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Plastid Transformation of Wheat Cultivar Using Plastid Expression Cassette Carrying Nitrogen Fixation Genes

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CHLOROPLAST transformation in wheat was achieved by bombardment of callus from immature embryos. A wheat chloroplast site-specific expression vector pMY-Wt-nif, was constructed by placing an expression cassette containing neomycin phosphotransferase II (nptII) and nitrogen fixation gene cluster from *Clostridium acetobutylicum* in the intergenic spacer between trnI and trnA of wheat chloroplast genome. Integration of nitrogen fixation gene cluster in the plastome was identified by polymerase chain reaction (PCR) analysis and Southern blotting using specific sequence of the cassette as a probe. Expression of nitrogen fixation gene cluster was examined by Western blot. The results strengthen the feasibility of wheat chloroplast transformation and also give a novel method for the introduction of important agronomic traits in wheat through chloroplast transformation.

Keywords: Chloroplast transformation, Expression vector, *Clostridium acetobutylicum*

The vast majority of nitrogen atoms in living organisms are derived from the atmosphere by certain species of bacteria and blue-green algae that reduce dinitrogen (N_2) to ammonia. This process, "biological nitrogen fixation," is of fundamental importance to the ecosystem because organic nitrogen is not completely recycled but is continually lost to the atmosphere as N_2 by the action of denitrifying bacteria. Relatively little biological nitrogen fixation occurs in conjunction with the agricultural growth of the major cereal crops-corn, wheat, and rice-or the major forage grasses. To compensate for this nitrogen deficiency, farmers throughout the world use 40 million tons of nitrogenous fertilizers each year (Young, 1992), which are manufactured by energy-intensive processes. Nitrogen fixation is widely but sporadically distributed among both eubacteria and methanogenic archaea (Young, 1992 and Raymond *et al*., 2004). The current understanding of nitrogenase diversity has been based largely on phylogenetic analyses of *nifH* and *nifD*, the nitrogenase structural genes (Zehr *et al*., 2003 and Henson *et al*., 2004). Recently, Raymond *et al*. (2004) performed genomic analyses of *nif* genes encoding the core components of nitrogenase, including the *nifH*, *nifD*, *nifK*, *nifE* and *nifN* proteins. The plastids of higher plants have their own 120-160-kb genome that is present in 1,000-10,000 copies per cell.

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Engineering of the plastid genome (ptDNA) is based on homologous recombination between the plastid genome and cloned ptDNA sequences in the vector. Manipulations of ptDNA include (1) insertion of transgenes in intergenic regions; (2) posttransformation excision of marker genes to obtain marker-free plants; (3) gene knockouts and gene knockdowns, and (4) cotransformation with multiple plasmids to introduce nonselected genes without physical linkage to marker genes. (Maliga and Svab, 2011).

The success of plastid transformation in any species depends largely on successful DNA delivery to target plastids, transgene integration into the plastid genome followed by selection and regeneration of transplastomic plants. Although plastid transformation is relatively straightforward in species such as tobacco, the process has proved to be less efficient and sometimes impossible in other more economically important crops; for example, with the exception a single report of heteroplasmy in rice (Lee *et al.*, 2006), chloroplast transformation in cereals has not yet been established. It has been hypothesized that this is mainly due to the plastid characteristics in combination with less efficient regeneration characteristics.

The monocistronic translation of nuclear messenger RNAs (mRNAs) poses problems in engineering multiple genes in plants (Bogorad, 2000). Expression of polycistrons via the chloroplast genome provides a unique opportunity to express entire pathways in a single transformation event, such as nitrogen fixation gene cluster. Additionally, chloroplast genetic engineering is an environmentally friendly approach resulting in containment of foreign genes and hyperexpression (Daniell *et al*., 1998).

In the present paper, we reported the production of fertile, homoplasmic, chloroplast-transformed lines in wheat using a transformation vector that targeted the trnI / trnA region of the wheat chloroplast genome. Stable expression of nitrogen fixation gene cluster in the leaf tissues was confirmed by PCR, Southern blot and Western blot from T1 progeny seedlings. The successful transformation of chloroplasts offered the potential to introduce the functional genes for improving agronomic traits in wheat.

