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# Photosynthetic Basis for Wheat Crop Improvement: Genetic Modification of Photosynthesis

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## ABSTRACT

A simple and effective genotype-independent method of wheat germ-line transformation by *Agrobacterium* pipetting into the spikelets of wheat before anthesis has being elaborated and patented. Using this method about 5000 transgenic wheat seeds of 20 varieties genotypes were produced, and a number of transgenic wheat plants of $T_1$-$T_3$ generations ($T_1$-$T_3$ – transgenic plants of first, second and third generations) were created. A 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, investigation of leaf anatomy, yield structure, PCR, real-time PCR and Southern blot analyses. Stable wheat transformation in the $T_2$ generation was confirmed by molecular techniques and high grain yield increased up to 25-50% in transgenic plants in comparison with wild types.

**Keywords:** increasing yield, photosynthesis, transformation, wheat improvement
**Abbreviations:** **$C_4$ photosynthesis**, photosynthesis type producing $C_4$ carbon acids; **$C_4$**, tropical plants with $C_4$-type of carbon metabolism (maize, sorghum, etc.); **$C_4$ wheat**, wheat with introduced genes encoding $C_4$ enzymes; **$C_3$ photosynthesis**, photosynthesis type producing $C_3$ carbon acids; **$C_3$**, terrestrial plants with $C_3$ type of carbon metabolism (rice, wheat, legumes); **$CO_2$**, carbon dioxide; **OPWPT**, optimal photosynthetic wheat plant type; **PEPC, PPDK, NADP-ME**, $C_4$ enzymes: phosphoenolpyruvate carboxylase, orthophosphate pyruvate dikinase, NADP-malic enzyme; **$T_1$-$T_3$**, transgenic plants of first, second and third generations

## INTRODUCTION

To meet the needs of feeding an increasing population on our 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. The most important metabolic process relative to crop productivity is photosynthesis, as it contributes to about 85% of the dry matter accumulated in cereal crops (Evans 1988; Edwards 2001), such as wheat and rice. Manipulation of photosynthesis in plants for increased photosynthetic capacity is a difficult goal but is the only way for crop improvement in the near future. Genetic modification of wheat photosynthesis for increased yield by application of DNA transformation technologies could be considered as a necessary step for wheat crop improvement in modern breeding. The advent of systems biology in combination with recent advances in genomic, metabolomic and phenomic technologies may now provide the means to engineer $C_4$ photosynthesis ($C_4$ photosynthesis – photosynthesis type, produced $C_4$ carbon acids) into $C_3$ plants ($C_3$ – terrestrial plants with $C_3$ type of carbon metabolism (rice, wheat, legumes) (Ruan and Teixeira da Silva 2010). An international project to engineer $C_4$ photosynthesis ($C_4$ photosynthesis – photosynthesis type, produced $C_4$ carbon acids) into rice has already been developed (Ku *et al*. 1999, 2001) while efforts to engineer $C_4$ wheat (wheat with introduced genes encode $C_3$ enzymes) are under discussion. The ultimate objective of our research was to improve the photosynthetic efficiency of wheat, which assimilates atmospheric $CO_2$ via the $C_3$ pathway of photosynthesis, by introducing some of the genes encoding features associated with $C_4$ photosynthesis.

At present wheat breeding should be transformed from an extensive to an intensive stage whose aims have 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).

In the 12 years since commercialization of the first genetically modified (GM) crop in 1996, farmers have planted more than 690 million ha (1.7 billion acres; James 2007). The first generation of biotech crops focused primarily on the single gene traits of herbicide tolerance and insect resistance. The next generation of biotech crops promises to include a broad range of products that will provide benefits to both farmers and consumers, and continue to meet global agricultural challenges. These products will most likely involve regulation of key endogenous plant pathways resulting in improved quantitative traits, such as yield, photosynthesis, and abiotic stress tolerance (e.g. drought, etc.; Martino-Catt and Sachs 2008; Raven 2008).

Radical improvements in crop productivity, such as engineering $C_4$ photosynthesis into $C_3$ plants, are necessary to ensure continued food security. $C_4$ photosynthesis can greatly enhance crop productivity over $C_3$ photosynthesis, like in the $C_4$ crops maize and sorghum, which evolutionarily are younger and the most adopted to increasing $CO_2$ levels, high solar radiation, high temperature, drought, etc., yet most crops directly consumed by people (rice, wheat and legumes) are $C_3$ species. The strategy of $C_3$-$C_4$ engineering is one of the grand challenges in plant biology (Sage *et al*. 2009). Introduction of genes encoding $C_4$ photosynthesis enzymes into $C_3$ crops (e.g. rice) has been successfully completed (Ku *et al*. 1996, 1999; Hausler *et al*. 2002). Transgenic rice expressing maize $C_4$ photosynthesis enzymes exhibited a higher photosynthetic capacity, better adaptation to stress conditions and higher grain yield (Ku 2001).

Wheat, another important $C_3$ cereal crop and one of three most important cereal crops in the world (Evans 1998;

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James 2007), is the main food crop cultivated in Kazakhstan and biotechnology provides a great promise for its metabolic engineering. Kazakhstan has very similar climatic conditions with the USA, Australia, South Africa and Argentina, including problems of global climate change, elevated $CO_2$ in the atmosphere, and increased drought. Increasing wheat productivity in Kazakhstan should be the result of coordinated integration of advances in the development of modern biotechnologies and conversion of ideas and recent achievements of plant metabolic physiology, biotechnology, genetic engineering into breeding, which have progressed rapidly in the USA and Australia. Solutions to achieve better crop yield, environmental stress protection, and safety are difficult to come by and require a fundamental scientific basis. A progressive and effective system for agriculture should combine economical, technological and scientific means to enhance crop yields (Evans 1998; Penna 2001; James 2007).

