ISSN 1022-7954, Russian Journal of Genetics, 2020, Vol. 56, No. 3, pp. 375–378. © Pleiades Publishing, Inc., 2020.
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SHORT COMMUNICATIONS

Differences in the Amylase Inhibitor Gene *SbAI* Expression in Potato during Long-Term Tuber Cold Storage and in Response to Short-Term Cold Stress

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Received April 3, 2019; revised May 21, 2019; accepted May 30, 2019

Abstract—Expression analysis of the amylase inhibitor gene *SbAI* in five potato cultivars (*S. tuberosum*) revealed *SbAI* downregulation in tubers during long-term (seven months) cold storage. Estimation of *SbAI* gene expression and starch content in leaves of *S. tuberosum* cv. Nadezhda and four wild potato species (*Solanum* sect. Petota) showed a dramatic increase in *SbAI* transcription accompanied by starch content drop in response to short-term cold stress.

Keywords: potato, *Solanum tuberosum*, wild tuber-bearing potato species, cold stress, SbAI amylase inhibitor, starch content, gene expression

DOI: 10.1134/S1022795420030163

In terms of production, potato *Solanum tuberosum* is the most important non-cereal starch-bearing crop in the world. After harvesting, potato tubers are stored at a temperature of 2–4°C to prevent sprouting, maintain moisture percentage, and reduce pathogenesis. However, the effect of low temperatures on tubers leads to the so-called cold-induced sweetening (CIS), which consists in the accumulation of reducing sugars as a result of starch and sucrose hydrolysis [1, 2].

In plants, starch accumulates in plastids: reserved—in amyloplasts of sinc organs; transitory—in chloroplasts of photosynthetic leaves [3, 4]. Starch degradation is mainly due to the hydrolysis, mediated by the activity of starch-hydrolyzing enzymes [5], primarily α-amylases (AMY, EC 3.2.1.1) and β-amylases (BAM, EC 3.2.1.2) [6, 7]. In the potato genome, ten *BAM* and five *AMY* genes were identified, which encode proteins specific to various substrates and cellular structures (chloroplasts, amyloplasts, etc.) [8, 9]. It was shown that low temperatures activate *StAmy23*, *StBAM1*, and *StBAM9* transcription in potato tubers [8, 10]. Corresponding enzymes, α-amylase StAmy23 and β-amylases StBAM1 and StBAM9, are involved in cold-induced starch degradation in tuber amyloplasts [8]. StBAM9 binds directly to starch grains, providing the release of soluble glucans, which hydrolysis continues in amyloplasts by StBAM1 and, further, in the cytosol by StAmy23 [8].

In turn, the amylase activity is regulated at a post-translational level by an amylase inhibitor (AI) [11, 12]. AI spatial structure is highly conserved among various plant species [13]. It was shown that AI is able to bind to amylase either by blocking the active site of the enzyme or by changing its conformation, thereby reducing catalytic activity [14, 15]. In potato, the gene encoding the amylase inhibitor was first cloned in *S. berthaultii* (*SbAI*) [16]. SbAI interaction with each of the amylases—StAmy23, StBAM1, and StBAM9, was experimentally confirmed [16].

SbAI expression was shown to be higher in tubers of CIS-resistant potato plants compared to CIS-sensitive plants, which confirms the negative correlation between the *SbAI* transcript number and the reducing sugar content [16]. *SbAI* overexpression in tubers of CIS-sensitive potatoes stored at low temperatures is accompanied by a decrease in the level of amylase gene transcription (including *StBAM1*, *StBAM9* and *StAmy23*) and starch degradation [16]. *SbAI* suppression in CIS-resistant potato tubers slightly upregulates the level of amylase gene expression and considerably increase the content of reducing sugars [16]. Thus, it may be suggested an inverse correlation between the expression levels of the inhibitor and amylase genes. Considering the post-translational inhibition of amylases by the SbAI, it was proposed that the reducing sugar concentration is a possible trigger of the amylase gene expression in potato tubers [16]. Given the

above, one of the SbAI functions is to suppress the cold-induced amylase catalytic activity.

The aim of this work was to determine the expression pattern of the amylase inhibitor gene *SbAI* homologs both in tubers of various *S. tuberosum* cultivars during long-term low-temperature storage, and in the leaves of different potato species (*Solanum*, section Petota) under short-term cold stress.

