Chapter 18
High Efficiency Wheat Transformation Mediated by *Agrobacterium tumefaciens*

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Abstract Wheat is one of the most important cereals for humans but quite recalcitrant in transformation. We have thoroughly examined every aspect of the wheat transformation protocols mediated by *Agrobacterium tumefaciens* and we were able to identify and optimize the key factors. Immature embryos isolated from healthy plants grown in a greenhouse were pre-treated with centrifuging and co-cultivated with *A. tumefaciens*. The frequency of transformation (independent transgenics/explant) was between 50 % and 60 % were routinely observed and higher than 90 % were recorded in the best cases. Not surprisingly, the key factors did not differ much from those in other cereal plants such as rice and maize. Both bar and hpt genes were good as selection markers. Fielder, a spring wheat cultivar, constantly showed high efficiency of transformation by our protocol. We have been able to obtain transgenic plants from the embryos harvested from the greenhouses throughout the year. Most of the transformed plants were normal in morphology and fully fertile. More than 40 % of the transformants had a single copy of the transgenes, which were inherited in a Mendelian fashion in most of the lines analyzed. Transgenic wheat has been generated at high frequency by several research groups by our protocol by now. Therefore, wheat has finally joined the list of cereals that can be efficiently transformed.

Keywords *Agrobacterium tumefaciens* • Cereal • Genetically modification • Transformation • *Triticum aestivum* • Wheat

Introduction

Transformation is an essential technology in both applied and basic studies in wheat. The first transgenic wheat was produced by particle bombardment method in the early 1990s (Vasil et al. 1992). Soon after efficient protocols of transformation mediated by *Agrobacterium tumefaciens*, which can generally transfer low copy

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numbers of large DNA segments with defined ends to plant chromosomes with few rearrangements, were developed in rice (Hiei et al. 1994) and maize (Ishida et al. 1996), wheat was also transformed by *A. tumefaciens* (Cheng et al. 1997) and quite a few reports followed. However, the progress thereafter made in wheat was slow (Przetakiewicz et al. 2004), while significant improvement in the efficiency of gene transfer and in the range of transformable genotype was made continuously in other cereals (Hiei et al. 2006; Ishida et al. 2003; Frame et al. 2006; Bartlett et al. 2008). Embryos and cultured cells of several wheat genotypes were tested, and various factors were examined in the early 2000s, however, the frequency of transformation was mostly less than 5 % of the inoculated tissue pieces. Even in the recent reports (He et al. 2010; Bińka et al. 2012), the frequency of transformation in wheat did not change much from those described in the early reports.

We examined any and all factors possibly involved in wheat transformation. Since we have an experience of development of efficient transformation protocol for rice and maize, we tried to include the factors, which were important in transformation of rice and maize and not tested in wheat. We were able to find a good combination of the parameters and to develop a highly efficient protocol for wheat transformation, which is hereby described.

Materials and Methods

Spring wheat cultivars, Bobwhite and Fielder, were cultivated in a greenhouse. Immature seeds were collected from panicles about 2 weeks after anthesis, sterilized with 70 % ethanol and 1 % sodium hypochlorite and then washed three times with sterilized distilled water. Immature embryos were isolated from the seeds under stereoscopic microscope.

Agrobacterium tumefaciens strain EHA101 and EHA105, and the vectors pIG121Hm (Hiei et al. 1994), pLC41bar and pLC41Hm were mainly used in this study. The vectors pLC41bar and pLC41Hm had the T-DNAs, which carried a gene for β-glucuronidase (GUS) that contained an intron in the coding sequence and a phosphinothricin (PPT) resistance gene for pLC41bar and a hygromycin (Hm) resistance gene for pLC41Hm respectively.

Isolated immature embryos were treated with centrifuging at various strength in the liquid medium and then inoculated with *Agrobacterium*. The embryos were placed on co-cultivation medium that contained 5 μM of $AgNO_3$ and/or $CuSO_4$ with the scutellum-side up and incubated at 23 °C in the dark for 2 days. Embryo axis was excised and the embryos were transferred to resting medium and incubated at 25 °C in the dark for 5 days. Some of the embryos after resting culture were examined histochemically for transient expression of GUS gene according to the procedure described by Ishida et al. (2007). The embryos without used for GUS assay were placed on the first selection medium that contained 5 mg/L of PPT or 15 mg/L of Hm and incubated for 2 weeks. Each of embryos was cut into two pieces, which were then transferred to the second selection medium that contained 10 mg/L of

PPT or 30 mg/L of Hm and incubated for 3 weeks. The cell clumps proliferated from the pieces were placed on regeneration medium including 5 mg/L of PPT or 30 mg/L of Hm and incubated at 25 °C under continuous illumination (35 μmol m^{-2} S^{-1}) for 2 weeks. Regenerated shoots were transferred to regeneration medium that contained 5 mg/L of PPT or 15 mg/L of Hm and incubated for 2 weeks. Regenerated plants were transferred to soil in pots and grown in a greenhouse.