We have worked on the photosynthetic basis of wheat crop improvement and drought resistance for many years (Kershanskaya 2000). It has been used as an integral complex approach to study the interactions between maximum crop productivity and characters of photosynthetic processes with studies ranging from the cellular to the whole plant level underlying diversity in structure and function for optimisation of wheat breeding. The mechanism of interaction between primary energetic processes in photosystems and chloroplast, high $CO_2$-fixation capacity in leaves and high crop productivity, ontogenetic changes in productive processes and the role of organs in accumulation and redistribution of biomass as well as special characters of photosynthetic metabolism ($C_3$, $C_3$-$C_4$, $C_4$ in wheat species and relatives) have also been investigated (Kershanskaya 2007). As a result, the concept of optimal photosynthetic wheat plant type (OPWPT) has been formulated and a conceptual OPWPT model with high productivity has been created. The OPWPT model can demonstrate which photosynthetic parameters need to be focused such that genetic modification in wheat further increases its productivity (Kershanskaya 2000, 2004, 2007). Among the main characters of photosynthetic metabolism that could be modified in a $C_3$ wheat plant by molecular metabolic engineering are photosynthetic enzymes.

Several attempts have been made in the past to express genes encoding the enzymes of the $C_4$ pathway in $C_3$ plants in an effort to tune their photosynthetic metabolism (Hatch 1987; Edwards *et al*. 2001). Why should we introduce $C_4$ photosynthetic genes into economically important $C_3$ crops? This is because $C_4$ crops, such as maize or sorghum, are evolutionary related but much more adopted to modern climate conditions (elevated $CO_2$ in the atmosphere, high solar radiation, high temperature, drought and desertification) than economically important $C_3$ crops, such as wheat and rice. $C_4$ plants do not spend energy on photorespiration and do not suffer from $CO_2$ saturation due to the development of two functionally and structural different photosynthetic cells, mesophyll and bundle shift cells, which in generally increase harvest. The link, in essence, lies between spending energy on photorespiration and decreasing harvest in $C_3$ crops while $C_4$ photosynthetic function depends on differentiation of photosynthetic cells.

The idea of expressing the genes encoding enzymes of the $C_4$ pathway in $C_3$ plants in an effort to tune their photosynthetic metabolism has been successfully realized in rice (Hudspeth *et al*. 1991; Imazumi *et al*. 1997; Edwards 2001; Ku 2001; Hausler 2002; Leegood 2002). Most importantly, these transgenic rice plants exhibited enhanced photosynthetic capacity and up to 30% gain in yield (Ku 2001). Manipulation of important regulatory genes involved in sucrose formation in rice and other important crops is also under way (Sage *et al*. 2009).

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. The most commonly used technology and approaches for genetic modification in wheat are: (1) wheat transformation via bombardment, (2) *Agrobacterium*-mediated transformation of wheat callus obtained from immature embryo or inflorescence, (3) microspore (immature pollen)-mediated transformation, (4) leaf mesophyll protoplast-mediated transformation into wheat, (5) wheat pollen transformation – electroporation, sonication, vacuum infiltration with $C_4$ DNA, or (6) pipetting *Agrobacterium* into the spikelets of wheat (Liu *et al*. 1988; Hess *et al*. 1990; Pukhalskii *et al*. 1996; Zeng *et al*. 1998; Danilova and Dolgikh 2005; Jones *et al*. 2005; Danilova *et al*. 2007).

Wheat transformation via bombardment and *Agrobacterium*-mediated callus transformation with bombardment are the most popular modern DNA techniques for effective wheat transformation, which have been shown to provide high levels of gene expression and stability of transgenes over several generations. The disadvantages of these approaches involve the tissue culture step, difficulties with establishment of an embryogenic culture and plant regeneration, since these consume time, chemicals and equipment.

We have developed a simple and efficient, natural, plant-friendly and rapid truly genotype-independent germline transformation technique by direct DNA delivery into regenerable cells in wheat for targeted gene introduction by *Agrobacterium* pipetting, similar to the pollen-transfection method for other cereals and close to conventional cross-hybridisation (Kershanskaya *et al*. 2007, 2008). No *in vitro* culture steps are required. The method of *Agrobacterium* pipetting transformation could be used for genetic modification of photosynthesis in wheat for introduction of maize genes encoding $C_4$ enzymes (PEPC, PPDK, NADP-ME; phosphoenolpyruvate carboxylase, orthophosphate pyruvate dikinase, NADP-malic enzyme, respectively), step by step. Results of introduction of the maize PEPC gene into wheat are reported in this paper. 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. Only results with low efficiency have been obtained thus far.

The main goal of our research was to investigate the advances in transformation technologies and thus to establish approaches for genetic modification of photosynthesis in wheat for increasing drought resistance and grain yield up to 30% through the introduction of maize genes encoding the $C_4$ photosynthesis enzymes into wheat since this has been successfully conducted in rice (Ku 2001).

The ultimate goal of this work was to produce new strains of wheat with enhanced photosynthetic capacity, drought tolerance and grain yield and to investigate the physiological consequence of genetic introduction of the PEPC gene into wheat using transgenesis as a model.

## MATERIALS AND METHODS

### Plant material

5 varieties of commercial USA spring and 15 Kazakhstan spring and winter wheat varieties with contrasting photosynthesis, productivity and drought resistance characters have been used as "wild types" to produce transgenic plants. About 5000 putative transgenic seeds with the PEPC gene were produced by the *Agrobacterium* pipetting method, and screened by antibiotic resistance. 17 wheat transgenic lines of $T_1$ and $T_2$ progenies (about 10-15 plants per each line) were confirmed by molecular biological techniques (described below) and were included in further physiological-biochemical, leaf anatomical and yield studies. The plants were cultivated using standard management practices for wheat crop (VIR recommendation 1995; Kuehl 2000). The maximum photosynthetic PFD at noon on a sunny day during the plant vegetation period was 1800 μmol $m^{-2}$ $s^{-1}$.

