Generating Marker-Free Transgenic Wheat Using Minimal Gene Cassette and Cold-Inducible Cre/Lox System

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Generating marker-free transgenic wheat using minimal gene cassette and cold inducible Cre/lox system

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Abstract

The precise elimination of selectable marker genes is highly desirable, when their function is no longer needed, because their presence raised worldwide public concerns against the release of genetically modified plants. This is the first report of simultaneous application of the minimal gene cassette and cold-inducible Cre/lox recombination system in wheat. The *bar* selection and *cre*-recombinase genes were eliminated from T_0 and T_1 transgenic lines with 44% and 51% efficiency. This approach provides a new, reasonably effective technique to produce selection gene-free transgenic wheat lines either immediately after tissue culture, or from the subsequent transgenic generation. The advantage of this method is that it does not require any additional cold treatment during the plant regeneration/growing because the transgene elimination is ensured by the vernalisation. Application of this method prevents gene flow by pollen and seed, because the selection and recombinase genes are eliminated before pollen development, therefore reducing the risk of GM plants.

Introduction

Genetic modification has become routine for a growing number of plant species. It is no question therefore, how to introduce foreign genes into the host genome. However precise incorporation and controlled expression still present a challenge, because the most efficient methods allow random introduction of transgenes into the plant genome generating unpredictable effects on both transgene and native gene expression (Gidoni et al. 2008). Beside to the appropriate gene expression, the risk of utilization of transgenic plant would be decreased by controlling gene integration and transcription and thus the public concerns would be appeased.

The most controversial parts of the transformation systems are the selectable marker genes, especially antibiotic and herbicide resistance genes which are used to differentiate transgenic from non-transgenic lines. Several alternative methods have been elaborated for marker free transgenic plant production, as positive selection, a strategy that gives transgenic cells a metabolic advantage over non-transgenic cells (Reed et al. 2001; Zhu et al. 2008). Another approach is the selective elimination of selectable marker sequences from the transgenic plant when they are no longer needed. Foremost the normal segregation of co-transformed selection gene and gene of interest (GOI) were used (Matthews et al. 2001). Because of its simplicity, this method is widely used in different plant species, but it is

laborious and time consuming because of the several selection steps in progeny generations. In addition, the co-integration of the selection gene and GOI into the same locus makes the selective elimination unfeasible. Another option for marker gene elimination is the transposon mediated removal, which however also requires further segregation steps (Cotsaftis et al. 2002). An additional method is the site-specific recombination which proved to be the most efficient way of marker gene elimination because of its relative simplicity. The natural function of these systems is the control of the precise excision or integration of defined DNA units into the host genome (Gidoni et al. 2008; Nandy and Srivastava 2012). The phage mediated recombination was successfully used in plant biotechnology (Kempe et al. 2010; Kittiwongwattana et al. 2007). The sifting characterised systems used in plants and other organisms are Cre/lox of bacteriophage P1 of *Escherichia coli* (Hoa et al. 2002; Hoess et al. 1985; Sternberg and Hamilton 1981), R/RS from the SR1 plasmid of *Zygosaccharomyces rouxii*, and FLP/FRT from the 2-μm plasmid of *Saccharomyces cerevisiae* (Akbudak and Srivastava 2011; Hu et al. 2008). The Cre and FLP recombinases are members of the tyrosine recombinase family (Gilbertson 2003). The accurate integration or excision requires a pair of identical recognition target sites. The structure of the sites are palindromic, 34 bp or 31 bp in loxP, FRT and RS, respectively, containing two inverted repeats (12-13 bp) which are linked by 7–8 bp asymmetric spacer region. The enzyme recognises the palindrom sequence and cuts on the linking region. The most frequently used recombination approach in transgenic plants is the Cre/lox system. Recombination events have also been achieved in animal and plant systems (Kopertekh et al. 2004).

The prolonged expression of *cre* recombinase gene may cause adverse effects in the plants, such as leaf chlorosis, growth retardation, chromosomal aberrations and reduced proliferation (Coppoolse et al. 2003), therefore the strict regulation of manifestation is crucial. The limited expression of the recombinase gene can be achieved by using tissue specific, developmentally regulated or inducible promoters in self-excising *cre*-recombinase vectors (Gidoni et al. 2008). Numerous transformation systems, based on inducible promoters, were constructed, and used successfully for transgene elimination experiments in many species (Chong-Perez et al. 2012; 2013; Liu et al. 2005; Zuo et al. 2001). One of the most frequently used promoter is the heat inducible promoter which also proved to be the most efficient one, although its application is limited because of the thermal optimum of a given species. On the other hand low temperature is the natural part of the lifecycle and necessary for floral induction in many species especially in temperate overwintering cereals. Therefore the adaptation of the plants to low temperature is critical because it is one of the main abiotic stress factors. It is a complex process which results sweeping changes in gene expression, and *de novo* biosynthesis of stress-protective compounds (Kosová et al. 2012; Thomashow 1999). The genetic background of the low temperature adaptation was intensely studied. Several low temperature-responsive cDNA clones were characterized from wheat (Limnin et al. 1995). One of the most important group of cold-inducible dehydrins is the WCS120 (wheat cold-specific) protein family (Sarhan et al. 1997), which is represented by seven members (WCS200, WCS180, WCS120, WCS66, WCS40 WCS726 and WCS80), and display cryoprotective activities (Kosová et al. 2007, 2011, 2013). The most abundant member of this family is the WCS120 protein in cold-treated wheat. The *wcs120* gene is specifically activated by cold (Houde et al. 1992; Vitámvás et al. 2008), and the promoter analysis revealed that its expression is regulated by both ABA-dependent and ABA-independent signalling pathways (Sarhan et al. 1997). Other abiotic factors such as light, and water-stress also induced the *wcs120* gene, although the level of WCS120 protein was substantially reduced compared to cold induction (Houde et al.1992; Shen et al. 2003). The induction temperature and induction time depend on the genotype and growth habit (Kosová et al. 2013).

