Breeding for a changing world and genetic modification of photosynthesis in wheat

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INTRODUCTION

Breeding for a changing world should be transformed from an extensive to an intensive stage which aims are changed from enhancement of ear size, harvest index, number of functional units in size and volume – into optimized function of productive processes (e.g. the complex of plant metabolic processes which results in productivity). Photosynthesis is the most important metabolic process relative to crop productivity, as it contributes to about 85% of the dry matter accumulated in the main cereal crops, such as wheat and rice. Genetic modification of photosynthesis in plants for increased photosynthetic capacity is a difficult goal but this is the only way for crop improvement in the near future. Green Revolution through conventional breeding during the 20th century has produced a vast number of crop varieties with higher grain yields and had won a temporary success in man's war against hunger (1). However, to meet the needs of feeding the 8.3 billion people projected to be on this planet at the end of this quarter century, both conventional technology and genetic improvement of food crops by application of recombinant DNA technologies will be required (1-4).

Recent advances in new technology greatly facilitate the analyses of the consequences of transgenic or genetic alterations on the expression of thousands or tens of thousands of genes simultaneously. Analogous analytical methods for cataloging the global effects of metabolic engineering on metabolites, enzyme activities and fluxes are also revealing. Methods for stable successful plant transformation via bombardment, *Agrobacterium*, pollen transformation almost elaborated. Introduction of genes encoding C_4 photosynthesis enzymes into C_3 crops (e.g. rice) has been successfully completed. Transgenic rice expressing maize C_4 photosynthesis enzymes exhibited a higher photosynthetic capacity, better adaptation to stress conditions and a higher grain yield (3). Now it's time to discuss extension of genetic modification of photosynthesis in wheat, another important C_3 cereal crop.
Wheat, one of three most important cereal crops (corn, rice, wheat) in the world, is the main food crop cultivated in Kazakhstan and biotechnology provides a great promise for its improvement. Kazakhstan has very similar climatic conditions with Australia, including problems of global climate change, elevation of CO_2 in atmosphere, and increased droughtness. Breeding important species such as wheat and rice utilizing the conventional C_3 pathway for carbon fixation suffer from O_2 inhibition of photosynthesis and the associated photorespiration and exhibit a lower photosynthetic efficiency, especially under high light, high temperature and drought conditions. Photosynthesis is more efficient in C_4 plants, which have special features that allow them to capture extra CO_2. Current atmospheric CO_2 levels (0.036%) limit photosynthesis in C_3 plants. Furthermore, photorespiration reduces net carbon gain and productivity of C_3 plants by as much as 40%. No closely related C_3 and C_4 crops can be hybridized by conventional cross-hybridization approach because of epistatic interaction between the alleles suppresses the expression of C_4 traits in the progeny, and genes for Kranz leaf anatomy and biochemistry of C_4 photosynthesis are not closely linked. Genetic modification of wheat photosynthesis for increased yield by application of DNA transformation technologies could be considered as a first step to wheat crop improvement in modern breeding. With the advancement in molecular biology, recombinant DNA technology has been used to introduce some of the C_4 features into C_3 crops.

The main objective of our research is to investigate the advances in transformation technologies and thus establish approaches for genetic modification of photosynthesis in wheat for increasing drought resistance and grain yield up to 30% through introduction of maize genes encoding the C_4 photosynthesis enzymes into wheat. The ultimate goal of this work is to produce new strains of wheat with enhanced photosynthetic capacity, drought tolerance, and grain yield for breeding for a changing world.