## Genetic material

The maize PEPC gene construct used was made available from Professor Ku's lab, WSU, USA (Ku 1999). The 8.8 kb maize PEPC gene fragment contains all exons, introns, and the promoter (from -1212) and the terminator (about 2.5 kb) sequences, selectable antibiotic resistance genes (kanamycin phosphotransferase, *npt*II; hygromycin phosphotransferase, *hpt*II) (Matsuoka 1989; Ku 1999). The $C_4$ photosynthetic gene encoding the key $C_4$ photosynthetic enzyme PEPC from maize has the following function in wheat:

(cytoplasm)
$PEP + HCO_3^- \rightarrow$ oxaloacetate + Pi

## *Agrobacterium* pipetting germ-line transformation technique

*Agrobacterium*-mediated transformation technique via pipetting *Agrobacterium* with the gene of interest into the spikelets of wheat before anthesis has been elaborated as a simple, natural and effective germ-line transformation method for wheat transformation. Strains, preparation, concentration of *Agrobacterium* with gene of interest; techniques of gene introduction into stigma; optimal stage for transformation, have been elaborated and patented (Kershanskaya and Dzhangalina 2008).

## Antibiotic screening technique

Investigation of transgene expression was evaluated after seeds were obtained by two screening steps: (1) resistance to hygromycin – by the *hpt*II selectable marker gene included in the PEPC gene chimerical construct; (2) resistance to kanamycin – by the *npt*II selectable marker gene by a new, elaborated and patented scheme. Seeds were screened in 2 steps: 1) for 5 days on antibiotic-containing medium at 50 mg/l followed by 2) 4 days on the same medium but in which the concentration of antibiotic was lowered to 25 mg/l; in both steps basal medium was MS (Murashige and Skoog (1962) medium, Sigma) agar medium; seedlings were then trasplanted to soil (patented conditions in Kershanskaya and Dzhangalina 2009).

## Molecular biological confirmation of stable wheat transformation: PCR, Real-time PCR, Southern blotting

Transgenic wheat plants of the first generation ($T_1$) were allowed to grow to the 2nd generation for further molecular biological confirmation of stable transformation and grain yield determination. DNA samples extracted from the young "flag" leaves of the stem-growing vegetative stage of untransformed "wild plants" using a phenol-chloroform and ethanol precipitation method (Chawla 2003) with purification with RNAase (Sigma) and antibiotic-resistant putative transgenic lines were used for PCR amplification with primers for the *npt*II (478 bp) and *hpt*II (400 bp) selectable marker genes included in the maize PEPC gene construct, as well as a probe of the PEPC gene as control. Primers for the *npt*II gene: forward primer (225-2) 5′-AAgCACgAggAAgCggTCAg-3′ (S) and reverse primer (224-1) CgACgTTgTCACTgAAgCg (US). Primers for the *hpt*II gene: forward primer 5′-GCTGCGCCG ATGGTTTCTACA-3′, reverse primer 5′-GCCCAAAGCATCAGC TCATCG-3′, from 155 to 554 bp (NCBI, Vector I Program).

PCR products were analysed by electrophoresis in agarose (A 9539, Sigma, USA) gels with 1 kb or 100 bp DNA molecular mass markers (Fermentas, UK).

Real-time PCR analysis was provided by the test-system "GMO-screening" with«ANK-32, GM-409-2» equipment, Synthol, Russia by the «GMO-screening» program. Detection of transgenic plants was performed by the identification of the *NOS*-terminator and *35S* promoter sequences using *FAM* and *ROX* fluorescent target probes, respectively (Test-system: "GMO screening", Cat. # GM-409-2, Instruction, Syntol, Russia, 2007).

Southern blotting was performed for probes which had been eluted from PCR products with *npt*II gene primers and a 1.1 kb PEPC maize genome *Eco*RI fragment (Ku *et al*. 1998). DNA was extracted and restricted by *Eco*RI and *Hind*III enzymes for 1 µg DNA – 1 U RE (Fermentas, UK). DNA was transferred to a nylon membrane (Amersham, UK) by alkaline denaturation buffer (0.4 M NOH + 0.6 M NaCl). The nylon membrane with transferred DNA was dried for 30 min and fixed under UV $\lambda_{265\ nm}$ for 5 min. Radioactive probe was prepared by a random prime labelling method with $^{32}$P-dCTP using the NEBlot Kit (New England BioLabs, UK). Hybridization was achieved in an Amersham oven (UK) for 16 hrs with 5 washes: 2 times in 1X SSC + 0.1% SDS at 50°C for 15 min and 3 times in 0.2X SSC + 0.1% SDS at 58°C. The rate of autoradiography was defined by an autoradiography meter (MKA, Russia). Membranes were exposed using X-ray HyperfilmTMMP (Amersham, UK) by placing in cassettes (Amersham) at -70°C.

## PEPC activity assay

The transgenic wheat plants obtained (about 15 plants from each of the 17 lines (def = inheritance from one seed)) were used for further characterization by phenotypic, photosynthetic, anatomical and productivity parameters.

PEPC activity was assayed according to the method of Ku *et al*. (1999). About 0.25 g leaf tissue was harvested from newly mature flag leaves at the end of stem-growing – beginning of flowering stage – from each plant in the light and quickly ground in 1.5 ml extraction buffer containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 2% (w/v) insoluble polyvinylpolypyrrolidone (PVP-40) and 10% glycerol (all reagents Sigma). After total maceration, the crude extract was centrifuged at 13,000 × *g* for 10 min at 4°C and the supernatant was used immediately to assay the $C_4$ enzyme. PEPC was assayed spectrophotometrically at room temperature in a mixture containing 50 mM Hepes-KOH pH 8.0, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 1.5 U NAD-malate dehydrogenase (MDH), 0.2 mM NADH and 20-50 µl enzyme extract (all from Sigma). The reaction was started by adding PEP at a final concentration of 2 mM. The change in NADH was monitored in a spectrophotometer (Ultraspec 2000, Pharmacia Biotech) at $\lambda_{340\ nm}$. Enzyme activity was expressed on a chlorophyll (Chl) basis. Chl concentration was determined after extraction in 96% ethanol spectrophotomertically by measuring the optical density (D) under λ = 665 and 649 nm using the 1985 Wintermans and De Mots formula (Kershanskaya 2007):

Chl *a* (mg/l) =13.70 × D665 + 5.76 × D649
Chl *b* (mg/l) = 25.80 × D649 + 7.60 × D665
Chl *a*+*b* (mg/l) = 0.60 × D665 + D649.

