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ALLELE VARIABILITY OF AMYLASE INHIBITOR GENE *AI* IN POTATO VARIETIES AND LINES

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Abstract

The economic efficiency of potato varieties includes not only yield characteristics, but also taste preservation during storage. Storing potato tubers at low temperatures leads to the degradation of starch and the accumulation of reducing sugars; the latter during heat treatment contribute to the deterioration of taste and participate in acrylamide synthesis. Starch degradation to simpler compounds is achieved in two pathways: hydrolytic and phosphorolytic. In the hydrolytic pathway, hydrolases, including α- and β-amylase, are responsible for cleavage of starch, and exhibit different activities depending on the tissue, organ type, cell localization, and plant species. Amylase activity is regulated at the post-translational level by an amylase inhibitor (AI), which binds amylase and blocks the active site of the enzyme, or changes its conformation, thereby reducing the catalytic activity. Although AI role in plant is very important, present data on the *AI* genes and encoded proteins in representatives of the genus *Solanum* are extremely limited. In this study, *AI* sequences were obtained and analyzed in 36 potato varieties and lines of domestic and foreign selection. Two types of *AI* coding sequence were identified, 621 and 630 bp, depending on presence of 9-bp insert GGTGCAWTT at the 3′-end of the cDNA. The analyzed gene was characterized by an extremely high polymorphism level: exonic sequences contained 134 SNPs (singe nucleotide polymorphisms) (21.3 %), which resulted in 69 amino acid substitutions (33.0 %) in the encoded proteins. Detected GAI/F_{202} insertion in the C-terminal region of some AI proteins resulted from the 9-bp 3′-gene insertion. Among the 69 amino acid substitutions identified, only 11 are radical and may lead to a change in the protein conformation. All of the analyzed potato accessions were heterozygous and possessed several allelic variants of the gene. In total, 70 allelic variants of the gene and 69 associated protein variants are identified. The largest number of single nucleotide polymorphisms is among the allelic variants of the gene in the varieties Lux (18 substitutions), Irbitskii (17 substitutions) and Gala (16 substitutions). The largest number of amino acid substitutions is in the AI proteins in the Gala (9 substitutions) and Gornyak (8 substitutions) varieties.

Keywords: *Solanum tuberosum*, potato varieties, amylase inhibitor, *AI* gene, allelic variants

Potato (*Solanum tuberosum*) holds one of the leading positions in agriculture of many countries as food crop, commercial crop and forage crop [1, 2]. In the Russian Federation the area of potato cultivation covers different climatic zones from North of the Arctic Circle to southern borders, and gross yield of this culture amounts to approximately 30 million tons, i.e., almost 10% of global potato production (380 million tons; data of FAO-STAT for 2017). Potato is one of four most important nutrient carbohydrate sources after wheat, rice and corn, and contains a large amount of vitamins and minerals [3-5].

The main nutrition value of potato is determined by the contents and qualitative composition of starch in tuber. Starch consists of a mix of two homo-

polysaccharides, line amylose and branched amylopectine, which differ in structure and biosynthesis pathways. Both amylose and amylopectine are α-1,4-glucane chains, where amylopectine consists of short chains linked between each other with α-1,6-glucosidic bonds in branching points [6-8]. Starch metabolism is well-studied. At least 40 enzymes are known that participate in carbohydrate metabolism in potato tubers and determine the contents and composition of starch and other carbohydrates [9-13].

Starch content in potato tubers can reach 25% [14)]. After crop harvesting the tubers are stored up to several months at low temperature (2-4 °C). Exposure to low temperature can induce cold-induced sweetening (CIS) of tubers, which manifests itself in strengthened starch hydrolysis and, consequently, in accumulation of reducing sugars [15-18]. During high temperature treatment reducing sugars interact with α-amino acids and carcinogenic acrilamide is formed, and taste deteriorates [19-21]. The cold-induced sweetening is also affected by tuber ripeness, mechanical damage, biotic and abiotic stress etc. [20, 22].

Starch cleavage into simpler compounds is achieved in two ways (hydrolytically and phospholytically). First, hydrolases, including α-amylases (AMY, EC 3.2.1.1) and β-amylases (BAM, EC 3.2.1.2) [23, 24], are responsible for starch cleavage. The amylases are endoamylolytic (α-amylases) and exo-amylolytic (β-amylases) enzymes, which specifically hydrolyze α-1,4-glucosidic bonds and form linear and branched maltooligosaccharides [25, 26].

Presently, 5 isoforms of α-amylase and 10 isoforms of β-amylase are known in plants, which can exhibit different activity depending on tissue, organ type, cellular localization and plant type [26-28]. Gene expression for nine amylases (*StAmy1*, *StAmy23*, *StBAM1*, *StBAM3*, *StBAM4*, *StBAM5*, *StBAM7*, *StBAM8* and *StBAM9*) was identified in potato tubers, where only three (*StAmy23*, *StBAM1* and *StBAM9*) have high expression under low temperature storage [26, 29].