The most frequently used vector during biolistic plant transformation is the circular plasmid, which comprises undesirable bacterial DNA fragments along with the transgene, which is responsible for several negative effects (Fu et al. 2000). Fu et al. (2000) achieved simple integration events with a low copy number and rear rearrangement by the application of the minimal transgene cassette, while realizing the same transformation efficiency as with the application of the whole plasmids. Moreover, the lack of the vector backbone sequences reduces the negative effect of transgene on endogenous gene expression (Fu et al. 2000; Vidal et al. 2006).

The aim of our research was to study whether the minimal transgene cassette based cold inducible Cre/lox recombination system is suitable for marker gene elimination from transgenic wheat directly after the tissue culture process before the transgenic plants produces any pollen, and whether there is any difference between the elimination efficiency in the T_0 or T_1 generation.

Materials and methods

Plant transformation and regeneration

Plant material and growth conditions

Donor plants of the cv. "Cadenza" spring wheat variety were germinated for 3 days under dark condition, covered by wet filter paper, then were potted to Jiffy-7 pellet (www.jiffygroup.com) and were illuminated with 200 $\mu molm^{-2}s^{-1}$ light for 14 hours a day. The seedlings (Zadoks scale 11) were vernalised at 4°C for 2 weeks under low light intensity (20 $\mu molm^{-2}s^{-1}$). After the vernalisation, seedlings were raised at a day/night temperature of 18/16°C with 16-h daylength in greenhouse (Global Glasshouse Venlo) under 200 $\mu molm^{-2}s^{-1}$ light intensity.

Vector construction

A transformation cassette (pKPK1) was assembled for biolistic plant transformation based on pGem-T-Easy vector (Promega). It was generated by sub-cloning the new DNA construct (Fig. 1.) into the single *Not*I restriction site of pGem-T-Easy vector. The new DNA construct is flanked by fragments originated from the 5' UTR of wheat *glu-1D-1d* gene, raising the possibility of PCR-testing. The sequence of these two fragments is identical to X12928.5 from Gene bank, from nucleotide 2602 to 4279. Other elements of the new DNA construct were ligated into the *Hind*III site of *glu-1D-1d* at base 3460, thereby separating the above fragment to two pieces of about 800 bp lengths. The new DNA construct contained the recombination cassette flanked by mutant lox sites, *lox66* and *71* (Albert et al. 1995). The recombination cassette included the coding sequence of *cre* recombinase gene (Gene Bank accession number: X03453.1) under the control of wheat *wcs120* promoter (AF031235**)** and the selectable marker gene *bar* under the control of maize ubiquitin promoter. The maize ubiquitin promoter-driven *uid*A (in this project it is considered as GOI) was inserted outside of the lox sites. All the three genes (*cre*, *bar*, *uid*A) were fused to *Agrobacterium tumefaciens nos* terminator. Linear DNA was used for biolistic plant transformation, similarly as it was published earlier by Loc et al. (2002). DNA fragment (Fig. 1.) was cut off from the pGem-T-Easy based construct by *Not*I digestion and it was separated on agarose gel and purified from gel.

Transformation

Immature grains were collected 12–14 days after flowering, and were surface-sterilized with 70% (v/v) ethanol for 2 min, then for 15 min in 10% (w/v) Domestos with a few drops of Tween 20 followed by three rinses with sterile distilled water. The scutella, 1.5 mm size, were isolated and embryo axes were removed to prevent germination. Explants were placed scutellum side up onto callus induction medium. The nutrient media were prepared as

described by Tamás et al. (2004) based on Sparks and Jones (2004) method. After keeping them in the dark for two days, the scutella were transformed using a PDS-1000/He particle gun according to the manufacturer's instructions. A 28 Hgmm vacuum was created in the chamber and the helium (He) gas was injected into the space above the macrocarrier at a pressure of 900 psi. Gold particles with a diameter of 0.6 μm were suspended in ethanol at a density of 20 mg/ml and coated with pKPK1 linear DNA as described by Sparks and Jones (2004).

Plant regeneration

After bombardment the scutella were placed in fresh Petri dishes at an equal distance from each other and incubated in the dark at 23°C for three weeks. Scutella exhibiting callus formation were then transferred to shoot regeneration medium and kept in the light for a further three weeks. Root and shoot regeneration were induced by illumination with low intensity cool white light (20 $\mu molm^{-2}\ s^{-1}$) at a constant temperature of 23°C. After three weeks, callies exhibiting plant regeneration were placed on shoot regeneration medium containing 2 mg/l phosphinotricin (ppt) for selection. The selection step was then repeated again with the same conditions. Efficiency of plant regeneration was evaluated in the 3^{th}–4^{th} week. Plant regeneration was expressed as the percentage of calli producing shoots compared with the number of embryos isolated. The survivor plants were planted onto Jiffy-7 pellet (www.jiffygroup.com).