Results

Preliminary Study

Agrobacterium strain EHA105 and LBA4404 were compared in the preliminary study. Because the GUS expression in the embryos infected with LBA4404 was generally weaker than that in the embryos infected with EHA101 and EHA105, LBA4404 was not examined further.

The preliminary study also revealed that centrifugation of the immature embryos at 5,000 × g or 20,000 × g for 10 min before the infection and the excision of the embryo axes from the immature embryos 2 days after the infection resulted in higher expression of GUS in the embryos after the co-cultivation. These conditions were taken into the design of the optimization experiments described below.

Immature embryos that were between 2.0 mm and 2.5 mm in length along the axis of Bobwhite and another cultivar, Fielder, were infected with EHA101 (pIG121Hm) in the optimization experiments. Firstly, further addition of salts to the co-cultivation medium was examined. Co-cultivation medium that contained 5 μM each of $AgNO_3$ and $CuSO_4$ was better than that without both or either one of the salts in the level of transient expression of GUS in and of callus formation from the embryos after the resting culture in both genotypes, employed in the rest of the optimization experiments, and taken into the recommended protocol.

Then, the timing of the removal of the embryo axes and the strength of centrifugation before the infection were revisited. The excision of axes 2 days after the infection and centrifugation at 20,000 × g were good in terms of both the transient expression of GUS in and the callus formation from the embryos after the resting culture in both genotypes. Thus, these processes were taken into the final protocol.

Production of Transgenic Wheat

Immature embryos that were between 1.0 mm and 3.0 mm in length along the axis of Fielder were co-cultivated with EHA105 (pLC41bar) or EHA105 (pLC41Hm) according to the protocol determined in the optimization experiments, and transgenic wheat was produced. In these experiments, the immature embryos in different size ranges were compared.

Table 18.1 Typical results of transformation

Vector	Size of immature embryo (mm)	Immature embryos inoculated (A)	Embryos produced resistant plants (B)	Frequency of transformation (B/A, %)
pLC41bar	1.5–2.0	17	8	47.1
	2.0–2.5	46	34	73.9
		90	67	74.4
		40	38	95.0
		17	13	76.5
pLC41Hm	1.0–2.0	14	3	21.4
	2.0–2.5	12	7	58.3
		17	14	82.4
		35	24	68.6
	2.0–3.0	47	28	59.6

Transformation of wheat was conducted efficiently under most of the combinations of the factors listed in Table 18.1, and the highest frequencies of transformation, ranging between 58.3 % and 95.0 %, were observed when the immature embryos of between 2.0 mm and 2.5 mm of Fielder were tested and the selection was made by the bar gene and hpt gene.

Characterization of the Transgenic Wheat

The transformants of wheat thus produced (T_0 generation) were all normal in morphology and fully fertile (Fig. 18.1). The GUS was well expressed in the tissues of leaves, roots, reproductive organs and the seedlings of the next generation of the transformants (Fig. 18.1).

Some of the transformants were analyzed by Southern hybridization. The integration of the T-DNA was clearly demonstrated, and the six transformants out of the 14 shown had a single copy of the transgene. The copy number of the transgene in the other lines was mostly two or three.

The inheritance of the drug resistance was examined in the T_1 generation. The hygromycin resistance segregated in 3:1 ratio in the progeny of all four Bobwhite transformants examined. The phosphinothricin resistance segregated in 3:1 ratio in the progeny of six Fielder transformants, in 15:1 in four lines and in 63:1 in three lines. One line showed none of these patterns, but, because presence of such a pattern at a low frequency among the transformants is quite normal, it was not investigated further to see whether the sensitiveness was linked to the absence of or loss of the expression of the transgene. Overall, it is evident that the expression of the transgenes was inherited to the progeny in Mendelian fashion.