## $CO_2$ gas-exchange measurements

The same matured flag leaves, which have the greatest impact on biomass during ear grain filling, were used at the flowering stage for all photosynthetic measurements and tolerance to photooxidation analysis. Photosynthetic rates and stomatal 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, USA) according to the method of Long *et al*. (1991) and Ku (2001) and calculated according to Farquhar *et al*. (2000) and Ku (2001). The photosynthetic responses to temperature and external $CO_2$ concentration were determined under different leaf temperatures or $CO_2$ concentrations at saturated light intensity (1200 µmol photon $m^{-2}$ $s^{-1}$), respectively. Optimal temperature for photosynthesis and carboxylation efficiency (the initial slope in response to $CO_2$) was then determined from these response curves, respectively. The photosynthetic $CO_2$ compensation point was measured at 30°C, 1000 µmol $m^{-2}$ $s^{-1}$, 360 µg/L $CO_2$ according to the method of Jiao and Ku (1996). Several intact flag leaves were sealed in a transparent plexiglass chamber and the $CO_2$ concentration in the chamber was withdrawn and analyzed by an infrared $CO_2$ gas analyzer. After reaching equilibrium, the $CO_2$ concentration in the chamber was used to determine the compensation point (µmol/mol) with the LI 6200.

## Leaf anatomy investigation

27 wheat (*Triticum eastivum* L.) transgenic lines were compared with the control wild type (non-transgenic form from the same

**Table 1** Wheat transgenic frequency resulted from screening on antibiotic resistance encoded by *npt*II and *hpt*II marker genes, which have been included into PEPC (*npt*II and *hpt*II) and ZOG1 (*npt*II) gene constructs.

| Gene | Antibiotic | Number of experiments / screened seeds | Number of regenerated plants | Transgenic frequency (%) |
|---|---|---|---|---|
| ZOG1 | Kanamycin | 9/2176 | 113 | 5.1 |
| PEPC | Hygromycin-B | 7/1927 | 158 | 8.2 |

Wheat transgenes were created by *Agrobacterium* pipetting method

cultivar) and *Zea mays* L. (approx. 20 different cultivars were employed, primarily 'Houis'). All plants were used to investigate leaf anatomy for the appearance of $C_4$ Kranz anatomy features by microscopic analysis (Gradchaninova 1978; Kirby 1985; Barykina *et al*. 2004; Wildon *et al*. 2004; Kirby 2007). Microscopic analysis and morphometric measurements of leaf anatomical structure were possible with the use of a light microscope (ZEISS AXIOSCOP 40 HBO100) combined with a 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 (Edwards *et al*. 2001; Voznesenskaya *et al*. 2001).

## Agronomical traits

Wheat transformation in $T_1$ and $T_2$ was confirmed by analysis of harvest parameters in transgenic plants in comparison with wild type lines. Thus 2419 transgenic plants of the 21 transgenic lines were investigated by assessing yield parameters: plant height, the main and lateral tillers length, number of stems or ears/plant, number of spikelets in a spike, main stem/lateral, number of grains/ear, grain mass/ear of the main and lateral stems, grain yield from 1 plant, transgenic effect in % of increasing grain yield/plant of transgenic lines in comparison with wild type in a minimum (average) of 15 plants (Kershanskaya 2004, 2007).

## RESULTS

A simple genotype-independent method of wheat germ-line transformation by *Agrobacterium* pipetting into the spikelets of wheat before anthesis has been elaborated. The method uses a unique indirect wheat pollen system that contains high quantities of flavonol glycosides which act as inducers of the *vir* region of the *Ti* plasmid. Strains, preparation, concentration of *Agrobacterium* with the gene of interest, techniques of gene introduction into the stigma, and optimal stage for transformation have all been elaborated and patented (Kershanskaya and Dzhangalina 2008, 2009). The method is very similar to wheat hand hybridization, is economical and does not require expensive and complicated tissue culture/regeneration steps. This method allowed us to produce putative transgenic wheat plants that express the maize PEPC gene for enhancing photosynthetic capacity.

In this study, foreign maize DNA was transferred into wheat, integrated and expressed. Transformants were fertile and showed no morphological abnormalities. *Agrobacterium* growth medium and conditions, time for pipetting, stage of plants, a technique of pipetting onto the wheat stigma as well as 2-step screening have been developed. This new, natural, cheap, germ-line method for efficient wheat transformation has been patented (Kershanskaya and Dzangalina 2008).

The two-step antibiotic screening technique has also been elaborated and patented (Kershanskaya and Dzangalina 2009). In total about 5000 putative transgenic wheat seeds of 20 varieties have been produced with an average transformation efficiency of about 5-8% (**Table 1**).