Material and Methods

Plant material and explant preparation

Mature caryopses of *Triricum aestivum* L., an Egyptian wheat variety with high yield production, were immersed initially in 70% ethanol for 1 min, followed by immersion in 30% commercial Clorox (containing 5.25% NaClO) supplemented with few drops of Tween 20 for 20 min. and washed several times with sterile distilled water before inoculation. Sterilized caryopses were aseptically germinated in 250 cm^3 jars containing 35-40 ml agar-solidified MS medium (Murashing and Skoog, 1962). All the operations and inoculations were

carried out under strict aseptic conditions in laminar airflow cabinet. The jars were kept in a cooled incubator with diurnal 16 hr light cycle. The temperature was regulated at 25±1°C. After ten days of seedlings growth, discs of approximately 3-4 mm in diameter from the leaves and cutting of 4-5 mm long sections of epicotyls were used for callus initiation.

Preparation of immature embryos explants

Immature embryos were isolated from field grown wheat (*Triricum aestivum* L.) under well-watered conditions (Agriculture Research Center, 2012). Spikes were collected from the plants 12-18 days post anthesis. Caryopses were surface-sterilized using 70% ethanol for 1 min. and 30% commercial Clorox supplemented with few drops of Tween 20 for 20 min. then Caryopses were washed four times using sterile distilled water. Immature embryos were isolated from the sterilized caryopses and used for callus initiation.

Induction and maintenance of callus cultures

Immature embryos and segments of leaves and epicotyls were cultured on sterile Petri dishes (9 cm in diameter) containing 20 ml of solid (0.8 g agarose) MS medium supplemented with 20 g/l sucrose, 150 mg/l L-asparagine and different concentrations of 2, 4-dichlorophenoxyacetic acid (1.0, 1.5, 2.0, 2.5 mg/l) alone or in combination with different 6-Benzyladenine concentrations (0.5, 1.0, 1.5 mg/l). Embryos were positioned with the epiblast exposed to the medium. The dishes were incubated in dark at 25±1°C for 13-15 days. After 30 Days of culture, callus induction frequency (CIF) was calculated as: CIF (%) = (No. of embryos produced calli) / (Total No. of embryos cultured) × 100. Sub-culturing was carried out after 2 weeks by cutting the induced callus into small pieces (150 ± 20 mg) and transferring them to fresh MS medium supplemented with 20 g/ sucrose, 150 mg/l L-asparagine and 2 mg/l 2,4-dichlorophenoxyacetic acid (2, 4-D).

Construct preparation for bombardment

60mg of gold particles (0.6μm) were placed into a 1.5ml microfuge tube. One ml of 70% ethanol was added to the particles, mixed for 5min at room temperature, and then incubated for 15min.The particles were collected by centrifugation at maximum speed for 5min, the ethanol was removed and the particles were resuspended in 1ml sterile H_2O, mixed for 1 min at room temperature, and then incubated for 1min. The gold particles were collected by centrifugation at maximum speed for 5sec. The H_2O wash steps were repeated three times, the supernatant after the fourth H_2O wash was removed, and then the particles were resuspended in 1ml of sterile 50% glycerol. While continuously vortexing the stock solution of gold particles, a 50ul aliquot were transferred to a fresh microfuge tube. The following additives were added: 2.5ul of plasmid DNA (2.5ug), 50ul 2.5M $CaCl_2$, and 20ul 0.1M spermidine. The microfuge tube continuously vortexed for 3min, the tube was incubated at room temperature for 1 min. to allow the particles to settle, and then collected by centrifugation at maximum speed for 15sec. The

supernatant layer was removed and replaced by 140ul of 70% ethanol without disturbing the pellet; the ethanol layer was removed and replaced by 140ul of 100% ethanol again without disturbing the pellet. The supernatant was removed and replaced with 50ul of ethanol.

Transformation of wheat plants

The microcarrier was placed in the metal holder of the gene gun apparatus, Biolistic PDS-1000/He Particle Delivery System (Bio-Rad Laboratories, Hercules,CA), using the seating device supplied by the manufacturer. The sheet was washed twice with 6ul of ethanol.