The amylase activity is regulated post-translationally by amylase inhibitor (AI), which binds to amylase and blocks active enzyme site or changes its conformation, thus reducing catalytic activity [30, 31]. The potato gene encoding amylase inhibitor was first identified in *S. berthaultii* (*SbAI*) [32]; however, its sequence is not provided in NCBI database.

In a number of studies Zhang et al. [29, 32, 33] showed that during low temperature storage potato tubers resistant to cold-induced sweetening as compared to CIS-sensitive tubers are characterized by higher *SbAI* gene expression; furthermore, negative correlation was identified between the number of *SbAI* gene transcripts and content of reducing sugars. Subsequent studies on transgenic plants showed that at low temperatures *SbAI* gene suppression in CIS-resistant potato lines results in increase in StAmy23, StBAM1 and StBAM9 amylase activity and increased volume of reducing sugars in tubers. At the same time, *SbAI* gene overexpression in CIS-sensitive potato lines at low temperatures caused suppression of such amylases [32].

In spite of essential significance of amylase inhibitors in potato cold-induced sweetening process, the information about variability of the aforementioned gene and its possible allele variants is lacking. For instance, GenBank NCBI (https://www.ncbi.nlm.nih.gov/) database contains information about full size gene *AI* (JX523608.1) and its mRNA (JX523606.1) only for anonymous sample of *S. tuberosum*. Furthermore, this database contains *AI* sequences of other representatives of *Solanum* genus, *S. lycopersicum* (XM_004233967.3, CP023759.1, HG975515.1) and *S. pennellii* (HG975442.1, XM_015211800.2).

In this study, we identified amylase inhibitor *AI* gene sequence in 36 domestic and foreign potato varieties and lines, and determined possible allele

variants of this gene and the protein it encodes for the first time.

The purpose of this study is to determine the sequences of amylase inhibitor (*AI*) gene and proteins encoded in cultivated potato varieties and lines, to evaluate their genetic variability, and to determine the allele variants of *AI* gene.

Techniques. The plants were collected in Lorkh *All-Russian Research Institute of Potato Farming* (Moscow Province, Russia). Gene sequence (JX523608.1) and mRNA sequence (JX523606.1) of *S. tuberosum* available in the GenBank NCBI database were used in a comparative evaluation of *AI* gene polymorphism.

The DNA was isolated from young leaves by a modified potassium acetate method [34].

Primers were developed based on *AI* gene sequences of *Solanum* genus members (JX523608.1, JX523606.1, XM_004233967.3, CP023759.1, HG975515.1, HG975442.1, XM_015211800.2) available in GenBank NCBI, which allows amplifying full-sized *AI* (SbaI_F 5′-ACTATGGCTTTTCATTACTCTA-3′; SbaI_R 5′-TTACATCAAAGAATAGTTGTATAAC-3′) and overlapping internal gene region (SbaI_in1R 5′-TCGTGAGAATAGTCTCTTGC-3′; SbaI_ex1F 5'-GTAACATGGCTCGCGTTC-3'; SbaI_ex3F 5′-AACAGAGGCTCCAAGTGC-3′; SbaI_in3R 5′-GGATAGTTTGAGCAACATAACTT-3′). The amplification was performed with a reagent kit (Dialat LTD, Russia). The reaction mixture contained in 15 μl 10× buffer, 1.5 μm $MgCl_2$, 0.2 mM of each dNTP, 0.5 μm of SbaI_F and SbaI_R primers; 0.2 U of BioTaq DNA of polymerase (Dialat LTD, (Russia) and 100 ng of genomic DNA. The reaction was performed as follows: denaturation for 40 s at 95 °C; primer annealing for 30 s at 54 °C, DNA elongation for 2 min at 72 °C (35 cycles); final elongation for 7 min at 72 °C (BioRad C1000 thermocycler, Bio-Rad, USA).

The resultant amplicons approximately 2 kbps long visualized in 1% agarose gel were cut out and purified with Zymoclean™ Gel DNA Recovery Kit (Zymo Research, USA). During cloning of full-size *AI* gene sequences of analyzed potato varieties and lines Quick-TA Kit (Eurogen, Russia) was used. The nucleotide sequences of fragments were determined on ABI 310 Capillary DNA Analyzer automatic sequencer (Applied Biosystems, USA) (Center for Collective Usage Bioinzheneriya RAS).

The resulting sequences were aligned and analyzed with MEGA 7.0 software [35]. Potential impact of amino acid substitutes on the structure and functions of proteins were evaluated using web-service PROVEAN (http://provean.jcvi.org/index.php) [36].