## Antibiotic screening

Different concentrations of antibiotics, number of days, screening steps as well as 5 sterilization protocols for 20 Kazakhstan winter and spring and 5 American spring wheat genotypes have been tested. 2176 putative transgenic seeds with the PEPC gene were screened on kanamycin and 1927 putative transgenic seeds with the PEPC gene were screened on hygromycin for assessing resistance to these antibiotics. After screening for 5 or 4 days on 50 and 25 mg/l hygromycin, respectively and 4 days on 25 m/l hygromycin on MS agar medium, 158 hygromycin-resistant plants derived from seeds with the putative PEPC gene were obtained, representing an 8.2% transgenic frequency or efficiency. Kanamycin-resistant plants were also obtained after screening on 50 and 25 mg/l kanamycin on MS agar medium with a transgenic frequency of about 5.1% (**Fig. 1**). As a result of antibiotic screening, hundreds of transgenic wheat plants of $T_1$ and $T_2$ generations were obtained.

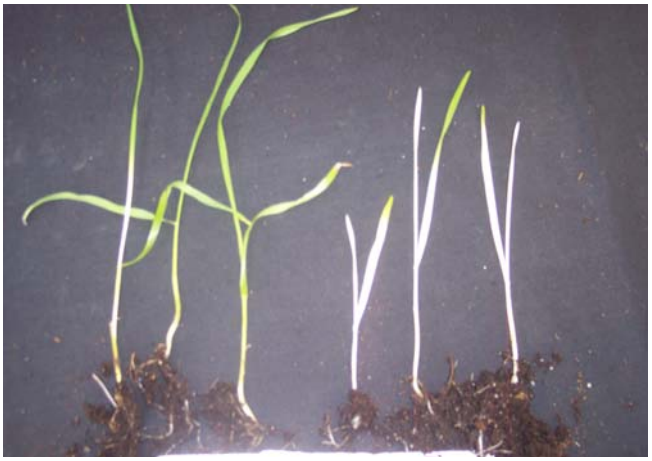

**Fig. 1 Antibiotic (50 mg/l kanamycin) resistant transgenic (left green) and wild (right white) wheat plants after screening (transformation frequency 5.1%).**

## Molecular biological confirmation of stable wheat transformation: PCR, Real-time PCR, Southern blotting

The next amplification conditions for PCR to identify the PEPC gene in putative wheat transgenic plants were optimized: denaturation temperature, duration of DNA synthesis, genomic DNA concentration resulting in the following protocol: 94°C for 4 min, 30X (94°C for 45 sec, 62 °C for 45 sec, 72°C for 45 sec) and a final extension at 72°C for 10 min. DNA was then maintained at 10°C indefinitely. The presence of the PEPC gene was confirmed by PCR in 20 of 84 putative antibiotic-resistant transgenic plants (**Fig. 2**). Transformation frequency based on PCR analysis was 23.8% from antibiotic-resistant plants and 2.3% when based on all putative transgenic seeds obtained by the *Agrobacterium* pipetting germ-line transformation technique.

The presence of transgenes (*NOS*-terminator and *35S*-promoter) in transgenic plants was confirmed by real-time PCR (not shown). Optimal DNA concentration was 100-200 ng/µmol DNA per probe.

DNA of 22 transgenic plants was confirmed by PCR and DNA of untransformed controls was analyzed by RT-PCR. Genetic transformation of 20 wheat lines of the first and second generations ($T_1$ and $T_2$) were identified by regulatory elements of the PEPC gene: the *NOS*-terminator and the *35S*-promoter.

Southern blotting (**Fig. 3**) was performed on 6 transgenic plants which were PCR- and RT-PCR positive (lines 4, 11, 16, 43, 48, 72) as well as on 1 positive (PEPC gene probe) and 1 negative (water) control. The PEPC gene probe (6.1 kb) and *npt*II gene (2.7 kb) were present in trans-

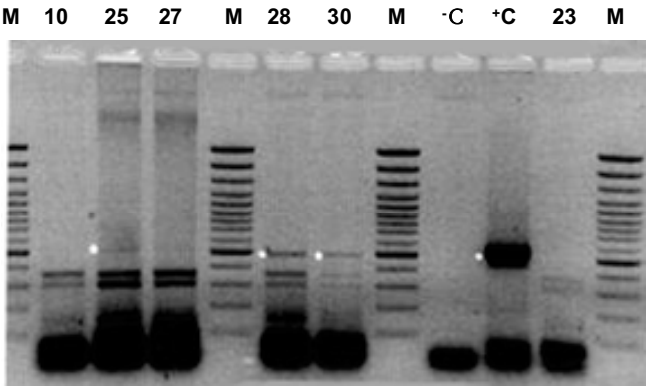


**Fig. 2** PCR profiles of antibiotic-resistant wheat plants of $T_2$ progenies obtained with PEPC gene: DNA markers (1 kb, Fermentas); (25, 27, 28, 30) transgenic wheat plants with band 500 bp; -C negative control, +C positive control (plasmid pSB 130/PEPC gene); 10, 23 – nontransgenic lines. Marker size is 1 kb.

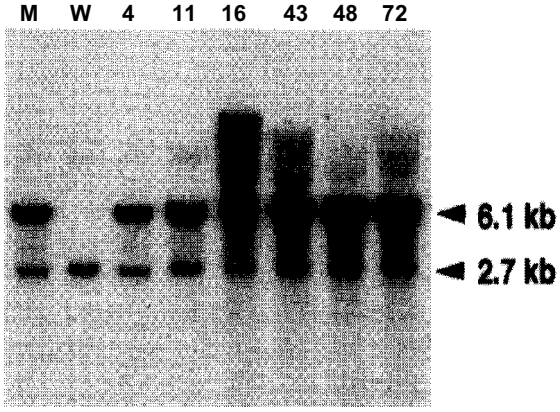


**Fig. 3** Southern blotting of antibiotic-resistant wheat plants of $T_2$ progenies obtained with PEPC gene: M positive control – mix of maize plasmid DNA corresponding to one copy in diploid genome PEPC gene 6.1 kb plus wheat genomic DNA - homologous PEPC gene 2.7 kb band, W - wheat, and (4 – 72) transgenic wheat plants with increasing copies number of maize PEPC gene.

genic lines 4, 11, 16, 43, 48, 72 (**Fig. 3**).