The prepared gold particle pellet was vortexed for 5sec, while suspension, 6ul of the particle slurry were quickly transferred and spread in the center of the microcarrier. The ethanol solution containing the DNA-coated particles allowed drying on the microcarrier. The microcarrier was loaded into the gene gun and then used to shoot the callus from a distance of 9cm.

Regeneration of transgenic plantlets

After bombardment, the calli were transferred to regeneration medium (4.4 g MS basal medium and 30 g sucrose, pH 5.8). When the medium cooled, TDZ (50 mg dissolved in 1 ml of DMSO) was added at different concentrations (0.0, 1.5, 2 and 2.5 mg/l). Calli were left to regenerate at 25°C under 16 hr photoperiod for 2 weeks. The regenerated shoots were transferred to the rooting medium (2.2 g MS basal medium and 30 g sucrose , pH 5.8). The recovery was performed at 25°C in the 12 h/12 h light/dark during regeneration and selection phases.

Confirmation of the vector integration and gene of interest expression

Transgene integration into wheat plastid genome was confirmed by PCR assay using internal primers I-F (which lands on the *npt*II gene) and I-R (which lands on the *nif* gene cluster). Another PCR assay performed using primers O-F (which land on the native chloroplast genome, 500bp upstream of the integration site), and O-R (which lands on the *npt*II gene). Both PCR reactions were performed in a total volume 50ul each containing 5ul (10x) Taq buffer contains 1.5 mM MgCl2, 1ul Taq enzyme (5unit/ul), 2ul dNTP mixture (2.5mM), 0.5ul I-F and I-R primer (100 Pmol/ul) or 0.5ul O-F and O-R primer (100 Pmol/ul), 5ul total DNA template and 36ul H_2O. The reaction mixture was subjected to amplification as following: Initial denaturation of DNA template at 95^{O}C for 2min, followed by 40 cycles of amplification with denaturing at 95^{O}C for 1min. annealing at 60^{O}C for 1min, the extension at 72^{O}C for 3min. and the final extension at 72^{O}C for 10min .

Southern blot analysis

Southern blot analysis was performed in order to investigate homoplasmy or heteroplasmy. The preparation of radioactive DNA Probe was performed using Amersham MegaPrime DNA Labeling Kit according to manufacturer instructions.

Blotting Procedure

Southern-blot analysis was performed using total genomic DNA isolated from untransformed and transformed leaves. DNA samples (5mg total DNA) were digested with *Mlu*I and *Nhe*I restriction enzymes and then were separated by gel electrophoresis on 0.8% agarose gels.

The hybridization probe was prepared by digesting the chloroplast transformation vector using *Mlu*I and *Nhe*I restriction enzymes.

Hybridization

The prepared probe was denatured by boiling at 100^{o}C for 5min, and then chilled on ice for 5min. The probe was added to 5ml hybridization solution, and then pre-warmed over night at 65^{o}C. The membrane was washed two times with 100ml of a pre-warmed solution of 1 x SSPE, 0.1% SDS at 65^{o}C for 20min, rinsed in 2 x SSPE, placed on 3MM paper wetted with 2 x SSPE and then sealed in a plastic bag. The pattern of hybridization was visualized on X-ray film by autoradiography using Kodak BioMax MS film.

Western blot analysis

The expression of the introduced *npt*II gene was authenticated using Western blot analysis The cell lysate from transformed and wild-type wheat leaves were extracted and prepared for analysis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Sambrook *et al*. (1989). The separated proteins were transferred onto a 0.2 µm Trans-Blot nitrocellulose membrane by electroblotting in Mini-Transfer Blot Module at 80V for 45 min. in Transfer. The membrane was placed in blocking solution and incubated for an hour at room temperature in a shaker. The membrane was hybridized with polyclonal anti-nptII serum and incubated for 2 hr at room temperature in a shaker. The hybridizing peptides were detected using horseradish peroxidase-linked secondary antibody. Blots were washed three times with PBST for 15 min. each time and then washed with only PBS for 10 min. Finally, 750 µl of 2x Stable Peroxidase Solution and 750 µl of 2x Lumi-Phos WB chemiluminescent reagent was poured over the membrane and a film was developed to visualize the bands.

