Comparative biochemical analysis of biotech cotton variety Porloq-4

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Abstract. Currently, the number of biotech plant varieties used in agriculture is increasing, which raises its efficiency. However, according to international requirements related to the development and application of biotechnology in science and production, the safety of the release of biotechnological varieties is considered. One of the key parameters for assessing the safety of genetically modified varieties is the assessment of significant equivalence, i.e. comparative biochemical assessment of genetically modified organisms (GMO). In this regard, the article provides information about the comparative assessment of key biochemical parameters for the Porloq-4 cotton variety, created by RNA interference of the phytochrome A1 gene. The research results have shown that the biotech cotton variety Porloq-4 is an equivalent analogue in terms of essential characteristics.

1. Introduction

For many years, people have been successfully applying the results of biotechnology, in particular, biotechnological methods in agriculture, pharmaceuticals, and industry. Improvement of the methods of isolation, reproduction, transfer, and expression of genes made it possible to overcome biological interspecies and intergeneric barriers and to obtain at the end of the last century the first organisms with artificially altered properties, where the existence of natural analogues and/or obtained by traditional breeding methods is virtually impossible. Currently, genetically modified varieties of agricultural crops with economically valuable properties occupy sown areas exceeding 180 million hectares, and food products made from such varieties are widely used in the national economy [1].

At the same time, imparting new qualities to agricultural plants ensures an increase in agricultural productivity, an improvement in nutritional properties, an easier processing of raw materials, as well as, in some cases, a decrease in the scale of sprayed chemicals, an increase in the profitability of farms and crop stability. At the same time, the acquired qualities of GMOs could also carry a certain risk to human health due to the unpredictable place of the recombinant DNA integration in the plant genome. Therefore, one of the main international requirements related to the development and application of biotechnology in science and production is the biosafety of genetically modified varieties with new efficient properties. In this regard, a number of international organizations, such as OECD, WHO and FAO, have developed basic principles for assessing the safety of genetically modified varieties, which are based on the concept of significant equivalence [2, 3].
According to this principle, it is not the level of safety of new food products as such that is assessed, but its change in comparison with traditional food analogues with a long history of safe use [2, 4, 5]. In this case, the equivalence is ascertained by the chemical composition of key compounds, the nature of metabolism, the composition of metabolites and nutritional value [3, 5, 6].

Thereby, this article deals with the comparative assessment of key biochemical parameters for the Porloq-4 cotton variety, created by RNA interference (RNAi) of the A1 phytochrome gene.

2. Materials and methods

The object of the study was gene-knockout and unmodified cotton varieties grown under phytotron conditions. The content of soluble sugars and starch in the collected extracts was determined by the anthrone method [7]. The sucrose content was analyzed in the resuspended supernatant according to the previously described methods [8]. Lipids were methyl esterified with a 0.4 M KOH solution containing methanol and a mixture of equal volumes of benzene and petroleum ether (1:1, v/v) [9]. Fatty acid methyl esters (FAs) were separated by gas chromatography on a gas chromatograph (Shimadzu GC-17A or similar) equipped with a hydrogen flame detector and an SP-2330 capillary column (15 m × 0.32 mm). The isothermal column temperature is 165°C, the detector temperature is 250°C. The composition of amino acids was analyzed by HPLC and pre-column modification using PITC (phenyl isothiocyanate) according to the method [10]. HPLC conditions: chromatograph Agilent Technologies 1200 with DAD detector, column 75x4.6 mm Discovery HS C18, 3 µm. Solution A - 0.14M CH3COONa + 0.05% TEA pH 6.4; solution B - acetonitrile.

Flow rate 1.2 ml/min, detection at 269 nm. The data were subjected to analysis of variance (ANOVA). The data are presented as mean ± standard error. The significance of differences between the means was determined with the Tukey test. Differences in P≤0.05 were considered significant.

3. Results and discussion

The analysis of significant equivalence presupposes, first of all, the compositional analysis of GMO and its analogues. It investigates the key components of the compared organisms/products that are most important for human health: nutrients and their antagonists; toxins, allergens, etc. [3, 5, 6]. Among the key nutrients, they are major (fats, proteins, carbohydrates) and minor (secondary metabolites). The nature and procedure of biochemical compositional analysis in assessing significant equivalence is carried out within the framework of special Consensus documents in relation to organisms most often used for genetic modifications, where groups of compounds that are necessary for the analysis of substantial equivalence are regulated [4, 5].