MATERIALS AND METHODS

5 varieties of commercial USA spring and 25 Kazakhstan spring and winter wheat varieties have been used as wild types to produce 2176 putative transgenic seeds with zeatin O-glucosyltransferase (ZOG1) gene (R. Martin, OSU, USA) and 1927 putative transgenic seeds with phosphoenolpyruvate carboxylase (PEPC) gene (3). Transgenes have been obtained by our patented simple natural effective genotype independent method of wheat germ-line transformation by *Agrobacterium* Pipetting into the spikelets of wheat before anthesis. Method uses wheat indirect pollen system containing high quantities of flavonol glycosides which acts as inducers of *vir* region of the Ti plasmid. Elaborated method is very similar to wheat hand hybridization, economic and does not require expensive complicated tissue culture step. 2-steps antibiotic screening technique has also been revealed, used and patented (4). Totally have been produced transgenic wheat seeds of 30 genotypes, and have been created number of transgenic wheat plants of T_1 – T_3 generations. 22 wheat transgenic lines of T_1 and T_2 progenies were included in physiology-biochemical, leaf anatomical and yield study.

Stable wheat transformation in T_2 has been confirmed by molecular biological techniques: polymerase chain reac-

tion (PCR), and polymerase chain reaction in real time (Real-time PCR). DNA extracted from leaves of untransformed wild plants and antibiotic - resistant putative transgenic lines was used for amplification with primers to the *kanamycin phosphotransferase* II (*ntpII)* (478 bp) *and hygromycin phosphotransferase (hptII)* (400 bp) selectable marker genes included into constructs of PEPC gene. Primers to *nptII* gene – 225-2 – AAGCACGAGGAAGCGGTCAG (S) and 224-1 CGACGTTGTCACTGAAGCG (AS). Primers to *hptII* gene – GCTGCGCCGATGGTTTCTACA, GCCCAAA-GCATCAGCTCATCG, molecule region from 155 to 554 bp. Analysis of PCR products has been provided by electrophoresis in agarose gels method using DNA molecular mass markers 1kb or 100 bp (Fermentas). Real-time PCR analysis has been provided by the test-system "Genetic modification organisms (GMO) - screening" with equipment «ANK-32, GM-409-2», Syntol, Russia by the program «GMO - screening». Detection of transgenic plants has been done by the identification of 35S promoter and NOS-terminator using FAM and ROX targets probes consequently.

High level of the maize C_4-specific PEPC gene expression in transgenic wheat plants was determined by assaying the activity of PEPC in leaf protein extract, followed by CO_2 gas-exchange and photorespiration measurements, leaf anatomy investigation, yield structure. PEPC activity was assayed by PEP decreasing spectrophotometrically at absorbance 340 nm according to the method described in (3). Enzyme activities were expressed on chlorophyll basis. Chlorophyll concentration was determined after extraction in 96% ethanol. Photosynthetic rates and stomata conductance of attached flag leaves were measured at 30 °C, 340 µmol/mol CO_2, 21% O_2 using a LI 6200 portable photosynthetic system (Li-cor, Lincoln, Nebraska) according to the method (3,4). Wheat transgenic lines in comparison with control wild type and maize were used for investigation of leaf anatomy structure by methods and analysis of C_4 Kranz anatomy features appearance (2). Microscopic analysis and morphometric measurements of leaf anatomical structure were provided using light microscope ZEISS AXIOSCOP 40 HBO100 combined with computer, Zoom Brouser EX software, camera Canon PC 1145, Canon A 610/A 620, total zoom – 150; and fluorescent microscope Hitachi H-600 (Hitachi Scientific Instruments, Nissei Sangyo America, USA) with silver coloring. 419 putative transgenic seeds of 21 transgenic lines have been investigated by harvest structure elements: plant height, the main/lateral tillers length, number of tillering/ears, number of spikelets in spike, main steam/ lateral, number of grains per ear, grain mass per 1 ear of the main steam/lateral, grain yield from 1 plant, transgenic effect % (4).

RESULTS

PCR confirmed the presence of the *nptII* selectable gene in 20 of 84 putative antibiotic resistant transgenic plants (Fig. 1). Transformation frequency by PCR analysis was 23.8% from antibiotic resistant plants and 2.3% from all number of putative transgenic seeds obtained by *Agrobacterium* Pipetting germ-line transformation technique.