Cold treatment for Cre/lox activation

In the first experiment the plantlets were regenerated then selected with 2 mg/l phosphinotricin (ppt). The T_0 plants were acclimated by illumination with low intensity (20 $\mu molm^{-2}s^{-1}$) cool white light at a constant temperature of 23°C for seven days in Jiffy pellet. After the acclimatisation, seedlings were raised at a day/night temperature of 18/16°C with 16-h daylength in greenhouse under 200 $\mu molm^{-2}s^{-1}$ light intensity. The plants (Zadoks scale 14) were cold treated for two weeks at +4ºC under low light intensity (20 $\mu molm^{-2}\ s^{-1}$).

In the second experiment, the T_0 plants were regenerated by the same method as in the first but the selection agent (ppt) was already applied from the callus induction phase. The plantlets were cold treated immediately after the *in vitro* regeneration (Zadoks scale 11) for two weeks at +4ºC under low light intensity (20 $\mu molm^{-2}\ s^{-1}$).

In the third experiment the T_0 plants were regenerated in the same way as in the first experiment, but after acclimatisation the plants were grown up without any cold treatment at a day/night temperature of 18/16°C with 16-h day length in greenhouse (Global Glasshouse Venlo) under 200 $\mu molm^{-2}\ s^{-1}$ light intensity.

The germinated T_1 seeds were potted to Jiffy-7 pellet and were illuminated with 200 $\mu molm^{-2}\ s^{-1}$ light for 14 or 16 hours a day. The T_1 seedlings (Zadoks scale 11) were vernalised at +4°C for two weeks under low light intensity (20 $\mu molm^{-2}\ s^{-1}$). After vernalisation, the seedlings were raised at a day/night temperature of 18/16°C with 16-h day length in greenhouse under 200 $\mu molm^{-2}\ s^{-1}$ light intensity.

Transgene detection

Assay for GUS activity

The GUS expression level was determined on explants sampled after 3 weeks of callus induction using the histochemical GUS assay as described by Wu et al. (2003). Explants were incubated overnight at 37°C in buffer containing 1 m*M* X-Gluc, 100 m*M* sodium phosphate buffer (pH 7.0), 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide and 0.1% (v/v) Triton X-100. Blue spots were visually observed under the microscope.

Transgene detection by molecular markers

DNA isolation

Genomic DNA was isolated from the leaf tissue of T_0 and T_1 plants before and after cold treatment by Qiagen DNA isolation kit. DNA quality and quantity were determined by NANODROP 1000 spectrophotometer.

PCR reaction

The presence/absence of transgenes was detected in three replications. PCR-reactions were carried out in 15 µl volumes containing 20-40 ng genomic plant DNA, 1×Taq buffer (Fermentas), 0.5-1 µM primers, 1 mM dNTPs, and 1 U Taq polymerase (Fermentas). PCR reaction of primer pair 1,3,5 and 6 was performed by the following program: denaturation at 94 °C(5 min); 30 cycles of amplification at 94 °C (40 s), annealing (30 s), elongation 74 °C (40 s) per cycle; and a final elongation step of 72 °C for 7 min. The *uidA* gene was detected either by primer pair 1, overlapping the *ubi* promoter – *uidA* transgene border, or by the primer pair 2 described by Wu et al. (2003). This latter primer pair covers the coding region of *uidA* gene from base 379 to 1431. For the *bar* gene detection, two primer pairs were also applied. Primer pair3, overlapping the *bar* gene and the nos terminator, while the primer pair4 described by Wu et al. (2003.), covering the coding region from base 61 to 504. The presence of *cre* gene was detected by the primer pair 5. The forward primer attaches to the 3' end of *wcs120* promoter, while the reverse primer links to the 5' end of *cre*-recombinase gene, and the product size is 600 bp. Site-specific recombination events were also detected by PCR, using primer pair 6. The amplified fragment overlaps the recombination region. The PCR-product expected to be amplified if recombination occurred is 350 bp otherwise a 5400 bp is amplified from the original KG1-Vec8 cassette in principle. This latter product is however highly unlikely to be produced because of the defaults of this PCR-program, i.e. elongation for 40 s. The sequences of all the primers are listed in Table 1 and their binding sites are shown in Fig. 1.

Excision footprint sequencing analysis

Vector-cloned fragments were sequenced by using Big Dye Terminator technology (Life Technologies). Sequences were compared to sequences deposited in Gene bank by using the Emboss program (http://www.ebi.ac.uk/Tools/emboss/)

Results

Plant transformation, regeneration and transgene detection

In order to study the efficacy of wcs120 promoter for the induction of *cre* gene in a selection marker gene excision system, a transformation cassette (called pKPK1) was assembled. This transformation cassette consisted of two DNA fragments. The so called recombination part was flanked by mutant lox sites (lox 66 and lox71), while the other part is considered to be the GOI. The recombination part included the *cre* recombinase gene under the control of the cold inducible *wcs120* promoter and the *bar* gene driven by the constitutive ubiquitin promoter. The ubiquitin promoter-driven *uidA* gene was chosen as the gene of interest in this experiment because it was easy to study its activity on the protein level. Plant material was produced for the recombination experiments, by transforming spring wheat cv "Cadenza" immature embryos with pKPK1, using biolistic method in three independent transformation experiments, differing in the way of selection. The competency for PCR of genomic DNA, extracted from the regenerated plants was tested using Ppd-D1 primer pair which attaches to the endogenous wheat *Ppd-D1* photoperiod sensitivity gene on the short arm of 2D chromosome. PCR-positive samples to this gene were included exclusively in further analyses. The presence of the *uidA*, the *cre* recombinase and the *bar* genes were

followed in the regenerated plantlets, by using primer pairs 1-2, 3-4, and 5, respectively (Table 1.).

