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HIGH-THROUGHPUT SEQUENCING TECHNIQUES TO FLAX GENETICS AND BREEDING

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❀ Flax (*Linum usitatissimum* L.) is an important oil and fiber crop. Using modern methods for flax breeding allows accelerating the introduction of some desired genes into the genotypes of future varieties. Today, an important condition for their creation is the development of research, that is based on next-generation sequencing (NGS). This review summarizes the results obtained using NGS in flax research. To date, a linkage map with a high marker density has been obtained for *L. usitatissimum*, which is already being used for a more efficient search for quantitative traits loci. Comparative studies of transcriptomes and miRNomes of flax under stress and in control conditions elucidated molecular-genetic mechanisms of abiotic and biotic stress responses. The very accurate model for genomic selection of flax resistant to pasmo was constructed. Based on NGS-sequencing also some details of the genus *Linum* evolution were clarified. The knowledge systematized in the review can be useful for researchers working in flax breeding and whereas fundamental interest for understanding the phylogenetic relationships within the genus *Linum*, the ontogenesis, and the mechanisms of the response of flax plants to various stress factors.

❀ **Keywords:** flax; genome-wide association studies; genomic selection; linseed; *Linum usitatissimum*; marker-assisted selection; RNA-seq; transcriptome; NGS-sequencing.

ВЫСОКОПРОИЗВОДИТЕЛЬНОЕ СЕКВЕНИРОВАНИЕ В ГЕНЕТИКЕ И СЕЛЕКЦИИ ЛЬНА

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❀ Лен (*Linum usitatissimum* L.) — важная масличная и прядильная культура. Применение современных методов селекции льна позволяет ускорять введение некоторых желаемых вариантов генов в генотипы будущих сортов. На сегодняшний день важным условием для их создания является развитие исследований, основанных на секвенировании нового поколения (NGS, Next Generation Sequencing). В данной обзорной статье обобщены результаты, полученные на основе применения NGS-секвенирования в исследовании льна. К настоящему моменту для *L. usitatissimum* получена генетическая карта сцепления с высокой плотностью маркеров, которую уже используют для более эффективного поиска локусов количественных признаков. На основе сравнительных исследований транскриптомов и микроРНК льна в контрольных и стрессовых условиях уточнены молекулярно-генетические механизмы ответов на биотические и абиотические стрессы. Была построена отличающаяся высокой точностью модель геномной селекции льна на устойчивость к пасмо. Также, благодаря применению NGS-секвенирования, удалось уточнить некоторые особенности эволюции геномов представителей рода *Linum*. Систематизированные в обзоре знания могут быть полезны для исследователей, ведущих работу по селекции льна, и представлять фундаментальный интерес для понимания филогенетических взаимоотношений внутри рода *Linum*, особенностей онтогенеза и механизмов ответа растений льна на различные факторы стресса.

❀ **Ключевые слова:** *Linum usitatissimum*; секвенирование РНК; RNA-seq; геномная селекция; лен масличный; лен-долгунец; маркер-ориентированная селекция; полногеномный анализ ассоциаций; NGS-секвенирование; транскриптом.

INTRODUCTION

Flax (*Linum usitatissimum* L.) is a valuable fiber and oil crop. Four subspecies are distinguished within the species: (1) common flax (L. *usitatissimum* L. var. *usitatissimum* sensu Rechinger (1974)), which is cultivated as a spring fiber and oil crop; (2) crown flax (L. *usitatissimum* var. *humile* (Mill.) Pers.); (3) linseed (L. *usitatissimum* L. var. intermedium (Czernom.) Kutuz.); and (4) dwarf crown flax (L. *usitatissimum* var. *nanum* Kutuz.) [1]. Flax is cultivated on all continents, except for Antarctica. Among crops used for fiber, flax cultivation area is ranked fourth in the world, and linseed is ranked tenth among oil crops [2].

In Russia, the flax cultivation objectives comply with global trends. However, special attention is paid to the development of varieties with higher ecological plasticity and adaptivity to unfavorable conditions, as well as to freezing tolerant crop forms with reduced photoperiodic sensitivity [3, 4]. Breeding of aluminum tolerant plant is important for flax cultivated in northern Russia [5, 6].

Compared to other crops, the use of the state-of-the-art technology (e. g., marker-assisted selection, MAS) for flax breeding is difficult because of insufficient data on economically important genes. However, the search for effective genetic markers for breeding continues. According to the number of publications concerning this topic, Russia is one of the five world leaders along with Canada, USA, India, and China (Supplementation 1).

Research on economically important genes in linseed exceeds three times that in fiber flax. Greater attention has been paid to the identification of the loci associated with the oil fatty acid composition than to loci associated with resistance/tolerance and seed yield (Fig. 1).

MAS is a popular method used for identification of markers associated with allelic gene variants. MAS is used for selection of traits with mono- and oligogenic control and can complement traditional breeding approaches to speed breeding for certain traits. The novelest approach for variety improvement by traits with mono- and