The *SbAI* expression pattern was determined in tubers of five domestic *S. tuberosum* cultivars: Barin, Krasavchik, Nadezhda, Severnoe Siyanie, and Utro. In 2017, plants were grown in the field (Lorch Potato Research Institute). In September, the tubers were collected and placed into a potato storage at 4°C. Expression analysis was performed by quantitative real-time PCR (qRT-PCR) in two biological replicates at three time points: in September (immediately after harvesting), in February (after five months of storage), and in April (after seven months of storage). Thus, the performed expression analysis reflects changes occurring in tubers during storage from the time of harvest to the time of sprouting.

The *SbAI* expression in response to short-term cold stress was determined in the leaves of *S. tuberosum* (cv. Nadezhda) and four wild potato species—*S. demissum* (CGN 20568), *S. kurtzianum* (VIGRR K-11969), *S. chacoense* (VIGRR K-3678), and *S. vernei* (VIGRR K-20832). These species were chosen because they differ in cold resistance level [17]. Plants were grown under normal conditions (23/25°C, 16/8 h day/night, greenhouse) and subjected to cold stress (4°C, 72 h, 16/8 h day/night, climate chamber). In the same leaves, the total starch content was determined. For this, 500 mg of leaf tissue was homogenized in 4.5 mL of a solution (33% v/v DMSO; 0.44 mM HCl), incubated at 60°C for 30 min, and cooled to 25°C. The mixture was diluted 1 : 5 with water (mQ), adjusted to pH 4.5, and filtered. The starch content in the filtrate was determined spectrophotometrically using a Starch kit (R-Biopharm, Switzerland).

Based on the *S. tuberosum* sequence homologous to *SbAI* (XM_006355976.2), specific primers were designed for gene expression analysis by qRT-PCR (SbAIrtF: 5'-TTGTAACATGGCTCGCGTTC-3' and SbAIrtR: 5'-TGTTGGTGAAGCACTTGGAG-3'). Tuber and leaf total RNA was extracted and used for cDNA synthesis (GoScript, Promega, United States) and determination of *SbAI* expression. qRT-PCR was performed with the "Reaction Mixture for RT-PCR in the Presence of SYBR GreenI and ROX" kit (Syntol, Russia) under the following conditions: 95°C for 5 min; 40 cycles (95°C for 15 s, 60°C for 50 s). Genes *ef1α* and *sec3* were used as references with the following primers: ef1αF (5'-ATTGGAAACGGATATGCTCCA-3') and ef1αR (5'-TCCTTACCTGAACGCCTGTCA-3') [18]; sec3F (5'-GCTTGCACACGCCATATCAAT-3') and sec3R (5'-TGGATTTTACCACCTTCCGCA-3') [19]. Statistical analysis of the obtained data was performed by GraphPadPrism 7.02 (https://www. graphpad.com).

Results of the expression analysis showed that during storage in the tubers of all studied potato cultivars, *SbAI* transcription level decreased (Fig. 1a). In freshly harvested tubers (September), the expression levels of *SbAI* varied significantly. Maximal *SbAI* expression was detected in tubers of the cv. Severnoe siyanie; minimal level was characteristic of cv. Krasavchik and Nadezhda. After five months of storage at 4°C (February), in Krasavchik and Severnoe siyanie tubers, *SbAI* expression sharply decreased, while in the case of cv. Barin and Utro, it decreased gradually; the expression of *SbAI* in cv. Nadezhda was almost unchanged. After seven months of storage (April), only trace amounts of *SbAI* transcripts were detected in all studied potato cultivars.

The data obtained are correlated with the fact that, during long-term storage at low temperatures, tubers go into a state of physiological dormancy, when biochemical processes in cells slow down. The degradation of starch, the main source of energy in tubers, occurs with low intensity, mainly for respiration. It was shown that, in 2–3 weeks at 10°C, tubers lose about 1 g of starch at a respiration rate of 3–5 mg CO_2 kg^{-1} h^{-1} [4]. Depending on the genotype and external conditions, the stage of physiological dormancy of potato tubers commonly lasts 6–12 weeks, but for a number of cultivars, it can reach 27 weeks [4]. The transition from the dormancy to sprouting is accompanied by an increase in respiration rate and glycoalkaloids, water loss, and the accumulation of reducing sugars [4]. Monosaccharides are formed as a result of starch hydrolysis and an increase in amylase gene expression [10, 20] and downregulation of amylase inhibitor gene *SbAI*, which is characterized in this study for all five *S. tuberosum* cultivars analyzed (Fig. 1a).

