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PhD thesis

FOOD SAFETY RELATED INVESTIGATIONS OF TRANSGENIC WHEAT (*Triticum aestivum* L.*)* TOLERANT TO TOTAL HERBICIDE

NAGY ANDRÁS

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1. INTRODUCTION

The purpose of producing genetically modified plants is to insert a gene responsible for some beneficial properties such as enhancing resistance, nutritional advantage or producing biologically active compounds by way of genetic engineering. Recently, the gene technological tools have undergone considerable development, their novel technological applications have raised a number of environmental, feed and food safety issues. The European Union requires the food safety assessment of genetically modified (GM) plants before market introduction, nevertheless that the post-market monitoring is compulsory for the approved traits to avoid unexpected risks for public health and environment on the bases of precautional principle, and also labelling of GM origin or contamination to promote consumers' free choice.

Conventional toxicological methodology cannot be applied for risk assessment of GM plants therefore international organisations have formulated recommendations. According to comparative safety assessment approach first of all it is necessary to identify the differences between the non-GM recipient plant and the GM plant by comparison of the main nutrients (Concept of Substantial Equivalence). Following there is need to assess the identified differences regarding the environmental, food safety (toxic, allergenic) and nutritional (short and long term exposure) impacts.

In the European Union comparative risk assessment is necessary before marketing a new GM plant, according to the EFSA guidance document. The key elements for such a safety assessment is the characterisation of the recipient plant (origin, history of safe use), the transgene donor (allergenic or toxic sequences), the transgenic technology (insertion of the donor gene and vector elements, characterization of the recombinant DNA), and the gene product (structure, origin, mode of action, toxicity, allergenicity). Beside these, the monitoring and surveillance of food safety parameters of the new GM plant are equally important (agronomic performance, composition of main nutrients, nutritional and safety analyses by using animal experiments), and their effect on the environment as well.

2. AIMS

- Comparative assessment of substantial equivalence of herbicide-tolerant spring wheat lines and non-GM control wheat to assess if there are substantial differences in nutritional composition as a result of GM technology, year of cultivation and herbicide treatment.

- Comparative study of allergenic proteins of herbicide-tolerant spring wheat lines and non-GM control wheat to assess, if GM technology, year of cultivation and herbicide treatment have caused such expected and unexpected changes on the levels of investigated proteins, which could increase the potential of allergenic risk.

- Investigation of the gut resistance of newly expressed gene product and selected marker proteins identified as potential allergens to explore the risks of resistant proteins getting into living organism.

- Comparative assessment of a selected spring wheat transgenic line and its non-transgenic counterpart used non-treated and heat treated in short term rat feeding trials to explore the risk if changes occurring on protein level have any harmful effect on protein utilisation.

- Suggestions based on my research experiences for promoting practical application of recommended methodical guidelines for food safety assessment of GM plants.

3. MATERIALS AND METHODS

3.1. Wheat samples

Six transgenic wheat lines (T106, T116, T117, T124, T128, T129) and the non-transgenic control *Triticum aestivum* L. spring wheat (CY-45) grown in experimental field in two subsequent years were used for the experiments. Experiments were extended to samples treated with total herbicide (Finale 14 SL; F) and conventional herbicide (Granstar; G) registered to wheat as well as to non-treated (weed removal, Ø) samples. The herbicide tolerant transgenic wheat lines were developed by the Cereal Research Non-Profit Company Ltd. (Szeged) using PDS-1000/He gene gun device. For the transformation pAHC20 plasmid molecule was used. Six independent transgenic wheat lines were reproduced on the bases of parental bread wheat in five green house cycles (generations). The wheat lines were homozygote for the inserted gene, in which the transgene was inherited stably. The transgenic wheat containing bacterial-derived (*Streptomyces hygroscopicus) bar* gene is regulated under the maize ubiquitin promoter. The gene in addition to conferring Finale resistance to wheat acts as a marker gene allowing identification of the GM plant by (PAT^+) selection.