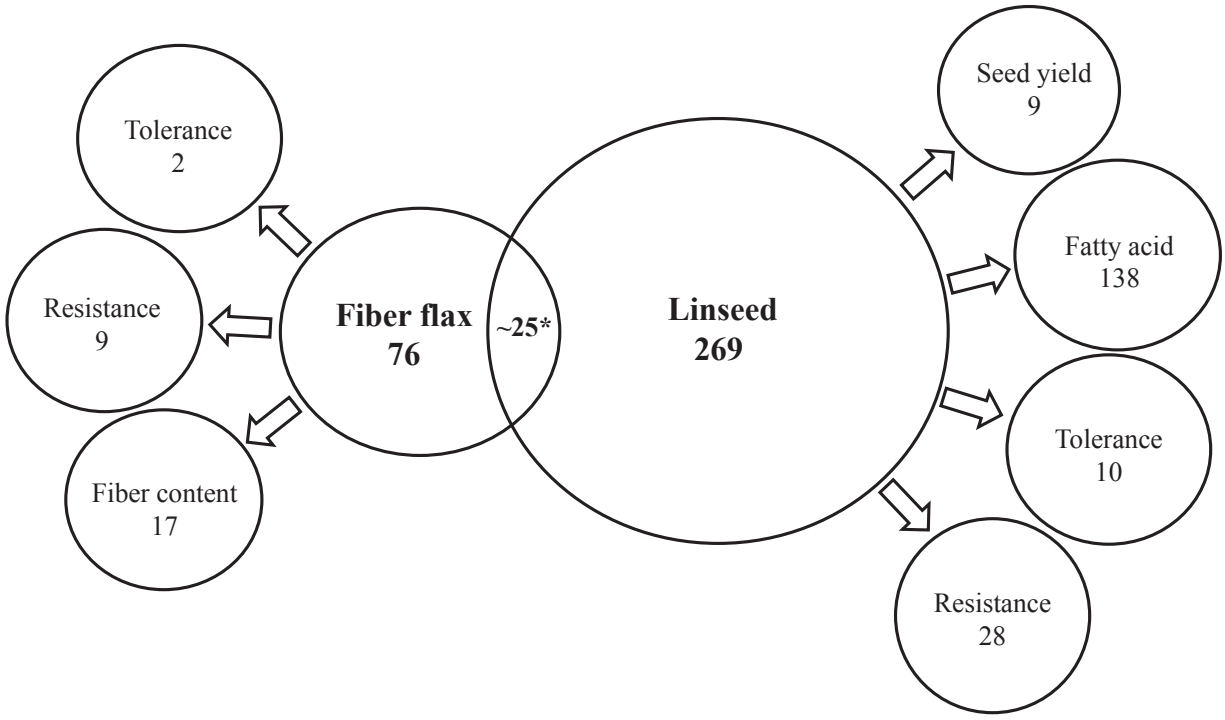

Fig. 1. The main directions of flax research using DNA markers. The number of publications related to the use of markers in the main areas in flax breeding is based on publications in the Scopus database (www.scopus.com, accessed September 13, 2019) at the intersection of the keyword "Marker" with "Fiber Flax" / "Linseed" and with "Resistance" (to biotic stress) / "Tolerance" (to abiotic stress) / "Fiber content" / "Seed yield") / «Fatty acid». * – not in all articles it is possible to determine which type of flax was described.

oligogenic control is genome editing. For example, Sauer et al. (2016) applied the CRISPR/Cas technology to create a glyphosate resistant genotype [7].

Genomic selection is a promising approach for crop improvement by traits with polygenic control. This is an alternative strategy that uses statistical models to predict the plants with the best phenotypes based on a large number of markers for individual selection [8]. This approach is used for flax genomic selection regarding resistance to pasmo (*Septoria linicola* (Speg.) Gar.) [9].

Next-generation sequencing (NGS) is frequently used to detect new genetic markers for MAS or target genes for genome editing, as well as for genomic selection.

The purpose of this review is to analyze and systematize data describing the application of NGS-based approaches in flax genetic studies and breeding, as well as assessment of the prospects for their further use in flax production.

SEQUENCING OF *LINUM USITATISSIMUM* GENOME

The chromosome number of the genus *Linum* L. representatives varies broadly from $2n = 16$ to $2n = 72$ [10]. The cultivated species *L. usitatissimum* has a diploid genome ($2n = 30$) according to the comparative analysis of the genomes of different representatives of *Linum* genus. These findings support the concept that *L. usitatissimum* passed during it evolution through allotetraploidy via hybridization of two ancestral diploid species [11–13].

In 2011, the first flax full genome physical map was presented by an international team headed by Canadian scientists under the Total Utilization Flax Genomics Project (TUFGEN). A library comprising 43,776 bacterial artificial chromosome (BAC) clones was developed for the CDC Bethune variety, based on which 87,552 BAC-end sequences (BES) were obtained [14]. The map consisted of 416 contigs covering ~368 Mb and 1 contig represented a chloroplast genome [14]. Among the known repeated fractions, ribosomal RNA encoding genes constituted ~13.8% and mobile genetic elements – 6.1%. Functions of 45.1% of sequences remained unknown [14].

In 2012, the genome of CDC Bethune variety was sequenced *de novo* via the whole-genome shotgun (WGS) sequencing [15]. Reads were assembled into 116,602 contigs (302 Mb) of which 88,384 scaffolds were linked together with a total size of 318 Mb, which corresponds to ~81% of the flax genome. More than 96% of the flax expressed sequence tags (ESTs) obtained from the database of the National Center for Biotechnology Information (NCBI), USA, were mapped to the genome sequence obtained. It was demonstrated that 20% of the genome is represented by transposons; the portion of microRNA genes was <1%. The authors detected 43,484 coding genes [15]. The assembly process detected 31 scaffolds containing chloroplast DNA, although an attempt to combine them in one molecule of the chloroplast genome failed [15].

The methods of direct and reverse genetics can be used to study variability by economically important traits. One such method is optical mapping based on direct identification of DNA restriction sites using laser confocal microscopy. The overall spectrum of the obtained DNA fragments serves as a unique "barcode" for the initial sequence [16]. The BioNano genome (BNG) optical map of flax used the BioNano IRIS platform, which allowed to improve the assembly quality and supplemented the genome sequence of the CDC Bethune variety based on physical and genetic maps [13–15]. The alignment of 211 BNG contigs (298.6 Mb, 94.2%) and 622 scaffolds (286.6 Mb, 94.9%) was performed by Wang et al. [15]. Based on the specified scaffolds obtained by You et al. [13] and the confirmed physical map of "CDC Bethune," 211 BNG contigs were combined into 94 supercontigs, which were then associated with the *L. usitatissimum* 15 chromosomes linkage maps. The total size of pseudo-molecules was 316 Mb, which covered 97% of the annotated flax genes [13].

Sequencing, assembly, and detailed analysis of the *L. usitatissimum* plastid genome was performed by Lopes et al. [17]. The plastid genome represented a DNA ring molecule of 156,721 bp with a typical structure consisting of four parts: two inverted repeats (31,990 bp each) between which the large single copy (LSC) and small

single copy (SSC) are located and unique areas including 81,767 bp and 10,974 bp, respectively. In total, 109 unique genes and 2 pseudo-genes were identified in the genome, along with the loss of conservative *clpP* introns and the complete sequence of *rps16* [17].

Thus, as a result of these studies, the *L. usitatissimum* genome constructed is available as a basis for quick and effective assembly of the genomes of other representatives of this species, as well as further related species.

Information on the assembly of the flax genome and associated data is provided in MAS 2.

APPLICATION OF NGS METHODS FOR ANALYSIS OF DNA POLYMORPHISM

Prior to the use of NGS for genotyping, there were about 20 methods of single nucleotide polymorphism (SNP) detection. With the development of NGS techniques it became possible to identify thousands of markers simultaneously [21]. Detected SNPs are used to assess the genetic diversity and to map genes or QTL (quantitative trait loci) for further marker-assisted selection (MAS) [22–31].

Pyrosequencing, an NGS approach based on the detection of released pyrophosphates, could be used for finding SNPs [32–35]. One of the first experiments performed with this approach examined the species *L. usitatissimum* and *L. bienne*. Using of a pipeline to identify *de novo* allelic variants in flax genome enabled the detection of 1,067 SNPs in 713 contigs. Most of those detected additional SNPs and indels (single nucleotide insertions and deletions) were confirmed by Sanger sequencing [36].