Thus, we have shown a decrease in *SbAI* expression during long-term tuber storage at low temperatures for seven months. Potato tuber is a specialized storage organ that, as a result of evolutionary adaptation, has acquired the ability to withstand prolonged exposure to low temperatures in a dormancy state. Therefore, we believe that the results obtained to a greater extent reflect *SbAI* behavior in response to the state of physiological dormancy of tubers rather than to cold stress. In this regard, to assess the *SbAI* response to low temperatures, the next experiment was performed on potato leaves, for which, in contrast to tubers, a temperature of 4°C is stressful.

Previously, the expression of the amylase inhibitor gene in potato leaves under cold stress conditions was not studied. Therefore, in this work, the dynamics of *SbAI* expression was determined in the leaves of one of the studied *S. tuberosum* cultivar Nadezhda before and after exposure to cold stress. At the same time, the starch content in the leaves was measured. As can be seen from Fig. 1b, in response to stress, a sharp (7–8 times)

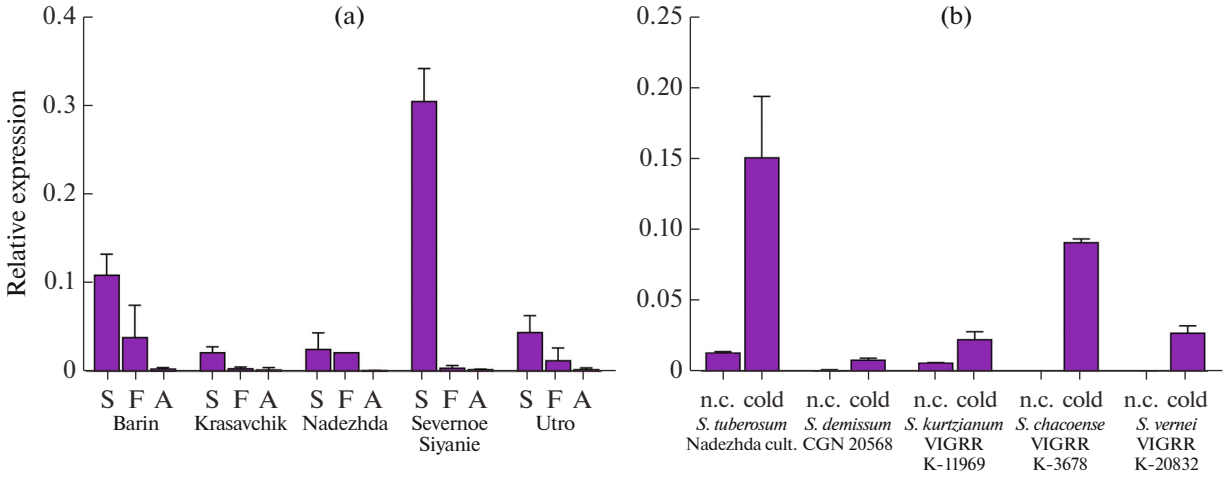

Fig. 1. Expression profile of amylase inhibitor gene *SbAI*: (a) in tubers of cultivated potatoes during long-term low-temperature storage (S—September, F—February, A—April); (b) in leaves of potato species under normal conditions (n.c.) and in response to cold stress (cold).

increase in *SbAI* transcription occurs, which is accompanied by the complete decay of starch (from 2.2 to 0 mg/g).

To understand whether the *SbAI* expression is species-specific or universal for the tuber-bearing Petota species, it seemed interesting to compare the data we obtained for *S. tuberosum* with the *SbAI* response to cold stress in other potato species [17]. For this, plants of four potato species (*S. demissum*, *S. kurtzianum*, *S. chacoense*, and *S. vernei*) with various degree of cold-resistance [17] were analyzed for *SbAI* expression pattern and starch content in leaves before and after short-term cold stress (Fig. 1b).