Thus, stable wheat transformation obtained by *Agrobacterium* pipetting was confirmed by molecular techniques – PCR, Real-time PCR and Southern blotting – in the second progeny of transgenic lines, $T_2$.

## PEPC activity assay

The level of expression of the maize $C_4$-specific PEPC gene in transgenic wheat plants was determined first by assaying the activity of PEPC in leaf protein extracts. A wide range of PEPC activity was detected among the transgenic plants; some of them had a high level of activity.

The activity of PEPC in the various transgenic lines as well as in the control (untransformed wild type wheat plants) was examined by the direct assay of PEPC enzyme activity (**Table 2**). The activity of this enzyme was low in control wheat cultivars ('Houis' and 'Wawawai'). In contrast, the activity of PEPC in transgenic plants was about 2-6 fold higher than that in control wheat plants reaching 5.47 μm PEP/min/mg Chl, with a concomitant increase in the amount of Chl.

Screening using the PEPC assay allowed us to obtained 18 plants with high PEPC activity from 71 putative transgenic wheat plants of the first generation $T_1$ that had been screened. 6 lines showed increasing of PEPC activity in comparison with non-transgenic control by 4-6–fold (**Table 2**).

Transgenic efficiency of screening by PEPC activity was 25.35% and 1.8% from the total number of putative transgenic seeds that had been screened on antibiotic-containing medium. Results of biochemical screening by the PEPC assay, in which expression of protein of the transferred PEPC gene was measured, were similar to results of the PCR analysis (transformation frequency by PCR analysis was 23.80% from antibiotic-resistant plants and 2.3% from all putative transgenic seeds).

## $CO_2$ gas-exchange measurements

The photosynthetic characteristics of PEPC transgenic plants were analyzed in detail. The transgenes exhibited higher light-saturated photosynthetic rates (40%), higher stomatal conductance (29%) at 1200 μmol photon $m^{-2}$ $s^{-1}$ and higher carboxylation efficiency than untransformed WT and photosynthetic rates reached 24 μmol $CO_2/m^{-2}$ $s^{-1}$ and stomatal conductance = 400 mol/$sm^{-2}$ $s^{-1}$. Most PEPC transgenic wheat plants exhibited enhanced photosynthetic capacity with an increase in photosynthetic rate up to 17% and up to 25% increase in stomatal conductance.

On the other hand, the photosynthetic $CO_2$ compensation points were slightly lower in the PEPC transgenic plants, indicating a stronger capacity of the plants to assimilate carbon under limited $CO_2$ conditions. The exact mechanism for the superior photosynthetic performance of these plants has yet to be determined.

## Leaf anatomy

Leaf anatomy in transgenic wheat plants in which a PEPC gene had been introduced was determined. $C_4$ transgenic wheat demonstrated some features of Kranz leaf anatomy: chloroplasts were located at the cell wall; bundle sheath cells began rolling and started to organize a circular structure similar to $C_4$ maize (Kranz); mesophyll cells formed a circle around bundle sheath cells and conducting bundles (**Fig. 4**), typical of Krantz anatomy. Differentiation of leaf cells anatomy was accompanied with an increase in photosynthetic capacity of $C_4$ transgenic wheat.

Except for a few plants, most of the transgenic wheat plants exhibited a normal phenotype and retained high fertility (85-90% vs 90% in untransformed plants). Transgenic plants had some advantage in ripeness – they started earring

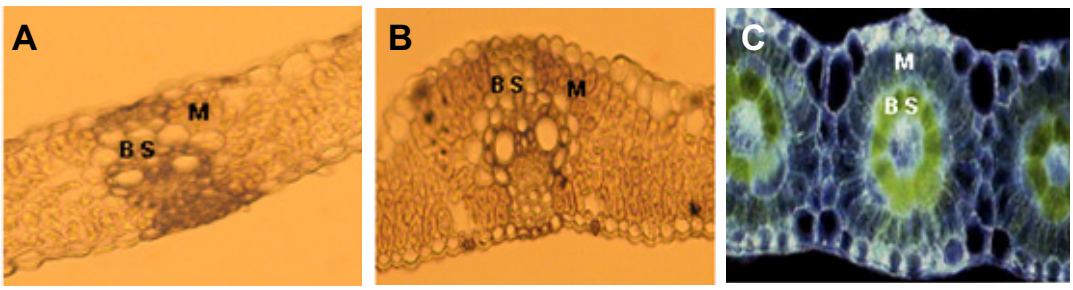


**Fig. 4** Anatomical profile of flag leaf wheat wild type Houis (**A**), transgenic line Houis-19 with PEPC gene from maize (**B**), and maize (**C**). Appearance of Kranz – anatomy of BS – bundle sheath cells and M – mesophyll cells in wheat transgenic line (**B**).

**Table 2** PEPC assay of putative transgenic wheat plants of first generation $T_1$ previously screened on kanamycin and hygromycin resistance. **Bold** data show increasing of PEPC activity in transgenic plants in comparison with control wild plants (*italics*).

