Transgenic approach to improve the nutritional quality of wheat (*Triticum aestivum* L.)

Ph.D. thesis

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the most important food crops for about half of the human population forming a staple diet in over 60 countries. Wheat whole grain contains several important vitamins, minerals, high amount of fiber, complex carbohydrates and substantial amount of proteins. The nutritional quality of the wheat is mainly determined by the low essential amino acid content of the storage proteins. Especially the arginine, hystidine, lysine and threonine are present in limiting amounts in the grain. The white flour contains even less lysine and threonine as a result of the milling process. With the removal of the aleurone and embryo tissues of the grain, high amounts of essential amino acids (4.8% and 8.3% lysine, respectively) are lost. Children require more essential amino acids than adults for their growth and development, and feed formulated for livestock also need proper protein composition.

Several attempts have been made to improve the amino acid contents, for example lysine content, of important crops by conventional breeding and mutagenesis. Recently, the achievement of wheat transformation techniques (Vasil et al., 1992) has opened a new approach to improve agronomic traits of transgenic crops, as well as the grain composition and quality. The aim of this work was to improve the essential amino acid content of the wheat flour via gene technology approach to express recombinant storage protein with the appropriate amino acid profiles in the wheat endosperm tissue.

It has been well known for several decades that the grain of the pseudo cereal *Amaranthus hypochondriacus* has not only high protein content (17-19% of seed dry weight), but its storage proteins are rich in essential amino acids such as lysine (5.0%, more than twice that of wheat), threonine (2.9%), tyrosine (~3.4%), and sulphur-containing amino acids (4.4%). One particular albumin type storage protein, called AmA1 was chosen to be expressed in the wheat endosperm, because its essential amino acid content is comparable to values recommended by the World Health Organization for a highly nutritional protein (FAO/WHO 1991).

The *ama*1 gene encoding the 304-amino acid polypeptide (Raina and Datta, 1992) was introduced into the wheat genome with biolistic transformation. The full-length cDNA of this 35 kDa protein has already been successfully expressed in potato tubers. To the best of our knowledge no report has been published so far on the successful expression of a protein with increased essential amino acid content in transgenic wheat.

OBJECTIVES

The main objective of this work was to improve the nutritional quality of wheat flour by enhancing the essential amino acids content of the endosperm tissue. In order to establish a transgenic line with this new property, the *ama*1 gene, driven by a powerful wheat endosperm-specific promoter (1Bx17 HMW-GS), was expressed in the wheat endosperm.
Detailed analyses were also carried out to detect the changes, if any, in the functional quality of the flour, influenced by the new protein composition. The expression of the pseudo cereal albumin storage protein AmA1 may play positive role on the end-use properties of the wheat flour, due to its interaction with the wheat storage proteins.

To achieve our goals we proposed:

1A. To establish efficient tissue culture method for plant regeneration from immature wheat embryos.
1B. To optimise tissue culture conditions for biolistic transformation.
2. To express an amaranth (*A. hypochondriacus*) albumin storage protein (AmA1) gene in the endosperm of transgenic wheat.
3. To confirm transgene expression at both nucleic acid and protein levels.
4. To investigate transgene inheritance in T_1, T_2 and T_3 generations.
5. To confirm tissue-specificity and proper functionality of the Bx17 HMW-GS promoter.
6. To analyse and quantify the effects of the foreign protein on the properties of the flour produced from T_2, T_3 and T_4 seeds.

MATERIALS AND METHODS

The tissue culture response of immature embryos from Hungarian winter wheat varieties 'Mv Emese', 'Mv Magvas', 'Mv Martina', 'Mv Pálma', 'Mv Emma' and three lines (B2, B8, B70) of 'Bánkúti 1201' were tested using standard MS media and culture conditions (Tamas et al., 1999). To improve plant regeneration frequency modified tissue culture conditions were applied: reduced incubation temperature (26±1 ˚C to 23±1 ˚C), reduced concentration of MS macroelements to half strenght, low and high light intensity. To optimise the method for biolistic transformation of the best performing Mv Emese and two spring wheat varieties (Bobwhite, Cadenza) embriogenesis inducing tissue culture conditions were used: preparing immature scutella (12-14 DPA) as explant source, short tissue culture phases, no glycin but $AgNO_3$ in the callus induction medium, $CuSO_4$ in the shoot regeneration medium (Sparks and Jones, 2004; Tamas et al., 2004).

In orderto establish stable transgenic wheat lines biolistic transformation method was carried out using the plasmid pAHC25, containing the selectable marker (*bar*) and the reporter gene (*uid*A) (Christensen and Quail 1996), was co-bombarded with the transformation cassette, called AmA1-pTLZ, carrying the *ama*1 gene. The transgene expression was driven by the endosperm specific 1Bx17 HMW-GS promoter. Equimolar amounts of plasmids precipitated onto gold particles were co-bombarded into two-day precultured scutella (Sparks and Jones 2004), using the PDS-1000 particle device (Bio-Rad). Selection and regeneration of the transformed cells were achieved using 4 mg/l L-phosphinotricine in the shoot- and root regeneration medium (Sparks and Jones 2004; Tamas et al. 2004).

