growth chamber showed high level of resistance, which was found to be distinct from the gene *Rz*. The new resistance gene was designated as *Rz 2* (3). Although the resistance transferred from *B. vulgaris* ssp. *maritima* is caused by one gene, this gene is not clear yet - *Rz 1* or *Rz 2*, or probably some other factors (17). Synthetic breeding populations (18) were studied over a long period. In 2002-2005, the hybrids-carriers of *Rz* were defeated by rhizomania in fields near the Imperial Valley (California, USA), which indicated the pathogen ability to overcome resistance caused by this gene. This fact was confirmed by laboratorial studies, field trials and greenhouse tests performed in the USA in 2004-2005 on sugar beet subjected to infecting with BNYVV the strain IV (19) and some other strains of this virus (20); it is possible that *Rz 2* and *Rz 3* of *B. vulgaris* ssp. *maritime* provide only partial resistance to these strains, which were significantly modified by so-called minor resistance genes of host plant.

Plants of the family C79-9 originated from WB 151 (sample PI 546397) (*B. vulgaris* ssp. *maritima*) demonstrated high resistance to strain IV BNYVV; the nature of inheritance and alleles of this trait have been established. Highly resistant plants were also detected in the hybrid population sugar beet × *B. vulgaris* ssp. *maritime*, which can be assessed by their reaction to BNYVV and strain IV BNYVV.

The modern task of breeding sugar beet - to form the bank of germplasm samples carrying high resistance to rhizomania along with improved agronomic traits, productivity and quality. This task is complicated by the emergence of new virulent strains of BNYVV and by need in intense studies on pathogen variability (21). Three strains (types) of the virus were identified in Europe using molecular markers RFLP and SSCP (single stranded DNA conformation polymorphism). So-called P-type isolates of BNYVV were initially described only in the region of Pitivier (France) and later in Kazakhstan (22). It has been also reported about this virus type detected in Japan, China and the UK (23). There's the actual risk of emergence and spread of new variants of BNYVV owing to reduced genetic diversity of cultivated beets and strong selective pressures exerted by resistance genes *Rz 1* and *Rz 2* (22).

It is well known that viruses rapidly mutate under the environmental impact. Though, it has been shown high conservatism of A-, B- and P- types, as well as the identity of nucleotide sequences to the samples described more than 15 years ago (22).

RNA 1, RNA 2 and RNA 4 sequences of BNYVV from Kazakhstan were identical to those of the P-type BNYVV from Pitivier. At the same time, the quite distinct RNA 5 was also isolated in Pitivier and in number of BNYVV sources from East Asia. There were some mutations of the virus accompanied by substitutions of amino acids in the coat protein CP, p25 and p26, leading to overcoming of host resistance genes. In particular, it has been experimentally shown (24) that overcoming of sugar beet resistance to rhizomania could be the result of selection of mutant RNA 3 (amino acid substitutions at positions 67-70 and 198 in the protein p25). Phylogenetic analysis of BNYVV isolates revealed a correlation between peculiarities of clusters in viral genome and geographical origin of samples (24). Rhizomania symptoms were detected since 2001 in France (near Pitivier), in other countries of Europe and in the United States, which can be caused by emergence of new variants of BNYVV, or the synergistic effect of mixed infection BNYVV and other pathogens.

All researches on genetics and breeding sugar beet abroad are currently performed at the international cooperation, and within a single country this is collaboration of research institutes, universities and private sugar plants. Coordination of research on genetics and selection of sugar beet is held by the International Board of Plant Genetic Resources (IBPGR) (Rome, Italy) and the International Institute for Sugar Beet Research (IIRB) (Brussels, Belgium). One of the problems is increasing the genetic bases of sugar beet, which is restricted owing to peculiarities of its breeding (based on CMS and monogerm seed). Much attention is paid to formation of germplasm collections of species, cultivars, hybrids and lines, to development of a uniform list of traits description in ancestors of varieties-populations, parental forms and their hybrids, to forming a unified database of source material for breeding. To develop the list of traits descriptions, the basis were the ones used by IBPGR and IIRB as they imply agronomic parameters important for commercial seed companies. The database of these descriptions is available in the information network of genetic resources (GRIN) (http://www.ars-grin.gov/cgi-bin/npgs/html//crop.pl?49). Studies of sugar beet germplasm resistance to biotic and abiotic stresses are supported by CGC (Committee on germplasm culture) and widely sponsored by NPGS (National Plant Germplasm System) (USA). Gene banks of cultivated crops, particularly species the genus *Beta*, were established in a number of countries. For example, the Federal Center of Breeding Research of Cultivated Crops (Brawnschweig, Germany) maintains a gene bank and an international database on beet containing data about more than 9000 samples from 20 banks in different countries. Carriers of corresponding genes are recorded in the journal "Crop Science" with assigned individual numbers.

In the past 20 years, NPGS has sponsored the establishment of germplasm collections of the genus *Beta* in the USA, Armenia, Belgium, Dagestan, Egypt, Greece, France, Ireland, Italy and Sardinia. Financial support of this work is also provided by the EU project GENRES ("Genetic Resources").

Thus, today, the main direction of breeding sugar beet abroad is increasing its resistance to pests, diseases and abiotic stresses through the use of genetic potential of wild species the genus *Beta*. For this purpose, international cooperation works on the establishment of germplasm collections of species and individual varieties. Investigation of samples includes using a complex of methods (primarily, molecular markers of DNA as the most numerous and informative for studying the genetic nature of organisms) aimed at identification and isolation of genes for commercially valuable traits. It has been reported about identification of genes for resistance to rhizomania, powdery mildew and nematodes in wild species and their introgression into sugar beet. All detected Mendelian genes are commercialized and information about them is available in the International Bank of genes (BAZ Gene Bank, Germany).

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