In 2015, Galindo-González et al. [24] demonstrated the effectiveness of Ion Torrent sequencing to detect mutations in certain areas of the genome for large populations of flax. The authors screened 768 mutants obtained via ethyl methane sulfonate (EMS) treatment with the use of microarrays. Out of the 29 potential mutations detected after NGS analysis, 16 were confirmed using Sanger sequencing [24].

NGS technology includes methods that reduced genome complexity, such as restriction site-associated DNA sequencing (RAD-seq) [37, 38], restriction enzyme-based reduced representation library sequencing (RRL-seq) [23, 39], genotyping-by-sequencing (GBS) [40], and specific locus amplified fragment sequencing (SLAF-seq) [29], which allows to detect of numerous SNPs in a short period of time at low cost.

Kumar et al. [23] performed RRL-seq of the eight flax varieties genomes using the Illumina platform (sequencing by synthesis via insertion of 3'-modified nucleotides with attached fluorescent tags) [23]. They detected 55,465 SNPs, 25% of which were located in the genes. The 4,863 SNPs, detected in the CDC Bethune and Macbeth varieties were confirmed by GBS of recombinant inbred lines (RILs). The average occurrence frequency of polymorphic loci was 0.17 per 1000 bp [23].

The SLAF-seq method is used to search for new SNPs, which reduces analysis costs and increases the possibility of effective deep sequencing [27, 41–46]. The method is an optimized version of RAD-seq and specifically intended for large-scale genotyping experiments based on a series of preliminary experiments [47]. In 2017, this method was used by Yi et al. [27] to detect 4,145 SNPs with an average distance of 0.64 cM, which were used to construct a marker-rich genetic map [27].

It is important that quantitative trait loci can be finally linked to certain scaffolds through SLAF-markers, which is the basis for preliminary conclusions about adjacent genes or genes with pleiotropic effects, which affect the flax economically valuable traits. For example, Wu et al. [31] developed a marker-rich map with QTL for economically important traits. Based on the analysis of F2 mapping population Diane × NY1 with 2,339 markers, they developed a genetic map (1483.25 cM) covering 15 linkage groups, with the average distance between the markers 0.63 cM. They described 12 loci associated with the flax economically valuable traits (e.g., plant height, seed yield, fiber content, and fiber yield) [31].

The quick introduction of high-throughput genotyping methods allowed to develop several genetic maps for one species, which are combined in the uniform consensus map integrating additional markers in the areas where QTL are

located [49]. For example, in 2013, Asgarinia et al. [49] used 143 SSR (simple sequence repeat) polymorphic markers – based on the analysis of mapping population NorMan/Linda and developed maps for all 15 linkage groups with total length 1,241 cM. They also conducted QTL-analysis for powdery mildew resistance and detected loci in the linkage groups 1, 7, and 9 associated with the SSR-markers mapped. The physical locations of these loci in the genome were determined by homology search in the whole-genome sequencing database, for which the information about nucleotide sequences of ESTs and BAC-clones was used [48, 54].

Thus, high-throughput sequencing can be effectively used to detect a large number of markers, as well as map the loci for economically important flax traits with simultaneous identification of their location on the genetic map and detect the physical location in the genome.

GENOME-WIDE ASSOCIATION STUDIES

Traditional mapping of QTL in plants is performed using bi-parental mapping populations [50], which have several limitations. The most critical problem is the absence of allelic diversity, as the population originates from two parents, and genetic variation is limited by the parent lines. Recently, genome-wide association studies (GWAS) have been used to detect candidate genes associated with economically important traits [51–53]. This method requires the using of large number of markers and is used to analyze a wide range of genotypes. Finally, it provides the base to understand molecular and genetic mechanisms of polygenic traits formation. Success of this method depends primarily on the genetic structure of the analyzed population as well as on quantity and distribution of molecular markers used [54]. GWAS becomes more accessible due to whole-genome sequencing data and development of high throughput genotyping methods.

Recently, GWAS has become an integral method for detecting new economically valuable flax genes [55–58]. The work started recently, and most experiments are aimed at identification of the loci, which determined the quality of the raw material obtained from flax.

SLAF-seq-based GWAS is an effective technology for identifying alleles, which was confirmed by Xie et al. [56, 57] by using a combination of these methods for genotyping of 224 flax varieties. Sequencing identified 584,987 SNPs, with an average occurrence frequency of 1 per 1.2 thousand bp.

Using efficient mixed-model association eXpedited (EMMAX), general linear model (GLM) and a mixed linear model (MLM) SNPs associated with oil content, plant height, number of branches and capsules and weight of 1,000 seeds were revealed, enabling the detection of candidate genes for these traits [56, 57] (Supplementation 3).

It is important that GWAS is implemented in addition to the search for traces of positive selection in the genome (for example, genome-wide selective sweep scan, GW3S). Such traces can be found as the reduction of nucleotide variations frequency in the regions bordering new useful mutations [60].

You et al. [59] described genome regions associated with the yield and oil content, detected using GWAS, which was completed by GW3S. Authors used GBS to investigate 260 lines assigned to three different bi-parental mapping populations, resulting in detection of more than 500 thousand SNPs. Based on the results of this analysis, the authors detected 33 QTLs for important flax traits, 7 of which were related to the maturity time, 5 – to plant height, and the rest were associated with the fatty acid composition of oil [59].

In 2018, Soto-Cerda et al. [55] detected loci influencing the mucilage and hull content in seeds of Canadian flax. A decrease of these parameters was desirable for increasing the feeding value of seeds in cattle farming. Seven loci were identified, which were associated with seed mucilage content and four loci were determined with a low hull content [55] (Supplementation 3).

GWAS is also suitable for analysis flax resistance to pathogens and factors of abiotic stress, which have complicated polygenic control. For example, He et al. [58] conducted GWAS for 370 flax accessions of Canadian collection based on the results of 5 years of field examinations concerning resistance to pasmo. Using GBS, they detected

258,873 SNPs distributed through all 15 chromosomes. Based on orthology with the genes of *Arabidopsis thaliana,* two candidate genes for flax resistance to this pathogen were detected [58].

Using GWAS based on 5-years field data, He et al. [9] conducted a search for loci associated with flax resistance to *Selenophoma linicola*. On the basis of the collection of 370 accessions as the training and test populations, they developed a high-throughput prediction model of flax's genetic resistance to this pathogen with an accuracy of 0.92, which is the most accurate genomic prediction model among all models for plant resistance to diseases [9].

Thus, GWAS concerning flax indicates the necessity of using several statistical models for loci identification, which promote a search for genetic markers for further use in breeding programs.

APPROACHES TO GENOMIC SELECTION

Genomic selection (GS) enables researchers to overcome the restrictions of MAS in terms of quantitative traits. GS aims at determining the genetic potential of an examined sample instead of identifying specific QTL [61].