Total genomic DNA isolated from leaf tissues, using the AquaGenomics procedure (MultiTarget Pharmaceuticals), was analysed by PCR for the presence of the marker (*bar*)-, reporter (*uid*A)- and the transgene (*ama*1) with the use of gene specific primers. To confirm the stable integration of the gene of interest in T_1 progenies of two transformant lines (28 and 58), plant genomic DNA, isolated from leaf tissues by CTAB method, was analysed by Southern-blot method. Transcription of the *ama*1 gene in the wheat endosperm was screened by RT-PCR in all primary transformants. To measure the amount of mRNA from developing endosperm (17, 24 and 31 DPA) of the T_3 seeds, Quantitative RT-PCR analysis was carried out following the method described by Terzi et al. (2005).

The *bar* and *uid*A gene activity was tested by herbicide resistance- and histochemical GUS assays in T_1 and T_2 generation, respectively. To monitor the proper function of the

tissue-specific promoter of the wheat storage protein, Western-blot analysis was performed on proteins purified from the developing endosperm (17, 24, 31 DPA) of the T_2 seeds of line 28.

The hardness index of mature T_2, T_3 and T_4 seeds from the *ama*1 PCR positive plants of transgenic lines was measured by Perten SKCS 4100 equipment. The meal and the bulked seeds were milled on laboratory Mini-mill then sieved through micro-sieves (METEFEM Ltd.). Total protein content of the flour samples was measured by the Kjeldahl method. To quantify the foreign AmA1 protein, expressed in the transgenic wheat endosperm, indirect ELISA was performed (Kang et al., 2003). The essential amino acid composition of the flour was measured following the protocol of Adebowale et al. (2007).

SE-HPLC (size exclusion high performance liquid chromatography) was used to determine the glutenin-to-gliadin ratio of the flour using the method of Batey et al. (1991). The amount of unextractable polymeric protein (UPP%) was determined according to Gupta and MacRitchie (1994). A modified RP-HPLC method (Marchylo et al. 1989) was carried out to determine the relative amounts of the HMW and LMW glutenin subunits. Analysis of variance (ANOVA) was performed on the mean values of HPLC data using the Statistica 7.0 program (StatSoft, Inc. 2006, USA).

Sedimentation value of the flour samples was measured with the use of the automated Sedicom System (Lab-Intern Ltd.), following the micro-scale method of Tömösközi et al. (2005).

The reological properties of the dough from the T_3 and T_4 transgenic flour samples were measured by micro z-arm mixer as described by Tömösközi et al. (2000) and Haraszi et al. (2004).

ACHIEVEMENTS

- An efficient tissue culture method was established for plant regeneration using immature embryos from the following wheat varieties (Mv Emese, Mv Magvas, Mv Martina, Mv Pálma, Mv Emma, Bánkúti 1201/B2, /B8, /B70).
- The optimised tissue culture conditions provided high plant regeneration frequency in all varieties investigated.
- The method was successfully optimised for biolistic transformation of the Hungarian variety Mv Emese, and two spring wheat varieties (Bobwhite, Cadenza) with the use of embriogenesis inducing tissue culture parameters.
- To the best of our knowledge this is the first report on successful expression of a foreign protein (AmA1 from *A. hypochondriacus*) with increased essential amino acid content in transgenic wheat endosperm.
- The amount of the accumulated AmA1 protein in transgenic wheat endosperm showed a close correlation with the increase in the essential amino acid contents (7.3% Lys, 4.0% Thr, 3.8% Tyr).
- The polymeric-to-monomeric (Glu/Gli) protein ratio and the size distribution of the polymeric proteins (UPP%) in the flour were found to be significantly higher in most of the transgenic lines compared to the control samples.
- This is the first report on successful expression of a non-wheat storage protein (AmA1) that had positive effect on functional properties and end-use quality of the transgenic flour.
- Most of the reological parameters of the doughs, produced in the micro z-arm mixer, were significantly higher, such as dough development time, peak resistance and dough stability. These data prove that stronger and more stable dough can be produced from these transgenic flour samples.

CONCLUSIONS

- Efficient tissue culture method can be established for plant regeneration from immature embryos of different wheat varieties by reducing both the incubation temperature to 23±1 °C and the concentration of macroelements in the regeneration medium to half strength. This modification significantly increased the average plant regeneration frequency (from 10% to 78%). Changes in the light intensity and the temperature raised the average frequency to 83%. The method is suitable to screen the regeneration capacity of other elite Hungarian wheat varieties.
- The optimised plant regeneration method from immature embryo derived calli, opened the way for successful biolistic transformation of either winter (Mv Emese), or spring wheat varieties (Bobwhite, Cadenza).
- The transgene (*ama*1) was stably integrated into the wheat genom and was transmitted to the next three generations. The transcription pattern of the foreign gene showed tissue specificity, which is in good agreement with the property of the 1Bx17 HMW-GS promoter.
- The amount of the *in vivo* expressed AmA1 protein showed close correlation with the increase in the essential amino acids content of the transgenic flour, while the total protein content had not changed significantly.
- The biolistic method of gene technology is suitable to produce transgenic wheat varieties with improved nutritional quality if foreign proteins, with appropriate essential aminoacid composition are expressed in the endosperm tissues.
- Our results indicate that the transgenic approach can be a method of choice to improve not only the nutritional quality of the wheat flour, but certain functional quality attributes in parallel, if the proper protein gene is introduced into the wheat genom.

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