In the first transformation experiment, 2400 immature embryos were transformed. In total, 63 plantlets survived both selections. DNA was isolated from these plantlets (Zadock scale 12). Not all the phosphinotricin selected plants proved to be transgenic according to PCR based screening. The specific bands of *uidA* and *bar* genes were visible in 40 lines which means 1.66% transformation efficiency. 38 of them were cold treated and two were used as untreated control (Table 2.). There were two transgenic lines ($1T_0/2$, $1T_0/5$) lacking *cre*-recombinase gene but harbouring both *uidA* and *bar* genes

In the second transformation experiment 1800 immature cv "Cadenza" embryos were involved. The selection was carried out at the beginning of the callus induction. After three weeks of callus induction, the *uidA* gene activity was scored by GUS histochemical assay. 64% of the calli showed positive reaction. Overall 67 plants were regenerated. DNA was isolated from the leaves segments after the plant selection (Zadoks scale 11) during *in vitro* plant regeneration. Twenty-nine of 67 plantlets survived the cold treatment, that were included in the further examinations. The transformation efficiency was 1.55% in this experiment.

The specific product proving the presence of the *uidA* gene was identified in 24 transgenic lines (Table 2). The whole transformation cassette was missing only from one line ($2T_0/3$). There was incomplete integration in further three lines. The *uidA* gene failed to incorporate into the $2T_0/4$ and $2T_0/25$ lines, while the recombination cassette was absent from the $2T_0/12$ line. Interestingly there are three progenies carrying the *bar* gene, where the *cre* recombinase is missing ($2T_0/1$, $2T_0/10$, $2T_0/11$). We used these lines as control in the further recombination experiments.

In the third experiment setup 1400 cv "Cadenza" immature embryos were transformed. Three days after the transformation the presence of *uidA* gene was monitored by GUS assay as described by Wu et al. (2003). Transient gene expression was detected in 26% of the calli. 1100 plantlets were regenerated and put through of ppt selection in two consecutive steps for three weeks. Altogether, 130 resistant plants were selected and potted out, but only 19 plants survived the acclimatisation. The T_0 plants were grown up in the greenhouse at a day/night temperature of 18/16°C to avoid the *cre*-recombinase gene induction.

DNA was isolated from the leaves at the two leaf-stage (Zadoks scale 12). The specific products of *uidA* and *bar* gene have been identified in 16 and 17 plants, respectively (Fig. 2.). No transgene incorporation was detected in two plants ($3T_0/3$, $3T_0/7$). Incomplete integration of the transformation cassette was observed in one plant ($3T_0/11$), where only the *bar* gene was detected. Sixteen independent transgenic plants were identified with both genes. The progenies of these lines were grown up in greenhouse without any cold treatment.

Elimination of *bar* selection and *cre*- recombinase genes by cold inducible Cre-lox system

In order to achieve selection marker gene removal, cold treatment was optimised for plants in different generations and developmental stages. Recombination frequency was determined by PCR using two different approaches. One was to detect the presence of *bar* and *cre* genes, while the other one was to screen for the recombination footprint. Primer pair 6. is able to bind outside of the recombination part (>4550 bp, Fig. 1) but due to the reaction conditions amplified fragment (~350 bp) can only be detected when recombination has taken place.

In the first experiment, 38 T_0 plants (Zadoks scale 14), after the seven days acclimatisation, were cold treated for two weeks at + 4ºC, and DNA were isolated from the leaves (Zadoks scale 16) two weeks after the cold treatment. Two transgenic lines carrying all the three transgenes were used as untreated controls. After the cold treatment the presence of all transgenes was studied by PCR primers (Table 1). The *uidA* gene was detected in all lines.

Both the *bar* selection and the *cre*-recombinase genes were eliminated from seven plants (17.5%). The lines, which failed to carry the *cre*-recombinase gene ($1T_0/2$, $1T_0/5$), did not lose the *bar* gene under cold treatment. (Table 2).

In the second experiment, 27 T_0 plantlets were cold treated for two weeks at + 4ºC immediately after *in vitro* phase (Zadoks scale 11) without any acclimatisation. DNA was again isolated two weeks later (Zadoks scale 14). Two transgenic lines carrying all the three transgenes were used as untreated controls (Table 2). The specific product of *uidA* (GOI) gene was identified in 24 lines as before cold treatment, and the histochemical staining was also positive (Online Resource 1). The *bar* selection and *cre*-recombinase genes were detected by 4 and 5 primer pairs in 12 and 15 lines respectively. Twelve transgenic lines lost both the *bar* selection and the *cre*-recombinase genes. The incorporation of the transformation cassette was incomplete in the $2T_0/4$ and $2T_0/25$ line, as only the recombination part of the cassette was detected before cold treatment. One of them ($2T_0/25$) lost the *bar* and *cre* under cold. In three lines ($2T_0/1$, $2T_0/10$, $2T_0/11$), which failed to incorporate the *cre*-recombinase gene, the *bar* gene still existed after the cold treatment. The recombination between the two lox sites occurred exclusively, in the cold treated plants carrying the *cre*-recombinase gene. Two weeks cold treatment at +4ºC induced the Cre/lox recombination system in 44.4% of the plants at one leaf stage (Zadoks scale 11).