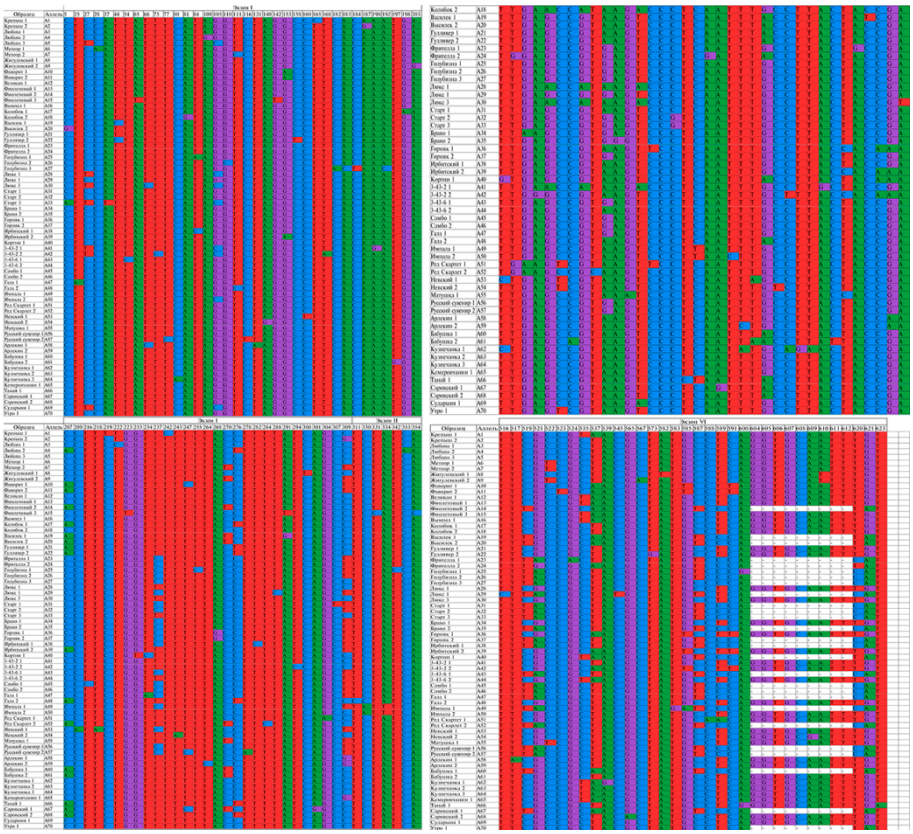
Results. Nucleotide polymorphism of amylase inhibitor gene. We amplified and later cloned amylase inhibitor (*AI*) genes in 36 potato lines and varieties of domestic and foreign origin (Table 1). Because modern potato varieties are tetraploid, we sequenced five clones of each analyzed sample to identify *AI* gene allele variants.

Complete nucleotide sequence of *AI* gene was determined for all analyzed potato samples as a result. The comparison of the obtained nucleotide sequences with data available in GenBank NCBI database for *AI* genes and mRNA identified their high homology (> 90%) in samples we studied and in other representatives of *Solanum* genus.

Analysis of exon-intron organization determined that, along with the other known plant amylase inhibitor genes, all *AI* gene sequences of *S. tuberosum* containe four exons. The length of *AI* gene of analyzed potato varieties varied from 1781 bps (in Meteor 1 variety) (hereinafter the numbers in variety name represent allele variant number) to 1872 bps (in Red Scarlett 2 variety). Very high polymorphism was identified in *AI* nucleotide sequences, i.e. it contained 530 var-

iable sites (single nucleotide polymorphisms, SNPs), and total degree of polymorphism amounted to 27.0%.

1. Allele variants of *AI* gene nucleotide sequence in studied potato lines and varieties



N o t e. The numbers in variety name represent allele variant number (see http://www.agrobiology.ru).

We identified two types of exon sequences with different length, 621 and 630 bps due to presence of a large number of samples of 9-nucleotide insertion (GGTGCAWTT) in 3′-end region. It has to be pointed out that exon sequence variability in samples that we studied turned out unexpectedly high. We identified 134 variable sites in encoding sequences, with a 21.3% polymorphism which is much higher than for other known carbohydrate metabolism genes. For instance, studies of specially selected polymorphic fragment of acid vacuolar invertase gene *Pain-1* (exon V-terminating codon) in *S. tuberosum* cultivars showed the variability of this region not more than 9% [37, 38]. When analyzing genes associated with starch phosphorylation in 192 potato lines from New Zealand, α-glycan-H_2O-dikinase (*GWD*) gene turned out to be the most polymorphic, and its variability was < 5%, whereas polymorphism of isoform starch-synthase gene (*SS I-III*) did not exceed 3.4% [39].