Evaluation of gene of interest expression

Percent leaf organic nitrogen on a dry weight basis was determined for each plantlet with a Kjeltec Auto 1030 Analyzer (Tecator). Dried leaves were ground and digested at 42ºC for 45 min in 8 ml of concentrated sulfuric acid and 2 ml of 0.2% selenium in hydrogen peroxide.

Results

Callus induction and selection

The trials for inducing calli from leaves and epicotyls segments failed for all

tested media. Necroses of epicotyls and leaves explants were observed after few days of culturing. For immature embryos explants, callus formation was completely inhibited by the presence of 1.0 mg/l 2, 4-D as the sole growth regulator or in combination with all 6-Benzyladenine (BA) concentrations. 1.5 mg/l 2, 4-D produced poor calli with brown or yellowish brown colors. The highest 2, 4-D concentrations (2.0 and 2.5 mg/l) alone or in combination with 0.5 and 1.0 mg/l BA gave the highest significant CIF values (83.96-89.48%). 2, 4-D at concentration of 2.0 mg/l was chosen for induction and maintenance of derived calli for the subsequent experiments. BA was excluded, as it did not confer any extra beneficial physiological responses and its exclusion would further reduce the cost of production (Fig. 1).

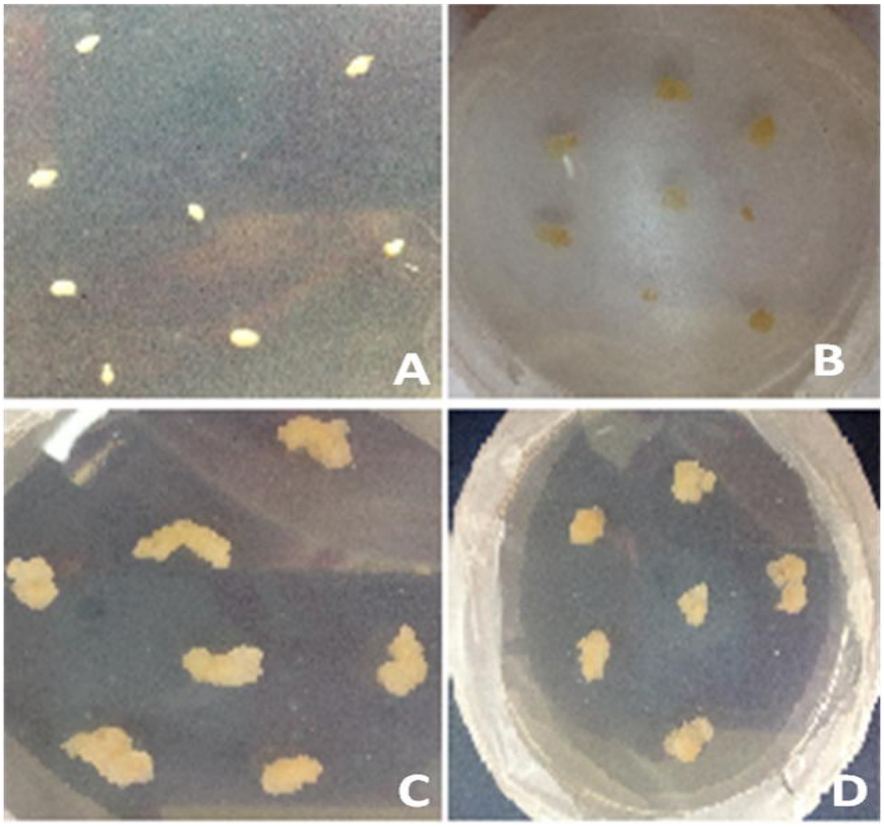

Fig. 1. Initiation and maintenance of callus cultures from *Triricum aestivum* L. embryos. (A) Wheat embryos cultured on callus induction media. (B) Induced callus from embryo explants. (C, D) Established embryos derived calli.