The estimation of Cre/lox system activation based on the recombination footprint was higher (70%) than based on the direct PCR amplifications of *cre*-recombinase and *bar* selection genes (44.4%). Seven plants produced both the specific product of the remaining part of cassette and the *bar* selection genes in the second experiment. A remarkable difference could be observed in *cre* gene activation based on the results of two recombination event detection approaches.

Thirty-four T_1 plants of three T_0 plants ($3T_0/1$, $3T_0/2$, $3T_0/4$) from the third transformation experiment were grown up in the greenhouse at 18/16 °C day/night temperature. In all the three transgenic lines the T_1 progenies originated from the main shoot and two side tillers. DNA was isolated form the plants in two leaves stages (Zadoks scale12), after that the wcs120 promoter-driven *cre*-recombinase gene was activated by two weeks cold treatment at + 4ºC. Two weeks after the cold treatment DNA were isolated again (Zadoks scale14). The presence of *uidA* gene was tested by primer pair 1 (Table 3). The specific 450 bp fragment was identified in three-quarters of offspring lines of $3T_0/2$ and $3T_0/4$ plants. The transgene segregation ratio was 3:1, which suggests that the gene was incorporated into the T_0 plant in one copy. In the case of $3T_0/1$, however 33% of the offspring lines carried the *uidA* gene. When the presence of the gene was examined in the descendants of the main shoot and the two side tillers separately, it turned out that three-quarter of the offsprings from the main shoot carried the *uidA* gene, but none of the tiller descendants contained it, which underline the chimeric nature of the $3T_0/1$ plant. Nevertheless the segregation of GOI for the progenies of the main shoot could again refer to one copy number of *uidA* gene. The presence or absence of the *cre*-recombinase gene was detected by primer pairs 5 in the T_1 lines containing the *uidA* gene. After the cold treatment the 600 bp long product was absent from the 66%, 46% and 40% of $3T_0/1$, $3T_0/2$ and $3T_0/4$ transgenic lines, respectively. The average efficiency of *cre*-recombinase system was 51% in T_1 progenies.

Sequencing the incorporated DNA after recombination

The sequence of the recombination footprint was determined in a recombination positive plant ($2T_0/8$). For this, the PCR product amplified with 6F and 6R primers was cloned into pCR2.1 vector (Invitrogen) and sequenced using M13 primers. The results indicated that a correct site-specific recombination has occurred in this plant (Fig. 3.).

Discussion

The most frequently used vector during biolistic plant transformation is the circular plasmid, comprising undesirable bacterial DNA fragments along with the transgene, which can be responsible for several negative effects (Fu et al. 2000). To avoid these problems, linear minimal cassettes have been used for biolistic transformation of wheat immature embryos in our experiments. The vector carried the *uid*A (GOI) gene outside of the lox-flanked region. Between the two lox sites, the recombination part, of the transformation cassette consisted of the cold inducible promoter-driven *cre*-recombinase gene and the *bar* selection marker gene, under the control of maize ubiquitin promoter. Although, only the minimal cassette was used in the present experiments, the results can be compared to our data published previously where the same genotype (cv. "Cadenza") and transformation method was applied. Transformation efficiency was slightly increased (~1.5%) using linear DNA rather than a circular plasmid, pAHC25 (0.8%) (Tamás et al. 2009). This result is in accordance with previously published efficiency data for biolistic transformation of wheat. It was reported to be around 1.6% in the majority of the transformation experiments published (Li et al. 2012) although it reached 12-20% in some case (Ogawa et al. 2008; Wright et al. 2001) or even exceeded 70% (Pellegrineschi et al. 2002), where whole circular plasmids were used. The transgene integration efficiencies were found to be the same (Fu et al. 2000) for the minimal cassette and for the whole plasmid transformation. On the contrary Uzé (1999) and Yao and co-workers (2007) observed increased transformation efficiency while using minimal cassette, instead of the whole plasmid (2.75 to 3.56% and 0.4 to 1.1%, respectively). Tassy and co-workers (2014) obtained 2.5% transformation efficiency by cassette in wheat.

In our experiments, the segregation of GOI was determined in the 7-18 progenies of only three independent transgenic lines. Although all of them have one copy of the transgene, based on the 3:1 segregation ratio according to the Mendelian fashion in T_1 generation, the accurate determination of the copy number would require higher numbers of the progeny of the independent transgenic lines. Simpler incorporation pattern and lower copy number were identified in other species also (Fu et al. 2000; Kumar et al. 2010; Loc et al. 2002). Yao et al. (2007) detected only one or two copies of transgenes in wheat. Tassy et al. (2014) identified only one copy of transgene in all transgenic wheat, bombarded by gen cassette. However there were no differences in the copy number either the plasmid or the minimal cassette was used in tomato and grapevine (Romano et al. 2003; Vidal et al. 2006).