3.2 Comparative investigation of non-transgenic control and transgenic wheat lines

- PCR technique was applied for detecting the presence of *bar* gene and sandwich ELISA for verifying PAT protein expression.
- Substantial equivalence was examined by determination of dry matter, crude protein, crude fibre, crude fat and ash content by standard methods.
- Changes in marker proteins were investigated in fractioned wheat proteins according to OSBORNE. After co-extracting albumin-globulin fraction (AGF) major wheat allergens (WGA, α –amylase inhibitor, serpin and amylases) were investigated. From the alcohol soluble fraction gliadins were investigated for wheat allergy and gluten sensitivity.
- For quantitative determination of wheat allergens WGA was used as a marker to which I have developed WGA-specific ELISA test. For quantitative determination of allergens responsible for celiac disease gliadin-specific ELISA was adapted.
- Changes occurring in protein fractions were monitored by SDS-PAGE and A-PAGE methods. To identify potential allergens human hyperimmune serum verified for clinical symptoms of wheat allergy and celiac disease were used for immunoblotting. For semi-quantitative determination of amylase inhibitors native PAGE was used carried out after protein separation by enzyme staining. Determination of amylases was carried out spectrophotometrically based on enzyme activity.

- For *in vitro* modelling of gut resistance of chosen marker proteins (PAT, WGA) I have developed a membrane-based digestion test, where the immunoreactive proteins resistant to pepsin digestion blotted on the membrane were identified by specific antibody after SDS-PAGE protein separation.
- For *in vivo* study of gut resistance an acute rat model was adopted, where the surviving immunoreactive proteins in biological samples were determined by PAT specific sandwich ELISA or WGA specific competitive ELISA.
- The *in vitro* nutritional value of the raw materials was characterised by Chemical Score (CS) which was calculated as the ratio of essential amino acid composition of the studied protein to a reference protein and expressed in percentage and by their relevant limiting amino acids. For amino acid determination ion exchange chromatography was applied.
- The short term effect of transgenic wheat line (T128) selected by safety issues was determined in short term rat feeding experiments, and was characterised by the body weight index (NPR) related to unit of protein intake and by nutritional indices of the protein utilisation (TD, NPU, BV) based on nitrogen balance.

4. RESULTS

- The *bar* gene insertion was detected by *bar* gene specific PCR method and the PAT protein expression was proved by PAT-ELISA, thereby verifying gene modification in all transgenic wheat lines.
- Concerning the composition of the main nutrients I found that the transgenic wheat lines were characterised by high variability, nevertheless they showed significantly higher protein contents in most of the cases in comparison to control wheat.
- I investigated if there was any meaningful difference in the main wheat allergenic profile concerning the albumin/globulin protein fractions (AGF) of wheat samples as WGA, α-amylase inhibitor, serpin and amylases. I did not found any difference in the protein bands, which might be related to any newly expressed protein after the electrophoretic separation of AGF. It was detected a significant increase in the immune reactive WGA contents, and a relative increase in the α-amylase inhibitor activities for most of the transgenic wheat lines. This fact could be beneficial for resistance breeding. However, concerning food safety aspects further investigations are necessary to define if there is any allergenic risk depending on the technological treatments and dietary exposure levels. The changes in total amylase activity were mostly attributable to changes in β-amylase activities. These activities showed significantly higher values for the non-treated transgenic wheat lines in the first year, however, this difference could not be detected in the samples with significantly increased total amylase activities due to year and herbicide treatments effects. I did not find any significant differences in the profile of gliadin allergens in the prolamin fraction.
- By investigating the gut resistance of the newly expressed PAT protein and chosen WGA marker proteins, I concluded that the PAT protein was fully digested in the rat gut, therefore did not represent any further allergenic risk for the organism. At the same time the immune reactive WGA was only partially digested in the rat gut and could be detected bound to the rat gut mucosal surface. Although the variability in individual responses of rats was high, the survival of WGA antigen can represent a potential allergenic risk for the organism.
- The series of short term rat feeding trials with parent wheat and the non-treated transgenic wheat line (T128Ø) selected by the food safety assessment aspects have shown that changes in wheat proteins did not reach the level to have any affect on the protein utilisation.

5. NEW SCIENTIFIC RESULTS

1. I was the first who carried out comparative assessment of the Hungarian engineered transgenic wheat lines carrying ppt tolerance and the non-transgenic control CY-45 spring wheat for substantial equivalence. I concluded that in certain transgenic wheat lines the protein content related to dry matter significantly increased (2001: T106G-F, T116G-F, T117G-F; 2002: T116Ø-F, T117Ø-F, T124Ø-F, T129F).