Regeneration of transgenic plantlets

After bombardment, the calli were transferred to regeneration medium. The supplement of growth regulators on media apparently facilitated the development of leaves from cultured calli. No adventitious leaves were produced when calli

cultured on MS media free from growth regulators supplementation. Levels of used hormone represented significant difference of regeneration. The most efficient induction rate and leaves development occurred on MS media containing 2 mg/l TDZ. As the concentration of TDZ increased over 2 mg/l, the leaves development potential of the calli tended to decrease. Four weeks later, the young shoots were transferred to the selective medium (Fig. 2).

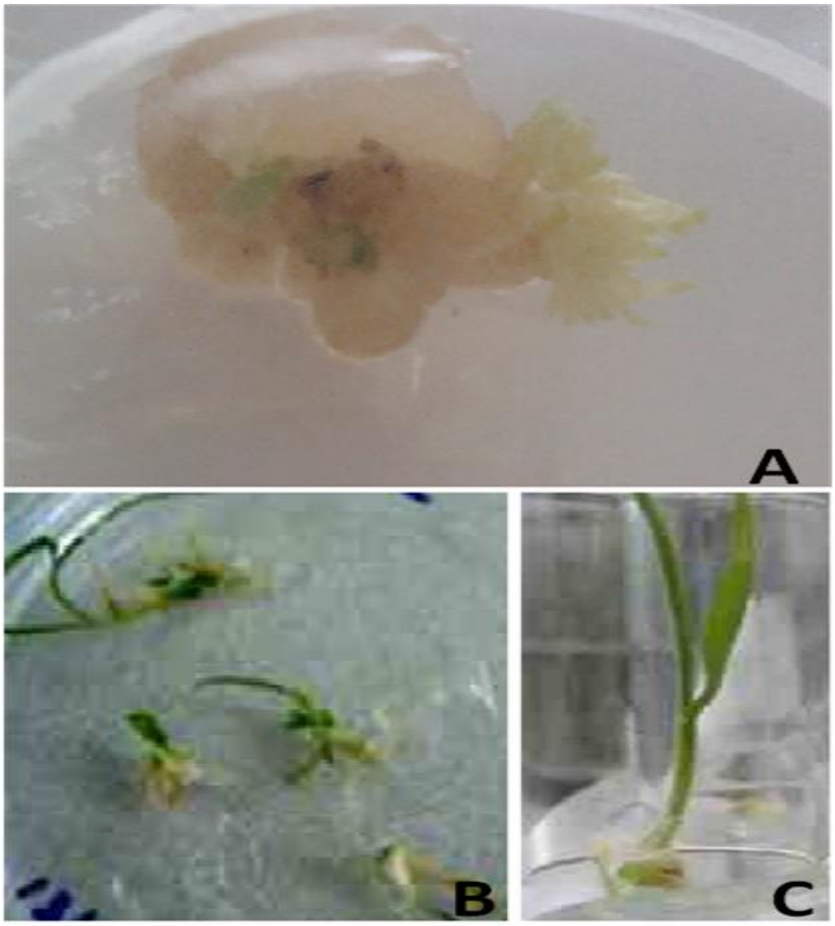

Fig. 2. *In vitro* plant regeneration from mature embryo of wheat after transformation. (A) and (B), leaves formation; (C), roots formation.

Confirmation of the vector integration and gene of interest expression

The transgene integration into wheat plastid genome was confirmed by PCR assay using internal primers I-F which lands on the *npt*II gene and I-R which

lands on the *nif* gene cluster, producing 2.6 kb PCR product (Fig. 3). In order to distinguish between nuclear and chloroplast transgenic cell lines, the external primer O-F which lands on the native chloroplast genome, 500bp upstream of the integration site, and O-R which lands on the *npt*II gene produced 2.6 kb PCR product.