Several GS attempts have been made on flax. In 2016, You et al. [62] conducted a selection of samples using GS according to their breeding value predicted by a statistical model based on the interaction between phenotypes and genetic markers. For seed yield, oil content, iodine value, and content of linolic and linolenic acids in each of the three tested parental populations, predicted accuracy and effectiveness of GS were performed relative to phenotypical selection based on three models: random regression best linear unbiased prediction (RR-BLUP), Bayesian LASSO (BL), and Bayesian ridge regression (BRR) [62]. Despite empirical results demonstrating that GS could increase the flax yield in breeding, further investigations are required to develop the optimal training populations and sets of markers for using GS in flax breeding [62].

Thus, increased using of GS remains a challenge because total analysis expenses are higher than for MAS. However, the development of technologies and enlargement of genome resources will ensure broader use of GS.

USE OF HIGH-THROUGHPUT SEQUENCING FOR PHYLOGENY OF *LINUM* GENUS REPRESENTATIVES

High-throughput sequencing methods have become the main approaches to analyze non model plant species for detection of their phylogenetic relations [63]. The RAD-seq and GBS methods are distinguished among all other methods, while whole-genome sequencing is used less frequently [40, 64–66]. Finally, phylogenetic reconstruction is based on SNP data that can be promptly obtained from any type of genome at low cost [67, 68].

In the study by Fu et al. [26], genome DNA from 18 flax accessions, which were attributed to 16 species of four sections, were sequenced using the Illumina platform. The experiment detected 6,143 SNPs in chloroplast DNA, 2,673 in mitochondrial DNA, and 19,562 in nuclear DNA. Phylogenetic trees constructed based on the analysis of nuclear, plastid, and mitochondrial markers reflect the divergence of four types of *Linum* that are similar in topology. This demonstrates the availability of congruent phylogenetic links of the four sections within the genus *Linum*. Three main branches detecting two main evolutionary stages that led to the cultivated flax (*L. usitatissimum*) were also identified. This species and its progenitor (*L. angustifolium*) formed an individual branch that was more genetically linked to *L. decumbens* and *L. grandiflorum* [26].

High-throughput sequencing of gene multicopy rRNA families enabled corrections in the flax phylogeny; the detection of intra- and interspecific divergence of the sequences of rRNA genes was also achieved. Based on sequence data, Bolsheva et al. [12] categorized the representatives of the section *Linum* into four branches: diploid species (*L. decumbens* and *L. grandiflorum)*; tetrapoloid species (*L. narbonense)*; tetraploid species (*L. usitatissimum* and its wild progenitor *L. angustifolium)*; and polyploid species (*L. marginale)*. It is assumed that *L. usitatissimum* and *L. angustifolium* could result from the hybridization of two diploid species ($2n = 16$) that are possible progenitors of the modern species *L. grandiflorum* and *L. decumbens*, or hybridization of the progenitors of *L. narbonense* with $2n = 14$ and diploid species with $2n = 16$ [12].

Variation in morphology, size, and number of chromosomes in karyotypes is determined by a set of repeated sequences. A comparative analysis of repeated sequences in the flax genome was conducted by Bolsheva et al. [69]. Based on low-covering whole-genome sequencing data for 12 flax species attributed to six sections they concluded that genomes differ by the number of repeated fractions but their sets are similar in all species [69]. The family of Ty3/Gypsy retrotransposons represented the major fraction of repeated sequences in all species analyzed. Evolutionary development of the flax forms was accompanied with waves of increasing numbers of tandem repeats (i.e., satellite DNA) and retrotransposons with long terminal repeats (LTRs). The least number of dispersed repeats and the largest amount of satellite DNA in flax genomes from *Linum* is probably the result of the allotetraploid origin of plants in this group [69].

Thus, NGS methods helped to reveal new details of evolution of *Linum* representatives which can be used in the studies of genomes organization and phylogenetic interaction within this genus.

TRANSCRIPTOME ANALYSIS

Access to updated and amended flax genomes sequences [13] will contribute to more effective breeding. However, information on flax genome only is insufficient. The extensive transcriptomics, proteomics, and metabolomics data are required because only the combination of all omics data will lead to better understanding of important physiological and molecular mechanisms unique for this plant species. High-throughput sequencing of RNA (RNA-seq) is effectively used to analyze transcriptomes and detect genes involved in the development of economically important traits, with special attention being paid to the resistance to biotic and tolerance to abiotic stressors [70–72]. The identification of differentially expressed genes (DEGs) currently provides better understanding of the mechanisms based on the development of the evaluated traits. The use of transcriptomics approaches in flax studies is in it's very beginning [73].

The central idea of transcriptomics is the comparison of gene expression in two contrasting conditions, for example, in the most favorable and least favorable environmental conditions. Especially effective is the comparison in different conditions a set of genotypes – resistant and susceptible to the tested type of stress. The identification of new genes of resistance/tolerance among DEGs will help to develop new diagnostic markers to speed breeding process.

Among abiotic stressors, unfavorable soil composition is a main factor limiting the flax cultivation areas. It is not surprising that one of the first experiments, conducted in this direction is considered to be the evaluation of the plant transcriptome under salinity-alkalinity stress by the RNA-seq method, where the expression patterns of five categories of genes were analyzed, including transcription factors, signal transduction proteins, phytohormones, antioxidant protection enzymes and molecular transporters. Among DEGs the genes belonging to the key regulatory families involved in response to abiotic stress, such as *WRKY*, *MAPKKK* (mitogen-activated protein kinase kinase), *ABA* (abscisic acid), *PrxR* (NADH-peroxiredoxin reductase), and genes encoding proteins included in ion channels were detected [74].

Searching for genes associated with osmotic stress in flax, Wu et al. [73] conducted sequencing of the transcriptome of seedlings grown in normal conditions and stressed conditions caused by polyethylene glycole 6000. The authors annotated 2,533 genes, out of which 239 were defined as DEGs. A significant number of genes were attributed to the families encoding transcription factors such as nascent polypeptide-associated complex (NAC), late embryogenesis–abundant (LEA), WRKY, ethylene responsive factors (ERF), and basic-leucine zipper (bZIP) [73].

In 2019, Wu et al. [75] applied high throughput sequencing of RNA to evaluate transcriptomes and detect flax genes for salinity tolerance. Out of the 2,582 co-expressed genes, 2,482 were annotated. It was demonstrated that the main mechanisms of flax salinity tolerance were associated with plant hormone signal transduction, photosynthesis-antenna proteins and biosynthesis of amino acids [75].