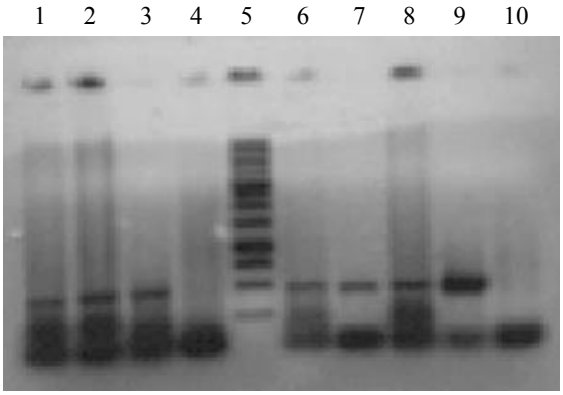

Fig. 1. PCR profiles of antibiotic-resistant wheat plants of T_1 – T_2 progenies obtained with primers 224/225 to the *ntpII* gene. (*1–4*) and (*6–8*) transgenic wheat plants; (*5*) DNA markers; (*9*) positive control (plasmid pBI 121); (*10*) negative control.

Identification of transgenic lines has been provided also by Real-time PCR technique. DNA of 22 transgenic plants confirmed by PCR, has been analyzed by RT-PCR. Genetic transformation of 20 wheat lines of first and second generations T_1 and T_2 has been proved by identification of regulatory elements of PEPC gene: NOS-terminator and 35 S-promoter.

The activity of PEPC was low in control untransformed wild type wheat cultivars. In contrast, the activities of PEPC in transgenic plants were about 2-6 fold higher than that in control wheat plants reaching 5.47 µm PEP / min · mg chl, a concomitant increase in chlorophyll amount (Table 1).

#, variety	µm chl / 40 µl ext	µm PEP / min · mg Chl.
Houis 4 (13)	7.39	**2.07**
Houis 5 (14)	9.81	**2.15**
Houis 6 (15)	**5.97**	**2.86**
Control (Houis 21)	*9.67*	*1.43*
***Wawawai* 14 (39)**	**7.92**	**2.31**
***Wawawai* 18**	**4.19**	**2.59**
***Wawawai* 19**	**5.13**	**2.90**
Max *Wawawai* 20	**7.15**	**4.38**
***Wawawai* 21**	**4.29**	**3.56**
***Wawawai* 22**	**4.06**	**3.56**
Max *Wawawai* 24	**3.17**	**4.44**
Max *Wawawai* 26	**3.87**	**5.47**
Max *Wawawai* 29	**1.06**	**4.93**
***Wawawai* 41**	**3.17**	**2.16**
Control (Wawawai 48)	*7.60*	*0.90*

Table 1. PEPC assay of putative transgenic wheat plants screened on kanamycin and hygromycin resistance

A wide range of PEPC activity was detected among the transgenic plants, some of them had high level of activity. Screening on PEPC assay has identified 18 plants with high PEPC activity from 71 screened putative transgenic wheat plants. 6 lines showed 4-6 fold increase in PEPC activity in comparison with non-transgenic control. Results of biochemical screening by PEPC assay on expression of protein of transferred PEPC gene were similar to results of PCR-analysis.

The photosynthetic characteristics of PEPC transgenic plants were analyzed in details. Transgenic plants exhibited higher light-saturated photosynthetic rates (40%), higher stomata conductance (29%) at 1200 µmol photon m^{-2} s^{-1} and higher carboxylation efficiency than untransformed wild type (WT) and photosynthetic rates got up to 24 µmol CO2 / m-2 s-1 and stomata conductance – 400 mol / sm^{-2} s^{-1} (Fig. 2). Most PEPC transgenic wheat plants exhibited an enhanced photosynthetic capacity: increasing photosynthetic rate up to 17% and increasing stomatal conductance up to 25%.