| #, variety | OD 665 (nm) | OD 649 (nm) | (µm chlorophyll / 40 µl extact) | OD 340 | (µm PEP / min · mg chlorophyll) |
|---|---|---|---|---|---|
| **Houis 4 (13)** | 0.542 | 0.204 | 7.39 | 0.48 | **2.07** |
| **Houis 5 (14)** | 0.838 | 0.235 | 9.81 | 0.66 | **2.15** |
| **Houis 6 (15)** | **0.402** | **0.176** | **5.97** | **0.53** | **2.86** |
| *Control (Houis 21)* | *0.709* | *0.267* | *9.67* | *0.43* | *1.43* |
| **Houis 9 (25)** | **0.580** | **0.234** | **8.22** | **0.52** | **2.03** |
| *Control (Wawawai 11)* | *4.84* | *0.186* | *6.67* | *0.27* | *1.30* |
| ***Wawawai* 12 (33)** | 0.574 | 0.272 | 8.94 | 0.56 | **2.01** |
| ***Wawawai* 13 (36)** | 0.530 | 0.237 | 7.97 | 0.48 | **1.94** |
| ***Wawawai* 14 (39)** | **0.512** | **0.240** | **7.92** | **0.57** | **2.31** |
| ***Wawawai* 15 (42)** | 0.506 | 0.235 | 7.79 | 0.46 | **1.90** |
| **Max *Wawawai* 4** | **0.703** | **0.342** | **11.13** | **0.295** | **2.13** |
| *Control (Wawawai 13)* | *0.660* | *0.282* | *9.67* | *0.13* | *1.08* |
| *Control (Wawawai 14)* | *0.632* | *0.280* | *9.46* | *0.13* | *1.10* |
| ***Wawawai* 18** | **0.339** | **0.106** | **4.19** | **0.135** | **2.59** |
| ***Wawawai* 19** | **0.386** | **0.139** | **5.13** | **0.185** | **2.90** |
| **Max *Wawawai* 20** | **0.531** | **0.196** | **7.15** | **0.39** | **4.38** |
| ***Wawawai* 21** | **0.355** | **0.106** | **4.29** | **0.19** | **3.56** |
| ***Wawawai* 22** | **0.351** | **0.096** | **4.06** | **0.18** | **3.56** |
| **Max *Wawawai* 24** | **0.281** | **0.073** | **3.17** | **0.175** | **4.44** |
| **Max *Wawawai* 26** | **0.352** | **0.086** | **3.87** | **0.263** | **5.47** |
| **Max *Wawawai* 29** | **0.108** | **0.020** | **1.06** | **0.065** | **4.93** |
| ***Wawawai* 41** | **0.218** | **0.092** | **3.17** | **0.085** | **2.16** |
| *Control (Wawawai 48)* | *0.452* | *0.242* | *7.60* | *0.085* | *0.90* |
| *Control(Wawawai 50)* | *0.500* | *0.237* | *7.67* | *0.085* | *0.90* |
| *Control (Wawawai 55)* | *0.775* | *0.345* | *11.63* | *0.140* | *0.97* |

**Table 3** Harvest structure and grain yield of wheat transgenic lines $T_2$ with PEPC gene and wild type Houis. **Bold** data shows increasing of grain yield parameter and transgenic effect of transgenic plants in comparison with wild type – control.

| Wild control and transgenic lines of cultivar Houis | Plant height (cm) | Number of tillerings/ ears | Number of spikelets in spike, main stem/ lateral | Number of grains per ear (g) | Grain mass per ear main stem/ lateral (g) | Grain yield from 1 plant (g) | Transgenic line - control grain yield effect (%) |
|---|---|---|---|---|---|---|---|
| Control | 78 | 1/1 | 27 | 1.5 | 0.56 | 0.56 ± 0.80 | 100 |
| H50PC-26 | 62.6 | 1.3/1.3 | 25.8/24 | 18/12.5 | 0.59/0.3 | **0.70 ± 0.10** | **125** |
| HPC-26 | 61.8 | 1.5/1 | 33 | 20 | 0.71 | **0.71 ± 0.21** | **127** |
| H 25PC-26 | 75.3 | 1/1 | 27.7 | 20 | 0.66 | **0.67 ± 0.19** | **120** |
| HPC-22 | 44.5 | 0.5/1 | 28 | 18 | 0.89 | **0.89 ± 0.25** | **159** |
| HPC-96 | 60 | 1/1 | 27 | 18 | 0.56 | 0.56 ± 0.20 | 0 |
| H 25PC-19 | 76 | 1.5/1.5 | 33/5 | 23/0 | 0.77 | **0.78 ± 0.14** | **139** |
| H 25PC-24 | 65.3 | 1/1 | 27 | 18.3 | 0.57 | 0.57 ± 0.15 | 102 |
| H 25PC-18 | 74 | 1/1 | 33 | 19 | 0.78 | **0.78 ± 0.18** | **139** |

and flowering early in comparison with the untransformed control. Some transgenic plants stayed green until completely ripe, and the leaf assimilation area of transgenes in $T_1$ and $T_2$ was much higher than in untransformed control, especially in flag leaves. This observation could show the productive advantage of these transgenes, as high leaf area in the wheat reproductive period provides high potential for photosynthetic activity and biomass formation. Maintenance of photosynthetically active leaves during grain filling will further contribute to greater yield potential (Evans 1998; Edwards *et al.* 2001; Kershanskaya 2007).

## Yield structure

We established 17 wheat transgenic lines $T_2$ with enhanced grain yield i.e., 25-50% higher than the WT (**Table 3**). Increasing grain yield by transgenes was caused by an increase in the number of grains per ear and grain mass.

## DISCUSSION

Wheat, a $C_3$ plant, is temperate in origin, and has one photosynthetic cell. Its photosynthesis is not saturated by atmospheric $CO_2$ levels and is characterized by high photorespiration. Maize, a $C_4$ plant is tropical in origin, has two photosynthetic cells (Kranz anatomy) and its photosynthesis is saturated at atmospheric $CO_2$ levels, with low photorespiration. Creating $C_3$-$C_4$ transgenic wheat would have many advantages for Kazakhstan, USA, Australia and wheat-growing countries the world over because $C_4$ plants are especially successful in areas that are arid or hot and in soils with high salt content.

The agricultural regions of Kazakhstan contain large arid zones and salty areas with increasing desertification, an unknown $CO_2$ balance in the atmosphere with a possible loss of carbon in soil up to 35-50%. Consequently, non-rational land is being exploited for agricultural uses. A promising advance in wheat crop improvement is to genetically modify its photosynthetic process by introducing $C_4$ photosynthesis genes into wheat and thus creating $C_3$-$C_4$ intermediate wheat with $C_4$ DNA.