The introns in studied sequences differed in length and variability significantly and, apart from a large number of nucleotide substitutes (396 in total), contained insertions and deletions. The size of *AI* gene intron sequences varied within range of 1151 to 1251 bps. The biggest differences were identified in intron I, which included extended insertions (up to 47 bps). In some cultivars in gene position 347-400 bps we identified a region containing nonhomologous insertions varying in length. The sequences of introns II and III had deletions not exceeding 18 nt (e.g. $GATATATTTCTCTY_{1406}$, $GTAT_{1452}$), and $TATACC_{1298}$ in-

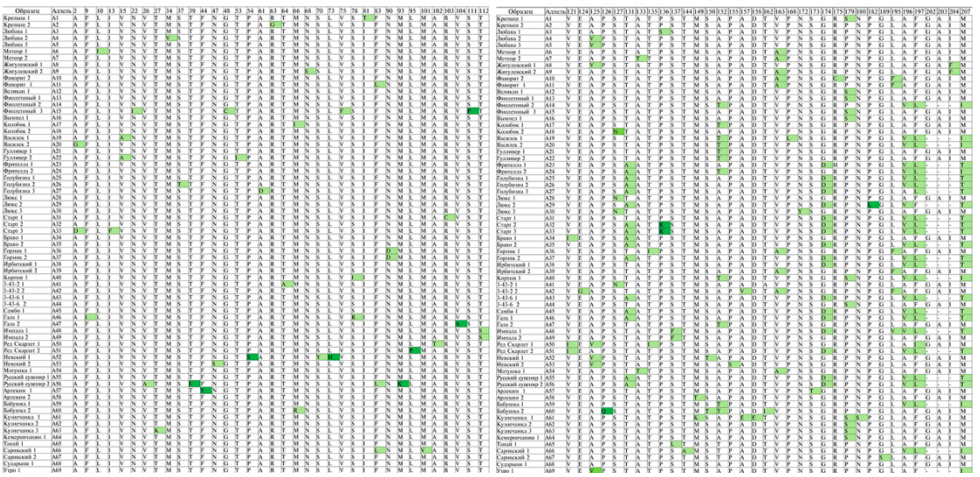
sertion.

As has been mentioned earlier, five *AI* gene clones were sequenced and analyzed for each sample, which allowed us to characterize homozygous/heterozygous status of this gene. All potato samples analyzed turned out heterozygous for the specified gene and several of its allele variants corresponded to them (see Table 1).

While analyzing exon sequences we identified 70 variants for 36 cultivars and lines. Earlier, only 11 allele variants for full-scale encoding sequence of acid vacuolar invertase gene had been reported for 19 cultivars [37]. It is noteworthy that as a result of the analysis we failed to identify an allele variant typical for the group of cultivars. All analyzed samples were characterized by specific allele *AI* gene variant. It has to be pointed out, however, that a number of allele variants differed from each other only by 1-2 nucleotide substitutions. The highest number of difference in terms of allele variants were displayed by the following cultivars: Luxe (18 SNPs), Irbitrskii (17 SNPs) and Gala (16 SNPs). This high gene variability and a large number of allele variants are not quite typical for plant genes and, in particular, for potato.

Amino acid polymorphism of amylase inhibitor. The resulting *AI* gene nucleotide sequences were translated. The corresponding amino acid sequences amounted to 206 and 209 amino-acid residue. The identified protein length differences were attributable to the presence of GAI/F_{202} insertion in terminal region due to GGTGCAWTT insert at 3′-end region. Out of 134 exon-specific SNPs, 69 resulted in amino acid substitution, whereas amino acid polymorphism amounted to 33.0%. We conducted PROVEAN-analysis and determined that 11 out of 69 substitutions are radical and can affect protein conformation. Therefore, the study identified 69 variants of amino acid AI sequence whose characteristics are shown in Table 2. AI sequences of Gala (9 substitutes) and Gornyak (8 substitutes) contained the highest amount of amino-acid residue substitutions.

2. Allele variants of amylase inhibitor amino acid sequence in studied potato lines and varieties



N o t e. The numbers in variety name indicate allele variant number. The variable amino acid sites are highlighted with green, whereas radical ones are highlighted with dark green (see http://www.agrobiology.ru).

To summarize, this study for the first time describes amylase inhibitor *AI* gene sequence in 36 cultivated potato lines and varieties, potential allele variants of this gene and encoded proteins. Furthermore, we identified a very high nucleotide (21.3%) and amino acid polymorphism (33.0%). It has to be pointed out,

however, that identified amino acid residue substitutions are in most cases (58 out of 69) neutral and, theoretically, should not result in conformational protein change. The findings allow us to continue searching for correlation between allele *AI* gene variants and sensibility of potato lines or cultivars to cold-induced sweetening.

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