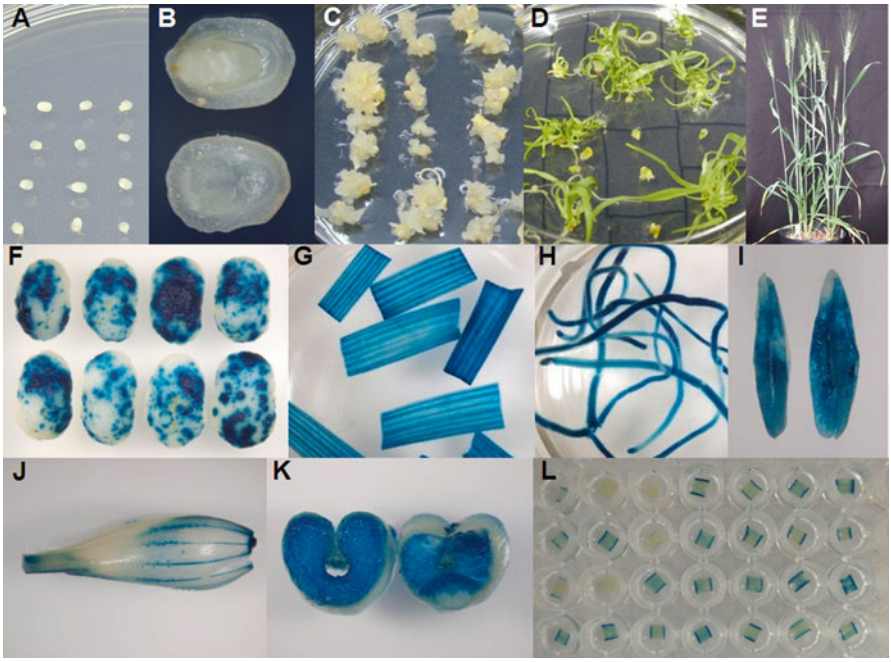

Fig. 18.1 Cells and plants derived from immature embryos of cv Fielder infected with *A. tumefaciens* EHA105 (pLC41bar) and expression of GUS gene in transgenic Fielder. (**a**) Immature embryos inoculated with EHA105 (pLC41bar). (**b**) Immature embryos 2 days after inoculation. Upper, before excision of embryo axis; Lower, after excision of embryo axis. (**c**) Immature embryos at the end of 2nd selection culture. (**d**) Plant regeneration and rooting from phosphinothricin (PPT) resistant explants under light condition**.** (**e**) Transgenic plant at flowering. (**f**) Transient expression in immature embryos after the resting culture. (**g**) Leaves detached from young regenerants. (**h**) Roots detached from young regenerants. (**i**) Spikelet. (**j**) Anthers. (**k**) Endosperm of immature seed. (**l**) Leaves detached from T_1 seedlings

Discussion

The transformation protocol hereby reported is quite efficient. The frequency of transformation of immature embryos, which was constantly over 50 % and as high as 95.0 % in the best case, was higher than the ones previously reported by roughly ten times. Therefore, one of the issues of wheat, which was a low efficiency of transformation methods, appeared to be resolved now.

Many factors needed to be examined and adjusted in order to achieve this much of improvement. The list of key factors per se in wheat transformation, including choice of genotype, quality of immature embryos, media composition, strain of *A. tumefaciens*, pre-treatment of embryos and handling of tissues, was not much different from those studied in rice and maize, but the details and specific parameters for the factors were quite different. For example, both centrifuging and heating as pre-treatments were effective in rice and maize, but only centrifuging was effective

in wheat. With respect to the handling of tissues, the process of the excision of embryo axes two days after the co-cultivation was not included in the protocols for transformation of other cereals.

The composition of the medium for co-cultivation of embryos and *A. tumefaciens* was a factor not well optimized before the present study, and a lot of experiments were needed. A combination good for wheat transformation was found by primarily looking after conditions suitable for stronger transient expression of the GUS gene in and the callus induction from the immature embryos after the co-cultivation and was further optimized.

It should also be noted that the windows of optimal ranges of parameters for the key factors in wheat seemed to be narrower than those in rice and maize. We experienced that, although the frequency of transformation in wheat was higher than that reported for maize when all the factors were optimal, the frequency dropped more drastically than maize when one or more of the factors became suboptimal.

The fact that too many factors with narrow optimal windows were involved in wheat transformation may explain both low frequency of transformation and low reproducibility of a protocol at a different laboratory before the present study. In addition, critical importance of the use of healthy immature embryos harvested at the right stage from wheat plants vigorously growing in a well-conditioned greenhouse, like any other cereal species, may explain them further. A greenhouse could be different in many ways from another no matter how similar they are and no matter how well the conditions are controlled. Therefore, a certain adjustment of the protocol by trials and errors may be inevitable for immature embryos from other greenhouses.

Commonly recognized advantage of *Agrobacterium*-mediated transformation, such as low copies of transgenes in the transformants and the stable Mendelian inheritance of the expression of transgenes, were well demonstrated in wheat. Thus, wheat has finally joined the list of cereals that can be transformed efficiently by *A. tumefaciens*. The next challenge for the present protocol is how reproducible it is at other laboratories. Chances are good because the many factors were well optimized now and the very high frequency of transformation was recorded in the present study. Finding of Fielder as a suitable genotype is another positive factor. In fact, the protocol recommended by the present study has already been tested successfully in more than ten leading laboratories in the world, and in this process, hands-on guidance provided by scientists experienced in wheat tissue culture was very helpful (personal communication). It is also likely that efforts will be made to develop methods for transformation of genotypes other than Bobwhite and Fielder, making use of the current protocol as a starting point. Therefore, the protocol must be very useful in basic and applied study of molecular biology, genomics, biotechnology and breeding in wheat.

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