The flax genome has high plasticity. Therefore, some accessions have genetic changes that have

been inherited by several generations in case of nutrient disbalance in the soil. Such genotypes are called genotrophs [76]. Dmitriev et al. [6] used transcriptome analysis to detect flax DEGs in the process of planting in unbalanced nutrients compared to optimal conditions. On the basis of high throughput sequencing authors selected 17 genes for the further analyses using quantitative PCR methods with extended group of accessions. In excessive nutrition conditions, gene expression changes were detected in genes encoding proteins of the WRKY family. In phosphate-deficient conditions, the expression of genes, which encoded proteins of the jasmonate ZIM-domain (JAZ), harbinger transposase-derived nuclease (HARBI1), and inhibitor of growth 1 (ING1) families, was changed [6].

In 2016, Dmitriev et al. [77] sequenced 16 transcriptomes of four flax accessions (two aluminum-stress tolerant and two sensitive ones) to evaluate plants resistance to aluminum toxicity. Based on the obtained sequencing data, genes with differential expression were detected, most of which had transportation functions. A significant increase of gene expression in glutathione-S-transferase and UDP-glycosyl-transferase allowed the authors to make the assumption that these genes are involved in protective mechanisms of plants against aluminum stress via scavenging of reactive oxygen forms and modification of the cell wall [77] (Supplementation 3). Later, Zyablitsin et al. [78] determined that three transcript variants of the gene *CAX3* are expressed in the roots of flax seedlings. Data of quantitative PCR correlated with the sequencing data of the same plants samples. Thus, CAX3-Ca^{2+}/H^{+}-antiporter could be involved in flax's reaction to high soil acidity and aluminum stress by Ca^{2+}-specified in tracellular regulation [78] (Supplementation 3).

Additionally, Dmitriev et al. [79] evaluated the reaction of flax varieties to reduced acidity and lack of zinc using transcriptome analysis. More considerable inhibitions of growth and changes in gene expression in comparison with the control group and the group grown in the conditions of zinc deficiency were detected in the tested flax varieties grown at pH 5.5. DEGs detected by the authors are involved in different processes, including ionic transport, cell wall biogenesis, oxidoreductase activity, and photosynthesis. These genes may play role in flax response to the tested stressors [79].

It is also important to evaluate the resistance of flax to biotic stresses. In 2016, GalindoGonzález et al. [80] were the first to apply transcriptome analysis to evaluate the mechanisms of resistance to *Fusarium oxysporum*. The CDC Bethune variety, which is moderately resistant to Fusarium wilt disease, was used for a complete examination of the transcriptome by RNA-seq. The results confirmed the known mechanisms of flax response to the pathogen to a great extent and amended the existing data, which allowed the authors to make an updated and more comprehensive model of flax resistance to *F. oxysporum* [80] (Supplementation 3).

Responses to Fusarium wilt disease in the flax hybrids resistant to it was examined by Dmitriev et al. [81]. For identification of candidate genes, a search was conducted among genes with elevated or reduced expression in resistant (in comparison with sensitive) varieties and populations during the ingress of infection. Among DEGs, special attention was given to the genes encoding salt response protein (SRG), UDP-glycosyltransferase, ATPase associated with different cell activities (AAA-ATPase), glucan-endo-1,3-beta-glucosidase, transcription factors -MYB, dehydrins, and auxin-sensitive protein SAUR. The authors assumed that the identified genes with specifically induced expression in response to *F. oxysporum* infection are the most optimal candidate genes for flax resistance to it [81].

Another direction in the area of transcriptomic research of plants relates to the evaluation of molecular genetic mechanisms of ontogenesis, for which comparison of gene transcripts from different parts of the plant and at different stages of differentiation is conducted. These results clarify known molecular genetic mechanisms and detect the new mechanisms of evolutionary and ontogenetic changes, as well as identify the potential target genes for the site-directed mutagenesis for the purpose of changing the type of growth and development, acceleration of flowering, maturation, etc.

RNA-seq was used to compare transcriptomes in two segments of the infertile shoot of a 14-day flax plant, in which all leaves were removed [82]. Detected differences in expression between the apical part of the shoot and the major part of the stem supported other approaches (e. g., screening of mutants) aimed at the evaluation of primary differentiation of phloem fibers [82].

In 2017, Mokshina et al. [83] examined the expression of genes of the cellulose synthase complex of flax (LusCESA) at different stages of development and in the process of gravitropic reaction of plants using RNA-seqand qPCR. Gravistimulation of plants temporarily increased the number of transcriptomes of the cellulose synthase complex. Thus, fibers of the inner (tertiary) cell wall have specific mechanisms of biosynthesis of cellulose and peculiarities of its regulation [83].

Using transcriptome analysis data, Shivaraj et al. [84] evaluated genes of integral membrane proteins, mostly presented by aquaporins. Analysis demonstrated high expression of proteins of the plasma membrane in numerous tissues, low expression of integral nodulin-26-like proteins, and expression of 17 integral tonoplast proteins specific for seeds. A large-scale analysis of the proteins provided better understanding of their physiological function in flax development [84].

Dash et al. [85] conducted assembly of transcriptome moderate tolerance to drought of the Indian flax variety T-397. The obtained data will help to identify loci and find SSR markers for selection of drought tolerant varieties [85].

Gorshkova et al. [86] conducted RNA-seq of the primary flax phloem for intrusively growing fibers obtained with laser microdissection methods. As changes in the levels of mRNA of the individual genes are not directly associated with protein synthesis and especially with the enzyme activity, transcriptome profiling was used as a high informative approach to detect metabolic pathways and proteins having a key role in the intrusive growth of the cells. It was determined that elongation of cells relates to the activation of photosynthesis and intense expression of chaperonins and thioredoxins localized in the chloroplasts. It was also defined that a great part of specifically activated genes encoded expansions, enzymes for the pectins modification, and several proteins localized in the cell wall [86].

Data available regarding the examination of flax transcriptome are provided in NCBI Sequence Read Archive [88] and NCBI Gene Expression Omnibus databases [73, 82, 85, 86, 88].

Importantly, in the area of flax transcriptomics, there are more publications regarding of its tolerance to abiotic factors than to resistance to the biotic ones. This is probably because of the breeding programs and the target properties of plants for specific regions. However, there exists a possibility for further prospective evaluation of DEGs under different types of stressors, as well as in the process of ontogenesis. Finally, the total data related to transcriptome analysis will allow to create a reference maps for the detection of resistance/tolerance additional genes useful for the programs of flax MAS or for genetic editing.

USE OF HIGH-THROUGHPUT SEQUENCING IN EVALUATION OF microRNA

MicroRNAs (miRNAs) are a class of small, noncoding RNA detected in different organisms, including plants. They consist of about 20–24 nucleotides and can control numerous biological processes by negative regulation of gene expression with specific binding and inhibition of the target mRNA [89]. The level of microRNA changes in stressful conditions caused by factors such as drought, hypoxia, cold, salinization of soil, availability of heavy metals, and high or low level of nutrients [90–95].