Taking these data into consideration, a comparative study of the biochemical composition of leaves of gene-knockout and unmodified cotton varieties was examined (Table 1). Initially, the content and composition of phospholipids was investigated. According to the results obtained, the fatty acid (FA) composition of different cotton genotypes is different (Table 1).

| Variety            | Saturated lipid content, % | Unsaturated lipid content, % |
|--------------------|---------------------------|------------------------------|
| Null segregant     | 23.86±0.64                | 76.14±1.34                   |
| Coker-312          | 31.57±0.95                | 68.43±0.82                   |
| T31-10             | 22.86±0.8                 | 77.14±1.6                    |
| T1-7               | 22.19±0.81                | 77.81±1.7                    |
| Namangan-77        | 25.13±0.93                | 74.87±1.58                   |
| Porloq-4           | 23.15±0.84                | 76.85±1.64                   |

Note: Lipid content is given in % from the total lipid content. p ≤ 0.01

The main FAs in all examined samples were palmitic acid (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3). These five FAs account for more than 95% of the total FA content in functional cotton leaves. At the same time, the FA composition in gene-knockout varietes
contained a higher proportion of unsaturated FAs (UFA) and a lower fraction of saturated FAs (SFA) compared to that in the control unmodified genotype Cocker-312.

Further, the content of carbohydrates in various cotton genotypes was investigated. As it is shown (Table 2), that the content of soluble sugars, sucrose, starch and the sucrose/starch ratio in stem leaves differ depending on the genotype under study and depends on the presence of a vector construct. At the same time, the RNAi cotton varieties have a higher content of soluble sugars and sucrose and, relatively, a higher value of the sucrose/starch ratio in comparison with the unmodified control variety Cocker-312 (Table 2).

**Table 2.** The content of carbohydrates in the leaves of modified and unmodified cotton varieties.

| Variety       | Content of water-soluble carbohydrates, mg/g dry weight | Content of sucrose, mg/g dry weight | Content of starch, mg/g dry weight |
|---------------|--------------------------------------------------------|------------------------------------|-----------------------------------|
| Null segregant| 59.7±1.6                                               | 34.2±1.4                           | 32.8±1.3                          |
| Cocker-312    | 45.4±1.2                                               | 22.8±1.1                           | 43.8±1.7                          |
| T31-10        | 72.2±2.1                                               | 44.8±1.7                           | 22.4±1.0                          |
| T1-7          | 68.9±1.9                                               | 41.6±1.6                           | 25.4±1.3                          |
| Namangan-77   | 53.7±1.5                                               | 30.5±1.3                           | 39.8±1.5                          |
| Porloq-4      | 62.1±1.8                                               | 35.7±1.5                           | 29.2±1.8                          |

**Note:** Carbohydrates content is given in mg/g dry weight. p ≤ 0.01

Then, the insertion effect of a gene knockout construct to the phytochrome A1 gene on the qualitative and quantitative composition of amino acids was examined (Table 3).