Cre/lox auto-excision system was successfully applied for eliminating the undesirable parts of the transformation vectors as the selection marker gene, in many species (Gidoni et al. 2008; Tuteja et al. 2012). For the induction of *cre*, in auto-excision strategy, many types of promoters have already been tested with different efficiency (Chakraborti et al. 2008; Chong-Perez et al. 2012, 2013; Russell et al. 1992). In our investigation a cold inducible promoter based Cre/lox mediated selection gene elimination system has been tested. Cold induction of wcs120 wheat promoter has been applied for *cre* gene activation, at one, two and four leaf-stage for two weeks at +4°C. This cold treatment proved to be sufficient for cre-recombinase gene activation, as the lox site flanked selection marker gene (*bar*) and recombinase gene (*cre*) were removed from transgenic plants, while the GOI (*uidA*) still remained in the wheat genome. We detected 17-60% efficiency of marker gene elimination by the Cre/lox recombination system. The highest activation was observed when the plants were cold treated at one or two leaf-stage, which was independent of the progeny generation (T_0 or T_1). Similar wide range of excision efficiency was reported in the literature using chemically, or heat shock induced or embryo specific Cre/lox systems (Chong-Perez et al. 2012; Liu et al. 2007; Zuo et al. 2001). Wang et al. (2005), however, reported lower variation in heat shock promoter activity. The efficiency of *cre* transgene activation may depend on the transgene

incorporation site or on the developmental stage of the transgenic plants when *cre* gene activity is induced (Russell et al. 1992). In our first and second experiments, the selection and recombinase genes were eliminated from several independent transgenic lines, indicating that the integration sites are probably of less importance.

Prolonged activation of cre-recombinase, causes aberration in plant development (Coppoolse et al. 2003), therefore the application of the transient expression or inducible promoters are required for *cre* gene activation. The Cre/lox recombination system based on a tissue specific promoter was successfully applied to eliminate selection gene with nearly the same efficiency as in our experiment (Bai et al. 2008; Hu et al. 2013). Mlynárová et al. (2006) and Moravčíková et al. (2008) reported efficiency as high as 99% and 96% using microspore- and seed specific cruciferin promoter controlled Cre/lox excision of the marker gene. The application of inducible promoters is another alternative of controlled gene expression. *Cre* induction had been demonstrated by expressing this gene under the control of a chemically inducible promoter with 29-66% efficiency (Sreekala et al. 2005). Ma and co-workers (2008) applied approximately the same recombination vector structure as in the present work with nearly the same efficiency (38%), but Cre/lox- recombination system was under the control of the salicylic-acid-regulated promoter. The heat shock-inducible Cre/lox system is the most extensively applied in many species. The efficiency of this system differed from our results. The lowest level has been detected in potato shoot internodes (5-14%) (Cuellar et al. 2006), Wang et al. (2005) have reached 70-80% Cre/lox activity in tobacco seed and leaf, while the excision rate has even reached 100% in tobacco seedlings (Liu et al. 2005). Heat shock can cause damage in wheat tissues therefore it is not the most proper way of inducing gene expression. Although there is no information about cold induced Cre/lox auto-excision system in the literature it seems to be an adequate way to induce site-specific excision of undesirable genes. Cold-inducible *cor15a* promoter was used with success in sugarcane transformation (Belintani et al. 2012). Quellet et al (1998) proved the strict cold regulation of *wcs120* promoter. In our study the same temperature was applied as in mentioned work of Quellet et al. (1998).

Estimation of Cre/lox system activation efficiency was higher (70%) based on the recombination footprint, than based on elimination of *cre*-recombinase and *bar* selection genes (44%). The explanation for this can be that the recombination was not complete in the whole plant in 26% of the transgenic lines. Recombination took place in some cells, while the Cre/lox system failed to activate in the others resulting in chimeric T_0 plants. Another source of ambivalent character of plant can be that the cells emerging before activation of the autoexcision system contain the *bar* and *cre* genes while the latter formed celles may lack of the *bar* and *cre* genes. The selection gene elimination by Cre/lox system resulted chimeric plants in other species as well. The ratio of chimeric plants may depend on the genome size, developmental stage (Russell et al. 1992) and the promoter of *cre* gene (Bai et al. 2008). In summary, the efficiency of the cold inducible Cre/lox recombination system presented here is comparable with the other constitutive, organ specific or inducible promoter based Cre/lox mediated transgene elimination methods.

The recombination activity in T_1 progenies was found to be around 50%, which was very similar to the level measured in the T_0 transgenic lines. Blechl and co-workers (2012) carried out a Bxb1 recombinase mediated site specific deletion in wheat. All T_1 descendants, carring Bxb1 recombinase, had recombinase activity underlining that the recombinase gene retains its activity in the subsequent generations. Moreover, we could induce the wcs120 promoter in the T_1 generation with the same efficiency as in the T_0 generation.

This is the first report of the simultaneous application of the minimal gene cassette and cold inducible Cre/lox recombination system in wheat. This approach provides a new effective technique to produce selection marker gene-free transgenic wheat lines immediately

after tissue culture or from the subsequent transgenic generation. The cold treatment necessary for activating the marker gene elimination did not require any additional steps in the plant regeneration/growing, because it took place parallel to the saturation of the vernalisation requirements of the cereals with winter growth habit. The application of this method prevents the gene-flow by pollen and seed, because the selection and recombinase gene are eliminated before pollen development, therefore reducing the risk of GM plants. Our system represents a stable and efficient biolistic wheat transformation system for generating transgenic wheat plants without undesirable sequences: the plasmid backbone and selection marker genes.

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Fig. 1: Schematic representation of the assembled minimal DNA-construct ligated into *Not*I site of pWBVec8. Primer binding sites are also shown. The new DNA construct contained the recombination cassette flanked by mutant lox sites, *lox66* and *71*, and included the coding sequence of *cre* recombinase gene (Gene Bank accession number: X03453.1) under the control of wheat *wcs120* promoter (AF031235**)** and the selectable marker gene *bar* under the control of maize ubiquitin promoter. The gene of interest (GOI), the maize ubiquitin promoter-driven *uid*A was inserted outside of the lox sites. All the three genes (*cre*, *bar*, *uid*A) were fused to *Agrobacterium tumefaciens nos* terminator.