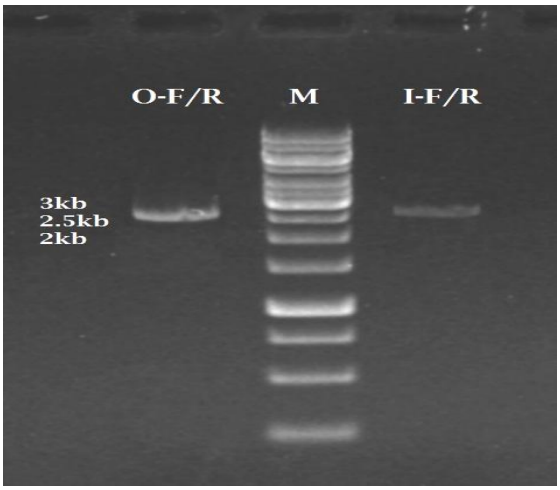

Fig. 3. PCR product (2.6 kb) from the external primer O-F which lands on the native chloroplast genome, and O-R which lands on the *npt*II gene. PCR product (2.6 kb) from internal primers I-F which lands on the *npt*II gene and I-R which lands on the *nif* gene cluster.

Southern-blot analysis

Southern blot analysis was performed in order to investigate homoplasmy or heteroplasmy, using total genomic DNA isolated from untransformed and transformed wheat plants generated from different transgenic cell lines. The hybridization probe resulted in 2.3 kb hybridization signal (Fig. 4).

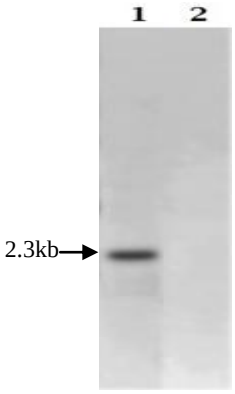

Fig. 4. Southern-blot analysis performed using total genomic DNA isolated from transformed wheat leaves (lane 1) and untransformed wheat leaves (lane 2). The probe used resulted in hybridization signal at the expected size 2.3 kb in the transformed plants only.

Western blot analysis

To further confirm the results of *npt*II gene activity in leaves, western-blot analysis was performed using crude extracts of transformed and untransformed wheat leaves. Fig. 5 shows the detection of *npt*II antigenic peptide.

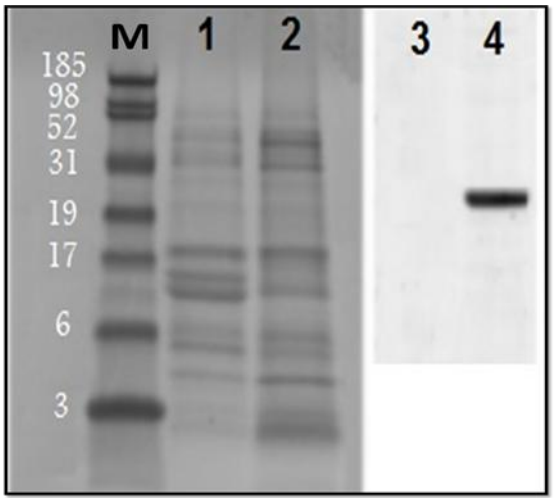

Fig. 5. Western blot analysis using polyclonal anti-*npt*II serum. Lane 1 and Lane 2 are SDS-PAGE for total protein of untransformed and transformed wheat leaves, respectively. Lane 3 and Lane 4 are *npt*II antigenic peptide detection in untransformed and transformed wheat leaves, respectively.

Evaluation of gene of interest expression

Percent of leaf organic nitrogen on a dry weight basis was determined (Table 1). The results indicate that the transplastomic wheat plants are able to form more organic- nitrogen than the control wheat plant.

TABLE 1. Determination of the percent of leaf organic nitrogen.

	Control	**Sample 1**	**Sample 2**	**Sample 3**
Flag leaf area (cm^2)	3	3	3	3
Leaf nitrogen (mmol m^{-2})	12+/- 3	19+/-1	18+/-3	17+/-5

Discussion

Our present efforts aimed to engineer nitrogen fixation machinery into wheat plant via chloroplast transformation system. In our lab, we constructed wheat chloroplast transformation vector (AlKhazindar *et al*., 2015). The chloroplast transformation vector, pMY-Wt-nif , was constructed by placing the expression cassettes containing nitrogen fixation gene cluster (*nif*) and the neomycin

phosphotransferase II gene (*npt*II) in the intergenic spacer between the *trn*I/*trn*A intergenic region.