**Table 3.** The content of amino acids in the leaves of modified and unmodified cotton varieties.

| Amino acid      | Null segregant | Cocker-312 | T31-10 | T1-7 | Namangan-77 | Porloq-4 |
|-----------------|----------------|------------|--------|------|-------------|----------|
| Aspartic acid   | 89.51±8.5      | 141.97±10.1| 159.18±10.1| 170.87±10.1| 143.14±10.1| 160.87±10.1|
| Glutamic acid   | 91.68±8.8      | 96.29±8.7  | 100.51±8.7| 112.12±8.7| 97.30±8.7 | 102.12±8.7 |
| Serine          | 25.2±6.2       | 47.65±6.1  | 29.79±4.1| 40.96±5.1| 48.95±5.1| 30.96±5.1 |
| Glycine         | 11.64±2.8      | 13.11±2.9  | 14.76±1.9| 25.89±3.1| 14.56±1.9| 15.89±3.1 |
| Asparagine      | 11.82±2.3      | 13.67±1.9  | 14.86±1.9| 26.23±1.9| 15.12±1.9| 16.23±1.9 |
| Glutamine       | 137.95±10.7    | 179.33±11.8| 349.96±12.8| 360.98±12.8| 180.96±12.8| 350.98±12.8|
| Cysteine        | 108.48±9.1     | 45.70±5.8  | 110.04±5.8| 122.15±6.8| 47.20±6.7| 112.15±6.8 |
| Threonine       | 137.70±10.6    | 62.28±6.4  | 105.22±6.4| 116.68±6.4| 63.82±6.4| 106.68±6.4 |
| Arginine        | 82.24±8.1      | 33.80±4.8  | 68.40±4.8| 80.09±5.8| 35.01±5.8| 70.09±5.8 |
| Alanine         | 88.50±8.7      | 104.05±8.9| 146.41±8.9| 157.85±9.9| 105.84±9.9| 147.85±9.9 |
| Proline         | 49.03±6.7      | 22.18±3.2  | 64.11±4.2| 75.96±6.2| 24.12±6.2| 65.96±6.2 |
| Tyrosine        | 83.95±7.9      | 48.53±4.2  | 82.48±6.2| 93.86±7.2| 49.78±7.2| 83.86±7.2 |
| Valine          | 9.06±1.9       | 24.15±2.8  | 25.24±3.8| 36.79±3.8| 25.78±3.8| 26.79±3.8 |
| Methionine      | 0              | 8.07±1.9   | 5.79±1.4| 17.36±1.4| 9.12±1.4| 7.36±1.4 |
| Isoleucine      | 0              | 10.21±2.0  | 12.52±1.5| 23.79±1.5| 11.14±1.5| 13.79±1.5 |
| Leucine         | 0              | 37.53±2.9  | 38.92±3.9| 50.23±3.9| 39.28±3.9| 40.23±3.9 |
| Histidine       | 0              | 42.94±3.0  | 36.89±3.9| 48.46±3.0| 44.42±3.0| 38.46±3.0 |
| Tryptophan      | 51.49±6.8      | 35.27±2.8  | 46.46±3.8| 57.82±4.8| 36.79±4.8| 47.82±4.8 |
| Phenylalanine   | 28.72±3.9      | 12.94±2.0  | 13.79±1.5| 25.17±3.0| 14.25±3.0| 15.17±3.0 |
| Lysine HCl      | 15.12±2.1      | 59.62±4.3  | 58.70±4.3| 69.93±5.3| 60.98±5.3| 59.93±5.3 |
| Total           | 1022.14±70.1   | 1039.3±65.6| 1484.13±100.3| 1713.30±104.6| 1067.60±68.9| 1513.30±102.4|

**Note:** Amino acid content is given in μg/g raw weight. p ≤ 0.01

All cotton varieties differ in both qualitative and quantitative composition of free amino acids, and the total amino acid content in gene-knockout varieties was higher than in the original unmodified genotype Cocker-312. In gene-knockout cotton, alanine, glutamic and aspartic acids, as well as their congeners amides, glutamine and asparagine, account for more than half of the sum of all amino acids (Table 3). There was also an increase in gene-knockout varieties T31-10 and T1-7 (compared to Cocker-312).
312) in the content of such amino acids as aspartic acid (by 78%), glutamic acid, asparagine, glutamine, and alanine (Table 3). Thereby, in modified varieties, an almost threefold increase in the content of valine and an almost 4-fold increase in the content of lysine HCl were noted (Table 3). The effect of hairpin insertion could be the influence of methionine and histidine in the leaves of gene-knockout cotton, in addition to isoleucine and leucine. Methionine and histidine were detected in approximately the same concentrations in the leaves of parental varieties, but were absent (or present in undetectable amounts) in the leaves of the original Cocker-312 (Table 3). Regarding cysteine, threonine, arginine, tyrosine and tryptophan, their content in gene-knockout varieties is lower than in Cocker-312, but much more than in parental varieties (Table 3).

4. Conclusion
Thus, to sum up, it should be noted that the presence of an RNAi construct for the phytochrome A1 gene in the genome of gene-knockout plants does not negatively affect the qualitative and quantitative composition of amino acids in cotton leaves. Moreover, the determination of the content of the main macronutrients (proteins, amino acids, fats, and carbohydrates) in gene-knockout cotton varieties in comparison with the unmodified control Cocker-312 variety and the parent variety Namangan-77 showed that the RNAi varieties are equivalent in terms of essential characteristics. This allows to conclude that the insertion of the RNAi construct has no negative effects on biochemical parameters.

In this way, according to the main guidelines, the varieties, obtained with the RNAi gene of phytochrome A1, could be considered safe and do not require a thorough safety assessment since they are significantly equivalent in composition and nutritional properties compared to existing analogues with a safe history of application [2, 4, 5, 11].

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