NGS is often used to evaluate microRNA in plants. Melnikova et al. [93] identified 96 conservative homologs of microRNA of 21 families, 12 of which were detected for the first time. The most spread family in flax is miR165/166, which according to the authors is involved in the regulation of genes expression, participates in the coordination of metabolism in plants under stress and in the specialization of the plant tissues. Expression of seven microRNAs (miR168, miR169, miR395, miR398, miR399, miR408, and lus-miR-N1) in the nutrient deficiency was assessed based on the sequencing data in the enlarged number of accessions using PCR in the online mode. In the conditions of phosphate deficiency, change in the

expressions of lus-miR-N1 and miR399 were detected. Negative correlation of the expression of lus-miR-N1 and its predicted target was detected, as well as of the gene of ubiquitin-activating enzyme E1, and miR399 and its predicted target – gene of ubiquitin-conjugating enzyme E2 [93, 96].

In flax response to aluminum stress, Dmitriev et al. [95] noted changes in the expressions of miR319, miR390, and miR393. They possibly play important roles in stress reactions of plant by regulating the growth processes [95].

In 2016, Yu et al. [94] examined the microRNA profile of flax in salinity-alkalinity stress conditions by using high-throughput sequencing. A total 124 microRNAs were attributed to 23 conservative families and 394 new microRNAs were identified. After assessment of the DEG profiles, 17 known lus-miRNA and 36 new lus-miRNA were selected, which were used to predict the target genes. Parallel analysis of the terminal reads of RNA and profiling of transcriptome demonstrated changes in the expression of 29 pairs of microRNAs under the applied stress conditions. It was suggested that the target miR398 gene coding for superoxide dismutase and miR530, which targets the transcription factors of the WRK family, could play significant roles in flax stress resistance [94].

Flax microRNA data are deposited in miRbase database. The resource contains repository that presents sequences of 124 microRNA of *L. usitatissimum* [97]. Primary and secondary structures, localization in flax genome of assembly v1.0, are also provided.

On the basis of published data, testing of tolerance to abiotic factors is highly interesting. However, examination of microRNAs and their functions in flax ares till in the initial stages and several key issues remain unanswered. The accumulation of knowledge about the mechanisms of microRNA regulation in plants will allow to develop artificial microRNA to use them as effective tools to control gene expressions.

CONCLUSION

The use of modern methods in addition to traditional breeding approaches makes development of flax varieties with desired traits more efficient (Fig. 2). The development of new genetic

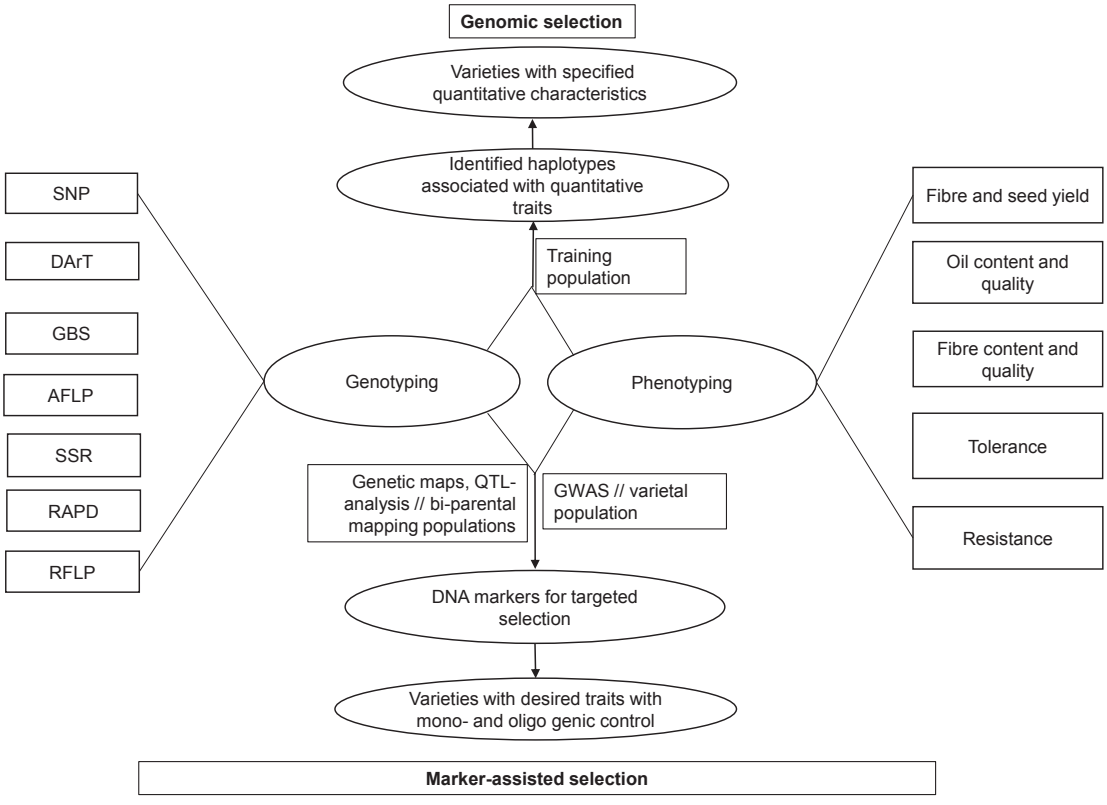

Fig. 2. Schematic representation of modern breeding approaches based on the use of a combination of approaches for marker-assisted and genomic selection

markers for breeding and detection of new target genes for genome editing will contribute to the enhancement of research based on the use of methods such as high-throughput sequencing in the quantitative genetics and transcriptomics of flax. The obtained results will also have fundamental relevance, leading to further understanding of the phylogenetic traits of the genus *Linum*, evolution of genomes representative of this genus, mechanisms of the ontogenetic development of plants and phenotypic variation, and variation in responses to different environment.