Most cereal crops exhibit natural spectinomycin resistance, because of the presence of point mutations in their 16S rRNAs sequences. In addition, the spectinomycin has been reported to hamper the transgenic callus regeneration in sugar beet chloroplast transformation (Francesca *et al*., 2009). Therefore, we could not consider *aad*A gene encoding aminoglycoside 3'-adenylyltransferase as the selection marker gene. The *npt*II gene has been widely used in the wheat nuclear transformation and has also been applied in tobacco and cotton chloroplast transformation. So, *npt*II gene was chosen as the selection gene in this study.

There is no effective regeneration system for monocots' leaves and petioles yet, which is definitely hampering the development of chloroplast transformation in monocotyledonous cereal crops. The calli from rice seed were bombarded for rice plastid transformation and resulted in heteroplasmic fertile plants (Lee *et al*., 2006). In wheat, we used the callus of immature embryos which were routinely used for nuclear transformation because of their easy *in vitro* regeneration and capability of obtaining fertile progeny (Jones, 2005). There were no adult chloroplasts in the callus of immature embryos, the protoplastids were spread in the cells and could be bombarded (He and Lazzeri, 2001). Therefore, the delivered foreign genes could be integrated into the genome of protoplastids along with the embryogenesis into adult chloroplasts. Our results are in agreement with Cuiju *et al*. (2011) who successfully managed to achieve a stable chloroplast transformation of immature scutella of wheat.

The homologous recombination between the two prrn regions and plastome specific regulatory regions in the transformation vector might eliminate the *nif* gene cluster and nptII expression cassettes that had been integrated into the chloroplast genome, but there were very few relevant studies about this explanation (Daniell *et al*., 1991 and Wei-Hong *et al*., 2014). Alternatively, in order to achieve highly efficient regenerable positive plants, it needed to improve the selection pressure in the earlier stages of the targeted tissues.

The marker gene and *nif* gene cluster integration were confirmed by different PCR assays revealing that our cassette had been integrated into wheat plastome successfully. The Southern blot assay was performed in order to define the heteroplasmy or homoplasmy of our cultivar, resulting in homoplasmic transgenic plants and the fertile transplastomic wheat plants were obtained. The expression of both genes was confirmed by Western blot assay.

In this study, we compared the percent of leaf organic-nitrogen on a dry weight basis for the control plant and three transformed plants, and we found that the transplastomic wheat plants do have a higher percent of organic-nitrogen in their leaves.

Our results indicate that the transformation machinery of wheat plastome is efficient and our transplastomic wheat is ready for the field studies.

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التحوير الوراثى للقمح باستخدام كاسيت جينى التعبير يحمل جينات تثبيت النتروجين

مها محمد الخازندار ، السيد طارق عبد السلام ، اسماء عدلان، يسري الصادي
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الفكرة الرئيسية تعتمد على تحوير بلاستيدات نبات القمح من خلال تعديل الآجنة الغير ناضجة باستخدام كاسيت جينى التعبير يحمل علي ناقلات. أنشئ كاسيت جينى التعبيرعن طريق وضع الجين المعبر نيوميسين ناقلة الفسفات الثاني (*npt*II) للمساعدة علي انتقاء السلالات التي نجح تعديلها الوراثي، والتتابع الجينى لمجموعة جينات تثبيت النيتروجين (*nif* gene cluster) من بكتيريا وكلوستريديوم أسيتوبيوتيليكم. كما يحتوي الكاسيت الجينى علي تتابع جينى (trnI & trnA) من جينوم البلاستيدات و التى تمثل قطري الكاسيت. تم التأكد من عملية التعديل بواسطة تفاعل البلمرة المتسلسل (PCR) و تفاعل(Southern)، و تم الكشف عن التعبير الوراثي للكاسيت من خلال دراسة البروتينات (Western blotting) المسؤلة عن تثبيت النيتروجين من العينات ايجابية التحوير، كما تم تقيم أستقرار التعبير الوراثي للكاسيت من خلال قياس النيتروجين الكلي لأوراق النبات المحورة .