As a result, it was found that, for all analyzed species accessions, as well as for *S. tuberosum*, *SbAI* expression level increased in response to cold stress (Fig. 1b). As for *S. tuberosum*, for the other four species, the increase in the *SbAI* expression level was accompanied by a sharp decrease in the starch content in the leaves: *S. demissum*, from 0.07 to 0 mg/g; *S. kurtzianum*, from 1.34 to 0.44 mg/g; *S. chacoense*, from 2.68 to 0 mg/g; *S. vernei*, from 1.49 to 0.14 mg/g.

It should be noted that the analyzed species differed quite strongly both in the initial starch concentration in the leaves, and in the degree of its after stress degradation. Thus, for *S. tuberosum* and *S. chacoense* after stress, starch was destroyed completely, while its initial amount in these species was similar and, at the same time, maximal among all analyzed accessions. *SbAI* expression levels in the leaves of *S. tuberosum* and *S. chacoense* after stress were statistically comparable and also characterized by maximum values. For *S. demissum*, *S. kurtzianum*, and *S. vernei*, other dynamics were observed. Transcription of *SbAI* in leaves of these species also increased in response to stress, but was significantly less than that of *S. tuberosum* and *S. chacoense* (Fig. 1b). A minimal increase in expression was observed in *S. demissum*, which obviously agreed with the smallest initial amount of starch in the leaves among all the analyzed accessions. Thus, there is a correlation between the initial amount of starch in the leaf and the expression level of the amylase inhibitor gene in response to cold stress. It can be assumed that exposure to low temperatures stimulates the accumulation of amylase activity and, as a result, enhanced starch degradation. This is consistent with the previously shown increase in amylase gene expression and the activity of the corresponding enzymes in response to low temperatures in leaves of potato, *Arabidopsis thaliana*, and other plant species [21–23]. In this case, the released sugars can, in turn, serve as a signal for increasing the *AI* transcription level, and amylase inhibitor synthesis that is able to prevent and/or regulate starch breakdown; the more sugars, the higher the expression of *SbAI*.

The present results, together with the previous data [2, 12, 22], will contribute to a deeper understanding of the molecular genetic mechanisms of the potato response to low temperatures both during long-term tuber storage (physiological state of rest dormancy) and during short-term cold stress.

FUNDING

This work was supported by the RFBR (grant #17-29-08017), the Ministry of Science and Higher Education of the Russian Federation, and the Federal High-Tech Program for Agricultural Development of the Russian Federation for 2017–2025 (subprogram "Development of Potato Selection and Seed Production in the Russian Federation"). Plants were grown using the experimental climate control facility (Institute of Bioengineering, Research Center of Biotechnology, RAS).