On the other hand, the photosynthetic CO_2 compensation points were slightly lower in the PC transgenic plants (data is not represented), indicating a stronger capability of the plants to assimilate carbon under limited CO_2 conditions. Taken together, these results suggest that the high PEPC coupled with enhanced stomata conductance confers the transgenic plants a higher capability to assimilate atmospheric CO_2. However, the exact mechanism for the superior photosynthetic performance of these plants remains to be determined.

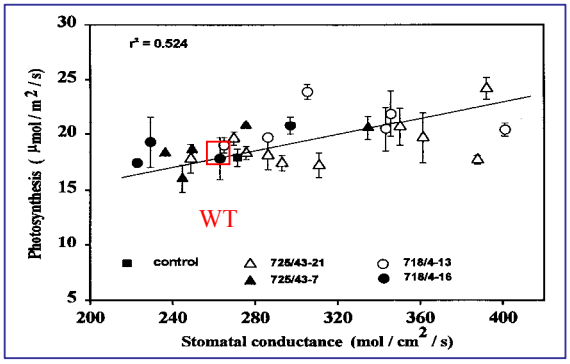

Fig. 2. CO_2 assimilation rates versus stomata conductance in wheat transgenic lines and wild type (WT).

Leaf anatomy in transgenic wheat plants with introduced PEPC – gene encoded phosphoenolpyruvate carboxylase from maize has been determined (Fig. 3).

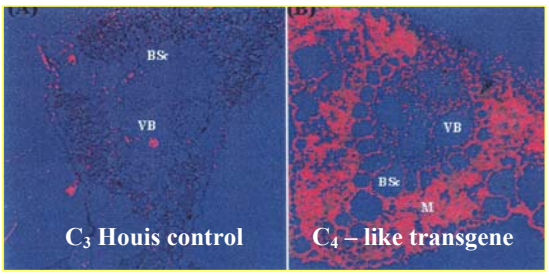

Fig. 3. Proper anatomy organization followed by maize PEPC gene expression in wheat transgenic leaves

In comparison with control, transgenes have obtained some features of C4 maize Kranz anatomy: chloroplasts have cell wall location; bundle shift cells have rolling shape and start to organize circle structure similar to C4 maize (Kranz); mesophyll cells organize circle around bundle shift cells and conducting bundles. Stable wheat transformation has been confirmed by grain yield increasing up to 25-50% in transgenic plants in comparison with wild types.

DISCUSSION

All enzymes involved in C_4 photosynthesis are found in leaves of C_3 plants. As in C_4 plants, they catalyze similar reactions in C_3 leaves, but do not contribute significantly to overall CO_2 assimilation. Introducing of the key enzymes of C_4 photosynthesis into C_3 wheat with proper leaf anatomy organization a limited C_4 acid metabolism may be installed for fixing atmospheric CO_2 directly via this pathway and partially concentrating CO_2 in the chloroplast.

CONCLUSIONS

Genetic modification of photosynthesis could be successfully provided in wheat by introduction of genes encoded C_4 enzymes via elaborated *Agrobacterium* Pipetting technique with transformation frequency 2.3%.

The maize C_4 genes can be efficiently expressed in the C_3 wheat. Transgenic wheat expressing maize C_4 acid metabolism enzymes exhibit increased photosynthetic capacit features of Kranz leaf anatomy and enhancement of grain yield up to 25-50%.

Totally have been produced and screened about 5000 wheat seeds of Kazakhstan's and commercial USA's varieties and have been created 20 transgenic wheat lines with enhance photosynthetic capacity and yield.

Genetic improvement of wheat photosynthesis for increased yield and drought resistance in wheat by application of recombinant DNA technologies could be considered as a first step to intensification of breeding for a changing world.

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Research was supported by: W. Fulbright Program, USDS USA; Scientific – Technical Program and Program of Fundamental Research of Republic Kazakhstan.