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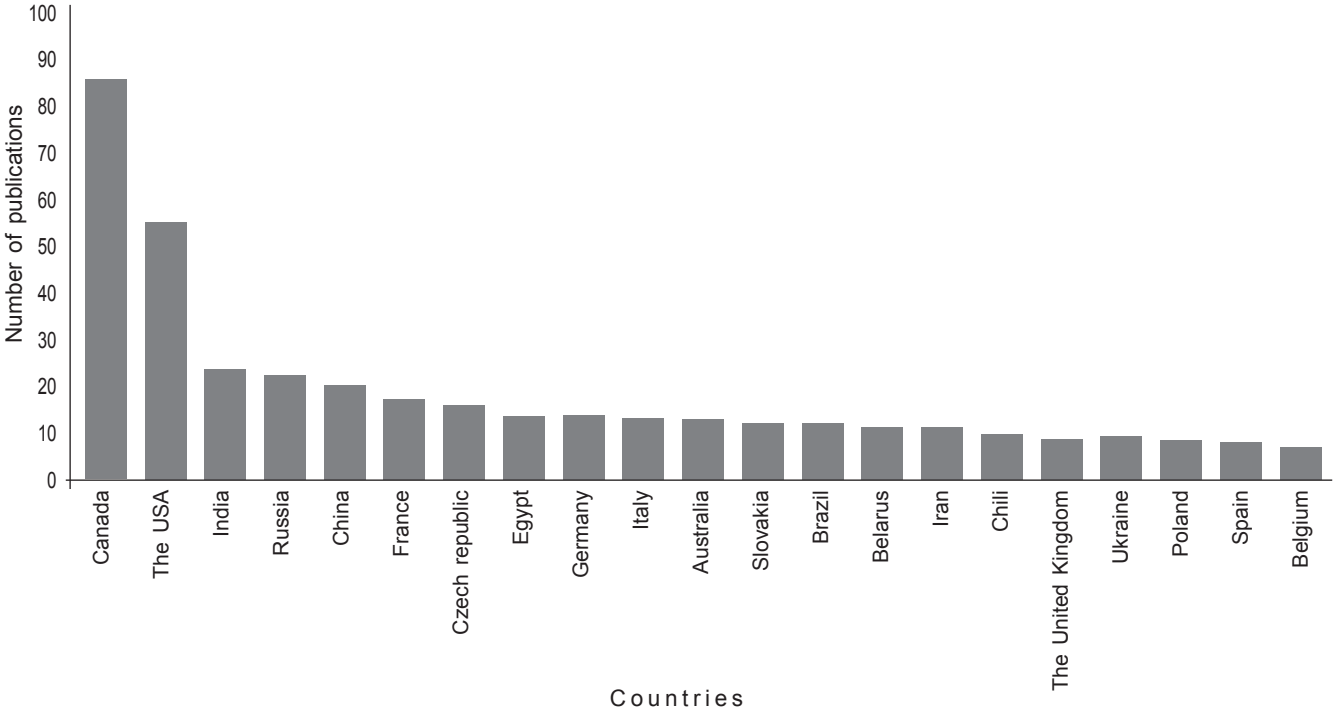
Conflicts of interest

The authors declare no conflicts of interest.

Supplementation 1

The use of flax DNA markers in studies in different countries is based on a search for publications in the Scopus database (www.scopus.com, accessed February 1, 2019) by the intersection of the keywords "Marker" and "Linum"



Supplementation 2

Electronic resources containing full genome flax sequencing data

Genome sequencing and assembly results, as well as recent assembly data for *L. usitatissimum* pseudomolecules, are available in the NCBI GenBank nucleotide sequence database under the identifier GenomeProject # 68161 [18]. Access numbers are from CP027619 to CP027633 for each of the 15 chromosomes. Sequences obtained on the basis of phasmids and BAC libraries were presented in GenBank under the numbers HQ902252, JN133299-JN133301 and JX174444-JX174449.

The annotated genome sequence and information about coding genes are also available in the Phytozome database [13, 15, 19]. The assembly characteristics are the total length of the assembled sequences is approximately 318.3 million bp, the number of scaffolds is 88,420, the number of contigs is 110390. Annotated genome contains 43471 genes and 43484 transcripts. In addition, the database contains ESTs from the NCBI GenBank mapped to the genome using PASA (the Program to Assembly Spliced Alignments) [15].

Some data on the genomes of flax species can be found in the European Archive for Nucleotide Sequences (ENA) [20]. For example, raw pair readings obtained by shotgun sequencing of wild flax species such as *L. leonii* (SRR1592650), *L. lewissii* (SRR1592654), *L. perenne* (SRR1592548), *L. narbonense* (SRR1592545) *L. grandiflorum* (SRR1592647), *L. decumbens* (SRR1592610) and *L. angustifolium* (SRR1592607).

Supplementation 1

Candidate genes associated with agronomic traits of flax

Trait	Candidate gene associated with trait	Encoding protein	Interpretation	Method	Reference
Palmitic acid content	*PIP5K (Lus10022606)* (*PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE*)	Phosphatidylinositol-4-phosphate 5-kinase	Playing a role in the inositol phosphate metabolism pathway, hydrolysis of phosphatidylinositol can serve as precursors for the synthesis of palmitic acid	GWAS	[56]
Plant height	*ABC* (*Lus10016125*) (*ATP-BINDING CASSETTE*)	ATP-binding cassette	Provide transport of lipids, sugars, amino acids, etc. thus plays an important role in the development and growth of plants	GWAS	[56]
	UGT (*UDP-GLYCOSYLTRANSFERASE*)	UDP-glycosyltransferase	The overexpression of *UGT84B1* and *UGT74E2* in Arabidopsis causes phenotypes with shorter stature and more branches. Affects the development of plants modulated the metabolic pathway of auxin	GWAS	[57]
	PL (PECTATE LYASE)	Pectate lyase	The *PL* gene is closely associated with the growth and development In rice and Arabidopsis by regulating the rate of cell division and the participation of cell wall modifications	GWAS	[57]
Fiber content	*Lus10016354*	Xanthoxine Dehydrogenase	Participates in transfer and signal transduction of phosphate from roots to the aboveground parts. Increased phosphate intake increases seed size	GWAS	[56]
Stearic acid content	*Lus10021171*	Protein phosphatase	In flax with reduced gene expression, an content of stearic acid is increased	GWAS, RNA-seq	[56]
1000-seed weight	*PHO1* (*PHOSPHATE PERMEASE*)	Phosphate permease	Participates in transfer and signal transduction of phosphate from roots to the aboveground parts. Increased phosphate intake increases seed size	GWAS	[56]
Number of branches	*GRAS* (*GIBBERELLIC ACID INSENSITIVE + REPRESSOR OF GA1-3 + SCARECROW*)	Transcriptional factor of GRAS family GRAS (GIBBERELLIC ACID INSENSITIVE + REPRESSOR OF GA1-3 + SCARECROW)	They play a key role in the development and signal transduction. LS and MOC1 are members of the GRAS protein family. The lack of *LS* gene expression inhibits the formation of axillary meristem and reduces the number of axillary buds. The absence of *MOC1* expression leads to an almost complete absence of tillering in rice because the gene product is involved in cell cycle regulation	GWAS	[57]
	XTH XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE	Xyloglucan endotransglucosylase/hydrolase	*XTH9* is expressed in Arabidopsis in the apical meristem of flower buds and stalks and is associated with an elongation of these parts of the plant; a decrease of the gene expression leads to a short interstitial length	GWAS	[57]

Supplementation (continued)