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Shepherd, L.V.T., Bradshaw, J.E., Dale, M.F.B., et al., Variation in acrylamide producing potential in potato: segregation of the trait in a breeding population, *Food Chem.*, 2010, vol. 123, pp. 568—573.
2. Chen, X., Song, B., Liu, J., et al., Modulation of gene expression in cold-induced sweetening resistant potato species *Solanum berthaultii* exposed to low temperature, *Mol. Genet. Genomics*, 2012, vol. 287, pp. 411—421.
3. Zeeman, S.C., Smith, M.C., and Smith, A.M., The diurnal metabolism of leaf starch, *Biochem. J.*, 2007, vol. 401, pp. 13—28. https://doi.org/10.1042/BJ20061393
4. Benkeblia, N., Alexopoulos, A.A., and Passam, H.C., Physiology and biochemistry regulation of dormancy and sprouting in potato tuber (*Solanum tuberosum* L.), *Fruit, Veg. Cereal Sci. Biotechnol.*, 2008, vol. 2, pp. 54—68.
5. Cottrell, J., Duffus, C., Paterson, L., et al., The effect of storage temperature on reducing sugar concentration and the activities of three amylolytic enzymes in tubers of the cultivated potato, *Solanum tuberosum* L., *Potato Res.*, 1993, vol. 36, pp. 107—117.
6. Preiss, J., Regulation of the biosynthesis and degradation of starch, *Annu. Rev. Plant Physiol.*, 1982, vol. 33, pp. 431—454.
7. Solomos, T. and Mattoo, A.K., Starch-sugar metabolism in potato (*Solanum tuberosum* L.) tubers in response to temperature variations, in *Genetic Improvement of Solanaceous Crops*, Razdan, M.K. and Mattoo, A.K., Eds., Plymouth: Sci. Publ., 2005, pp. 209—234.
8. Hou, J., Zhang, H., Liu, J., et al., Amylases StAmy23, StBAM1 and StBAM9 regulate cold-induced sweetening of potato tubers in distinct ways, *J. Exp. Bot.*, 2017, vol. 68, no. 9, pp. 2317—2331. https://doi.org/10.1093/jxb/erx076
9. Van Harsselaar, J.K., Lorenz, J., Senning, M., Sonnewald U., and Sonnewald, S., Genome-wide analysis of starch metabolism genes in potato (*Solanum tuberosum* L.), *BMC Genomics*, 2017, vol. 18, no. 1, p. 37. https://doi.org/10.1186/s12864-016-3381-z
10. Zhang, H., Hou, J., Liu, J., et al., Amylase analysis in potato starch degradation during cold storage and sprouting, *Potato Res.*, 2014, vol. 57, pp. 47—58.
11. Liu, X., Cheng, S., Liu, J., et al., The potato protease inhibitor gene, *St-Inh*, plays roles in the cold-induced sweetening of potato tubers by modulating invertase activity, *Postharvest Biol. Technol.*, 2013, vol. 86, pp. 265—271.
12. Mckenzie, M.J., Chen, R.K., Harris, J.C., et al., Post-translational regulation of acid invertase activity by vacuolar invertase inhibitor affects resistance to cold-induced sweetening of potato tubers, *Plant Cell Env.*, 2013, vol. 36, pp. 176—185.
13. Lay, F. and Anderson, M., Defensins—components of the innate immune system in plants, *Curr. Protein Pept. Sci.*, 2005, vol. 6, pp. 85—101.
14. Barbosa, A., Albuquerque, E.V.S., Silva, M.C.M., et al., Alpha-amylase inhibitor-1 gene from *Phaseolus vulgaris* expressed in *Coffea arabica* plants inhibits alpha-amylases from the coffee berry borer pes, *BMC Biotechnol.*, 2010, vol. 10, pp. 44—51.
15. Kumari, B., Sharma, P., and Nath, A.K., α-Amylase inhibitor in local Himalyan collections of *Colocasia*: isolation, purification, characterization and selectivity towards a-amylases from various sources, *Pestic. Biochem. Physiol.*, 2012, vol. 103, pp. 49—55.
16. Zhang, H., Liu, J., Hou, J., et al., The potato amylase inhibitor gene *SbAI* regulates cold-induced sweetening in potato tubers by modulating amylase activity, *Plant Biotech. J.*, 2014, vol. 12, no. 7, pp. 984—993. https://doi.org/10.1111/pbi.12221
17. Machida-Hirano, R., Diversity of potato genetic resources, *Breed. Sci.*, 2015, vol. 65, no. 1, pp. 26—40. https://doi.org/10.1270/jsbbs.65.26
18. Lopez-Pardo, R., de Galarreta, J.I.R., and Ritter, E., Selection of housekeeping genes for qRT-PCR analysis in potato tubers under cold stress, *Mol. Breed.*, 2013, vol. 31, no. 1, pp. 39—45.
19. Tang, X., Zhang, N., Si, H., and Calderón-Urrea, A., Selection and validation of reference genes for RT-qPCR analysis in potato under abiotic stress, *Plant Methods*, 2017, vol. 13, article 85. https://doi.org/10.1186/s13007-017-0238-7
20. Davies, H.V., Carbohydrate metabolism during sprouting, *Am. J. Potato Res.*, 1990, vol. 67, pp. 815—827.
21. Scheidig, A., Fröhlich, A., Schulze, S., et al., Down-regulation of a chloroplast-targeted beta-amylase leads to a starch-excess phenotype in leaves, *Plant J.*, 2002, vol. 30, no. 5, pp. 581—591. https://doi.org/10.1046/j.1365-313X.2002.01317.x
22. Sitnicka, D. and Orzechowski, S., Cold-induced starch degradation in potato leaves—intercultivar differences in the gene expression and activity of key enzymes, *Biol. Plant.*, 2014, vol. 58, no. 4, pp. 659—666. https://doi.org/10.1007/s10535-014-0453-2
23. Thalmann, M. and Santelia, D., Starch as a determinant of plant fitness under abiotic stress, *New Phytol.*, 2017, vol. 214, no. 3, pp. 943—951. https://doi.org/10.1111/nph.14491