Fig. 2: The presence or absence of *uidA* (GOI) and *bar* selection genes in T_0 transgenic lines from third experiment.

Fig. 3: Sequence of the recombined region (upper lines) aligned to the expected sequence (lower lines).

Figure1
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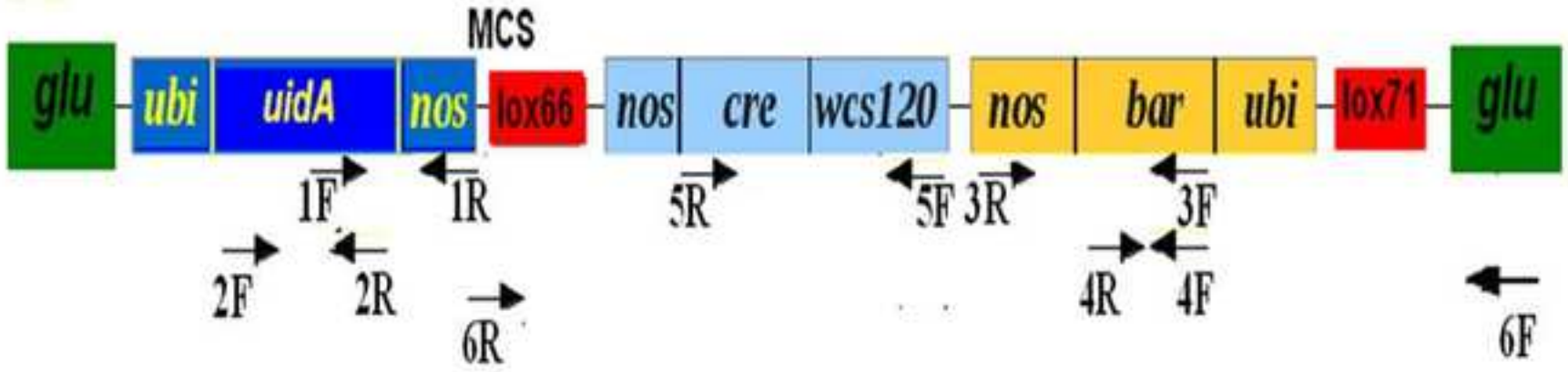

Figure2
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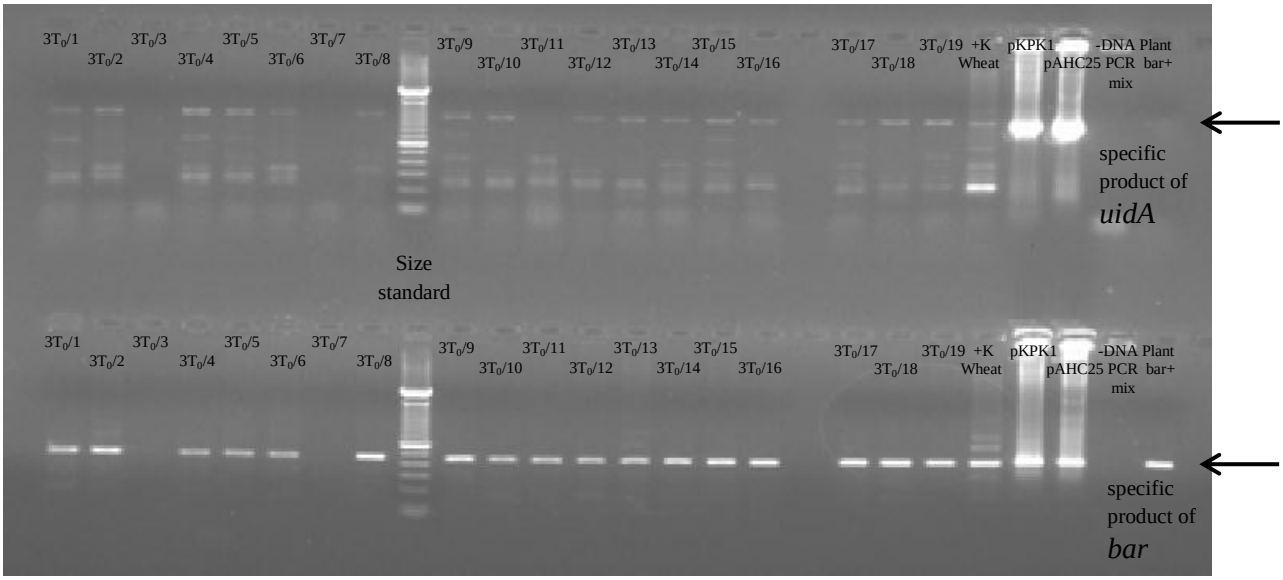