Trait	Candidate gene associated with trait	Encoding protein	Interpretation	Method	Reference
Mucilage content	*GATL5* (*Lus10009311*) (*GALACTUROSYL TRANSFERASE-LIKE 5*)	Galacturosyl transferase-like 5	Involved in rhamnogalacturonan I (RG I) backbone synthesis.	GWAS	[55]
	MUM4 (*Lus10009288*) (*MUCILAGE-MODIFIED 4*)	UDP-L-rhamnos synthase	Important for production of rhamnose, a key substrate for mucilage biosynthesis	GWAS	[55]
	PME36 (*Lus10009287*) (*PECTIN METHYLESTERASE 36*)	Pectin methylesterase 36	Plays role in pectin synthesis and cell wall modification	GWAS	[55]
	SBT1.7 (*Lus10009313, Lus10007083*) (*SUBTILISIN-LIKE SERINE PROTEASE*)	Subtilisin-like serine protease	Запускает активацию ферментов, модифицирующих клеточную стенку для выделения слизи	GWAS	[55]
	TT8 (*Lus10007101*) (*TRANSPARENT TESTA 8*)	TT Transcription Factor Family	Part of a transcription factor complex that, along with GLABRA2 (GL2), regulates *MUM4* gene expression	GWAS	[55]
Hull content	*AGL62* (*Lus10035456*) (*AGAMOUS-LIKE MADS-BOX PROTEIN*)	AGL Transcription Factor Family	AGL62 mutants can initiate embryo and endosperm formation but are not able to form the seed coat	GWAS	[55]
	GH17 (*Lus10018306*) (*GLYCOSYL HYDROLASE FAMILY 17*)	Glycosyl hydrolase family 17	Coexpressed with TT12, AHA10, and BAN, that might process glycosylated flavan-3-ol monomers, leading to accumulation of PAs in the seed coat	GWAS	[55]
	UGT79B1 (*Lus10026926*) (*UDP-GLUCOSE FLAVONOL 3-O-GLUCOSYLTRANSFERASE*)	UDP-glucose flavonol 3-o-glucosyltransferase	A key enzyme that catalyzes the final stage of anthocyanin biosynthesis	GWAS	[55]
Response to High Soil Acidity	*CAX3* (*CA^{2+}/H^{+}-EXCHANGER*)	Ca^{2+}/H^{+}-exchanger	Participates in the regulation of plant growth and assimilation of nutrients, as well as in the regulation of phosphate transport. CAX3 is in tonoplast. CAX3 mutants of arabidopsis are susceptible to stress caused by increased salinity and acidity. *CAX1* and *CAX3* are involved in the regulation of pH in the apoplast	RNA-seq	[78]

Supplementation (continued)

Trait	Candidate gene associated with trait	Encoding protein	Interpretation	Method	Reference
Response to aluminum stress	*GST* (*GLUTATHIONE S-TRANSFERASE*)	Glutathione-S-transferase	Participates in stress response, including oxidative stress, and can act as glutathione-dependent peroxidase. The effect of aluminum on plants leads to the formation of reactive oxygen species and lipid peroxidation at low pH. GST expression increases both in resistant and toxic-sensitive effects of this substance in corn, arabidopsis and others under increasing of aluminium content. An increase in GST expression was observed in flax under Al stress, especially in resistant varieties	RNA-seq	[77]
	CAX3 (*CA^{2+}/H^{+}-EXC*)	Ca^{2+}/H^{+}-exchanger	The increased regulation of the *CAX3* gene was revealed in flax tolerant to aluminum. Activation of *CAX3* gene promotes the compartmentalization of Ca^{2+} in vacuoles. It is possible mechanism to ensure flax resistance to stress caused by increased aluminum concentrations	RNA-seq	[78]
	UGT (*UDP-GLYCOSYLTRANSFERASE*)	UDP-glycosyltransferase	Participate in the biosynthesis of secondary metabolites, hormonal homeostasis, detoxification of xenobiotics and in the reaction of plants to stress. Changes in UGT expression upon exposure to elevated aluminum concentrations were observed in rice, flax, corn, and other crops. Genes participate in lignan biosynthesis and cell detoxification from secondary metabolites of reactive oxygen species, which may be a mechanism of flax resistance to Al stress	RNA-seq	[77]
Resistance to *Fusarium oxysporum*	*WRKY3*, *WRKY70*, *WRKY75*	Transcription factors of WRKY family	Overexpression of these genes in *A. thaliana* is observed under biotic stress. The transcription of *WRKY75* orthologs is enhanced in *Brassica napus* after infection with *Sclerotinia sclerotiorum* and *Alternaria brassicae*	RNA-seq	[80]
	MYB113, *MYB108*	Transcription factors of MYB family	Ранее показано, что у *A. thaliana MYB113* индуцируется при инокуляции *F.oxysporum* и имеет решающее значение для производства антоцианов, которые включают специфические стадии метаболизма фенилпропаноидов	RNA-seq	[80]

Supplementation (continued)

Trait	Candidate gene associated with trait	Encoding protein	Interpretation	Method	Reference
Resistance to *Fusarium oxysporum*	*ERF1, ERF14* (*ETHYLENE RESPONSE FACTOR*)	Ethylene response transcriptional factor	Increased expression of ERF family genes is observed in infected plants. In plants which inoculate by *Fusarium*, reactivation of ERF genes was observed. ERF1 is probably one of the most important genes involved in the protection of plants from fungal pathogens, and is associated with resistance of *A. thaliana* to *F. oxysporum sp. conglutinans* and *F. oxysporum sp. lycopersici*	RNA-seq	[80]
	CYP79B2, CYP79B3 (*CYTOCHROME P450*)	Cytochrome P450	CYP450 promotes the conversion of tryptophan to indole-3-acetaldoxime (a precursor of indole-3-acetic acid — auxin). Activation of the auxin pool is crucial for the germination and growth of plants, which can also protect plants against pathogens	RNA-seq	[80]
	PRX52 (*PEROXIDASE 52*)	Peroxidase involved in the lignin formation	Participates in the formation of lignin. Gene expression increased more than 40 times in *A. thaliana* under the infection of *Verticillium longisporum*	RNA-seq	[80]
	CHS (*CHALCONE SYNTHASE*)	Chalcone synthase	A key enzyme in the biosynthesis of flavonoids, which have a high antioxidant ability. This is used to create forms with increased resistance to *F. oxysporum* and *F. culmorum* by constructing a transgenic flax with multiconstruction, including chalcon synthase (CHS), chalconisomerase (CHI) and dihydroflavonol reductase (DFR) of petunia	RNA-seq	[80]
	DFR (*DIHYDROFLAVONOL REDUCTASE*)	Dihydroxyflavone reductase	One of the key enzymes for the synthesis of anthocyanins, is used, like CHS	RNA-seq	[80]
	RIPK (*RPMI-INDUCED PROTEIN KINASE*)	RPMI-induced protein kinase	The gene is an ortholog of the *A. thaliana ATG05940*, which encodes a protein kinase that activates effector immunity	RNA-seq	[80]

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