It is hoped that by introducing some of the key enzymes of $C_4$ photosynthesis into $C_3$ plants with proper intercellular compartmentation 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.

All enzymes involved in $C_4$ photosynthesis are found in the 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. This may play an important role in carbon and nitrogen metabolism or energy supply (e.g. release of ATP from PEP catalyzed by PPDK) in reproductive tissues, and enhanced expression of the enzyme may boost seed development and grain productivity.

Enzymes involved in $C_4$ photosynthesis, albeit low in $C_3$ plants, may also play important roles in plant defense responses to biotic and abiotic stress. Metabolic alterations in response to stress allow plants to adapt to adverse conditions. Thus, increased expression of some $C_4$ photosynthesis enzymes in $C_3$ plants could confer enhanced tolerance under stress conditions.

High $CO_2$ favors the carboxylase reaction and thus net photosynthesis; whereas high $O_2$ promotes the oxygenize reaction leading to photorespiration. As a byproduct of photosynthesis, $O_2$ accumulated in the atmosphere and reached the current level (21%) hundreds of million years ago. On the other hand, the atmospheric $CO_2$ levels had decreased throughout geologic time. 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%. This renders $C_3$ plants less competitive in certain environments - high light, high temperature and drought conditions. With some modifications in leaf anatomy, some tropical species (e.g. maize and sugarcane) have evolved a biochemical "$CO_2$ pump", the $C_4$ pathway of photosynthesis, to concentrate the atmospheric $CO_2$ in the inner bundle sheath cells where Rubisco is located and overcome photorespiration. Thus $C_4$ photosynthesis is not limited by the current levels of atmospheric $CO_2$ (references required).

Based on our classic knowledge of $C_4$ photosynthesis, the presence of Kranz leaf anatomy is essential for its function and the enzymes involved in the $C_4$ pathway are compartmentalized between the mesophyll and bundle sheath cells. So it is unclear as to how a high level of the maize PEPC (a key enzyme in $C_4$ photosynthesis) alone could lead to increased photosynthetic capacity in transgenic wheat. Several possibilities may account for these effects. Earlier data showed that the higher photosynthetic rates of the PEPC transgenic plants may be due to increased stomata conductance (Ku *et al*. 2001). PEPC is known to be involved in organic acid metabolism in the guard cells, which is important for build-up of turgor pressure and opening of stomata. Increased stomatal conductance to $CO_2$ diffusion can lead to a higher internal $CO_2$ concentration and consequently reduce photorespiratory loss of assimilated carbon. In fact, transgenic wheat expressing PEPC tend to have higher photosynthetic rates with increased stomatal conductance, like transgenic $C_4$ rice (Ku *et al*. 2001).

In engineering the $CO_2$ concentrating mechanism of $C_4$ photosynthesis, there are two important components to be considered: the biochemical pathway (enzymes) and the specialized leaf structure. The coordination of two specialized leaf cells in $C_4$ leaves, namely mesophyll and bundle sheath cells (together termed Kranz leaf anatomy), is important for pathway function. Our first goal was to engineer the key enzymes involved in $C_4$ photosynthesis in wheat without Kranz leaf anatomy. *Hydrilla verticillata* is known to shift from $C_3$ to $C_4$ photosynthesis and assimilates atmospheric $CO_2$ via the $C_4$ pathway without Kranz leaf anatomy (Bowes and Salvucci 1989). Inorganic carbon is first assimilated into the $C_4$ acid malate in the cytoplasm via PEPC, and subsequently malate serves as a donor of $CO_2$ to Rubisco in chloroplasts by the decarboxylating enzyme, NADP-malic enzyme (Ku 1999). It is possible that this archetypal version of $C_4$ photosynthesis, which does not depend on Kranz compartmentation, can be engineered to function in terrestrial $C_3$ plants.

Transgenic wheat plants expressing the maize PEPC exhibit a higher photosynthetic capacity (up to 40%) than untransformed plants. The increased photosynthetic capacity in these plants is mainly associated with enhanced stomatal conductance and a higher internal $CO_2$ concentration. The results suggest that PEPC plays a key role in organic acid metabolism in the guard cells to regulate stomatal opening. It is known that under photoinhibitory and photooxidative conditions PEPC transgenic plants are capable of maintaining a higher photosynthetic rate, a higher photosynthetic quantum yield by PSII and a higher capacity to dissipate excess energy photochemically and non-photochemically than untransformed plants. Preliminary data from field trials show that relative to untransformed plants, the grain yield is about 25-35% higher in selected PEPC wheat plants, due to increased tiller number. Taken together, these results suggest that introduction of $C_4$ photosynthesis enzymes, in this case PEPC, into wheat has a good potential to enhance its tolerance to stress, photosynthetic capacity and yield.

## CONCLUSIONS

Maize $C_4$ genes were efficiently expressed in a $C_3$ plant, wheat, by an elaborate *Agrobacterium* pipetting technique with a 2.3% transformation frequency. Such an approach of successful wheat transformation is beneficial for biotechnology and breeding, as this method is natural, cheap, simple and effective.

About 5000 seeds of Kazakhstan and commercial US wheat varieties were produced and screened; in total 17 transgenic wheat lines with enhanced photosynthetic capacity and with the appearance of features of Kranz leaf anatomy and increasing yield were created.

Stable wheat transformation in the $T_2$ generation has been confirmed by molecular biological techniques and high grain yield increased up to 25-50% in transgenic plants.

Transgenic wheat plants expressing the maize PEPC gene exhibited a higher photosynthetic capacity (up to 40%), increased PEPC-enzyme activity (up to 6-fold), appearance of $C_4$ Kranz leaf anatomy structure, and higher grain yield (up to 20-50%) than untransformed plants.

Implementation of new biotechnological techniques and new wheat strains could bring credible impact into agriculture of the main food strategic crop – wheat.

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