Figure3

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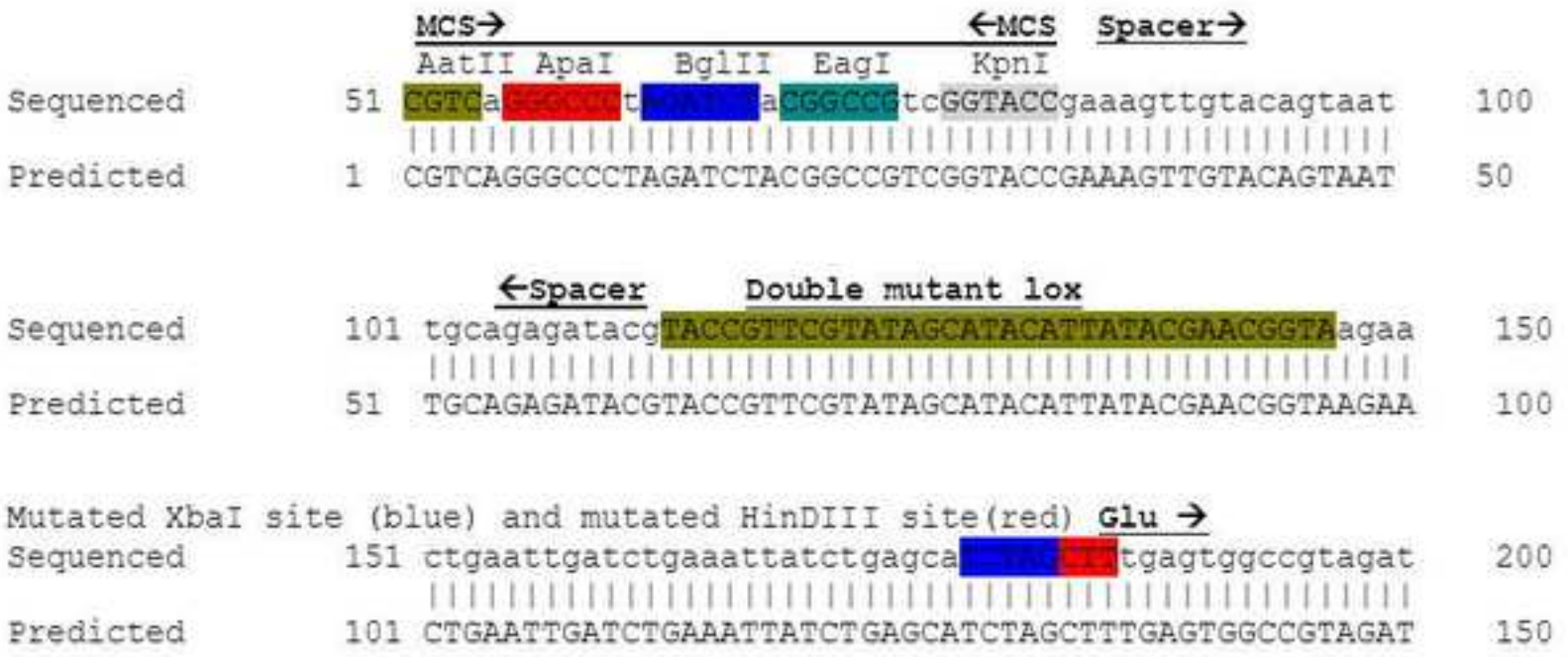

Table1
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Table 1: PCR primers for *uidA* (GOI), *bar* selection, *cre*-recombinase genes and mutant lox site detection.

Gene	Primer	Primer sequence	Anealing temperature	Product size
uidA	*1F*	5'-ACTGGCATGAACTTCGGTG-3'	58 °C	450 bp
	1R	5'-TAACATAGAT GACACCGC-3'	58 °C	
	2F	5'-AGTGTACGTATCACCGTTTGTGTGAAC-3'	62 °C	1051 bp
	2R	5'-ATCGCCGCTTTGGACATACCATCCGTA-3'	62 °C	
bar	*3F*	5'-CACGCTCTAC ACCCACCTG-3'	58 °C	500 bp
	3R	5'-ATAATTTATCCTAGTTTGCG-3'	58 °C	
	4F	5'-GTCTGCACCATCGTCAACC-3'	57 °C	444 bp
	4R	5'-GAAGTCCAGCTGCCAGAAAC-3'	57 °C	
cre	*5F*	5'-TAGTAGATTTCCCGAGTGAG-3'	50 °C	600 bp
	5R	5'-TATCTTTAACCCTGATCCTG-3'	50 °C	
mutant lox site	*6F*	5' GATTTGCTGCTCGTATTGTC3'	55 °C	350 bp
	6R	5' TCGGTTGGAAAAGCGCAG 3'	55 °C	
Ppd-D1	*F*	5'-GATGAACATGAAACGGG-3'	56 °C	320 bp
	R	5'-GTCTAAATAGTAGGTACTAGG-3'	56 °C	

Table2
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Table 2: Presence of *uidA* (GOI), *bar* selection and *cre*-recombinase genes before or after cold treatment in the transgenic lines.

Experiment	**No. transgenic lines**	**Presence of transgenes before cold treatment**			**Presence of transgenes after cold treatment**			**Recom-bination**	**No. *bar* and *cre* gene free plants**
		uidA	*cre*	*bar*	*uidA*	*cre*	*bar*		
1	38	38	36	38	38	29	31	7	7
2	27	24	22	25	24	10	13	19	12
3	19	16		17					

Table3
Click here to download Table: Table 3.docx

Table 3.: Result of cold treatment in T_1 offspring lines

Ancestor lines	**Treated T_1 plants**	***uidA* + plants**		**Plants lost *cre*-recombinase**	
		No.	**% of total**	**No.**	**% of total**
$3T_0/1$	9	3	33.33	2	66.66
$3T_0/2$	18	12	77.77	6	45.15
$3T_0/4$	7	5	71.42	2	40.00