2. From the results of the comparative assessment of allergenic proteins of transgenic wheat lines carrying ppt-tolerance and non-transgenic control CY-45 spring wheat I concluded that substantial changes only occurred in AGF proteins, whereas in gliadins there were no significant changes.

3. In most of the transgenic wheat lines (2001: T116Ø-G-F, T117Ø-G-F, T124Ø-G-F, T128Ø-G-F, T129Ø-G-F; 2002: T106G, T117G-F, T124G T129Ø-G-F) I found significant increase in WGA content, and on the other hand GM lines showed high variability when compared to each other.

4. In case of α-amylase inhibitors, I detected higher level of enzyme activity in the examined lines (T128Ø-G-F, T129Ø-G-F), which was further increased by herbicide treatment. Although these changes can increase resistance of GM lines against pathogens, they may increase the potential of allergenic risk.

5. I adapted an acute rat model for gut resistance of newly expressed gene product (PAT) in GM lines and the selected marker protein (WGA) identified as potential wheat allergen From studying gut resistance I concluded that the PAT protein was digested in the gut, whereas it cannot be excluded that WGA lectin which was specifically bound to the intestinal epithelium could represent a potential risk of getting into the organism.

6. For *in vivo* investigation of WGA in rat gut I applied specific competitive indirect ELISA, and for *in vitro* investigation I developed an immunoblotting method preceded by membrane bound pepsin digestion.

7. From the comparative assessment of T-128Ø transgenic wheat line selected by food safety aspects and non-transgenic CY-45Ø control spring wheat based on the short term feeding trials I concluded that changes occurred at protein level did not reach the level to affect protein utilisation.

6. CONCLUSIONS AND SUGGESTIONS

- The high variability within GM lines caused difficulties in statistical evaluation of my research results, which can be attributed to the effect of transformation technology (e.g. random integrations, somaclonal variability during plant regeneration) and environmental effects.
  The transgene needs to be characterised in more detail on molecular level, thus besides expected effects unexpected effects could be predicted as well.

- The expression level of PAT enzyme is very low compared top plant protein, therefore I purified albumin/globulin fractions rich in gene products and other biologically active proteins for protein investigations.
  If risk assessment of gene product is to be carried out using recombinant proteins, then it is necessary to insure homologous structure to which application of conventional methods are needed. For comparative characterisation of potentially unsafe proteins of difference, utilisation of omix techniques (e.g. proteomics) and development of databases are necessary.

- PAT enzyme of bacterial origin does not show sequence homology to any known allergens in the databases, thus to determine functional homology random serum tests should be carried out. In reality, however, it cannot be executed, since there is no available hyperimmune serum specific to the given bacterium and hyperimmune serum from group of patients consuming GM protein in a controlled way, showing allergic clinical symptoms.
  To predict allergenic risk by functional homology development of such physiological markers are needed, which can be used for monitoring of authorised GM plant after marketing and after verifying clinical symptoms for collecting such hyperimmune sera in a sera bank.

- Since functional homology studies are not executable, examining gut resistance was necessary. For examinations structural identity of proteins (recombinant and purified) should be maintained. The WGA studied, due to its specific sugar-binding capacity, can influence the immune response and metabolism of the gut by entering into direct contact with the gut. However, the animals' individual responsiveness showed such variability, which made certified validation of biological relevance impossible.

Besides the in vitro examination of lectins with specific sugar-binding capacity (e.g. WGA) by pepsin digestion, I consider it important to test them in acute rat models. From the viewpoint of biological relevance of results from animal models, development of appropriate predictive statistical models is needed, which can account for the variability arising from individual responsiveness.

- As I experienced differences on protein level, animal trials was used for testing short term biological effect. Under the given experimental circumstances, changes occurring in protein level did not cause detectable biological effect. Considering Hungarian eating habits, it can be assumed that wheat transformed by *bar* gene does not generate risk for consumer in the short term.

It would be practical to develop planning of animal models (number of animals, composition of diet, critical dose of active ingredients, etc.) into a standard protocol.

PUBLICATIONS RELEVANT TO THE SUBJECT OF THE DISSERTATION

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