Chapter 6

Floral Transformation of Wheat

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Abstract

A method is described for the floral transformation of wheat using a protocol similar to the floral dip of *Arabidopsis*. This method does not employ tissue culture of dissected embryos, but instead pre-anthesis spikes with clipped florets at the early, mid to late uninucleate microspore stage are dipped in *Agrobacterium* infiltration media harboring a vector carrying anthocyanin reporters and the *NPTII* selectable marker. T1 seeds are examined for color changes induced in the embryo by the anthocyanin reporters. Putatively transformed seeds are germinated and the seedlings are screened for the presence of the *NPTII* gene based on resistance to paromomycin spray and assayed with *NPTII* ELISAs. Genomic DNA of putative transformants is digested and analyzed on Southern blots for copy number to determine whether the T-DNA has integrated into the nucleus and to show the number of insertions. The non-optimized transformation efficiencies range from 0.3 to 0.6% (number of transformants/number of florets dipped) but the efficiencies are higher in terms of the number of transformants produced/number of seeds set ranging from 0.9 to 10%. Research is underway to maximize seed set and optimize the protocol by testing different *Agrobacterium* strains, visual reporters, vectors, and surfactants.

Key words: Pre-anthesis wheat spikes, *Agrobacterium*, nuclear transformation, floral dip.

1. Introduction

Early reports of *Agrobacterium*-mediated gene transfer to the floral tissue of hexaploid wheat (*Triticum aestivum* L.) set the stage for the merry-go-round of hopeful and discouraging reports of germ-line transformation in wheat. Seventeen years ago floral transformation of wheat was reported, but the transformation events were apparently not stable and were rearranged or lost in subsequent generations *(1)*. Later it was reported that *Agrobacterium*-mediated germ-line transformation experiments in wheat, barley, and maize produced artifacts on Southern

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blots in the T1 generation when the transgenes were analyzed and these 'artifacts' were not stably transmitted to the next generation *(2)*. They were thought to be due to bacterial contamination and/or transformation of endophytic bacteria *(2)*. The target for these early attempts was purportedly the pollen *(1, 2)*, and rudimentary *Agrobacterium* infiltration medium was applied into cut florets at or near anthesis. Subsequently, particle bombardment protocols of dissected embryos or embryogenic calluses were devised *(3–5)* that did not risk bacterial contamination.

Wheat transformation is both an art and a science, and developing a protocol that consistently generates transformants is challenging. An additional complication is that there are technical difficulties in working with a large genome species. The hexaploid wheat genome is 17,000 Mbp per haploid nucleus *(6)*, and molecular manipulations in this crop are not trivial. The choice of selectable markers and reporters may also determine success, as many screens (e.g. herbicide resistance) can be influenced by the environment. In addition, selecting for a transformation event in monocotyledons is not as clear-cut as in dicotyledonous plants. We are currently optimizing floral transformation of wheat to generate a protocol that consistently produces transformants (*see* **Note 1**).

2. Materials

2.1. Plant Materials

1. Crocus or Chinese Spring wheat seeds are available from the USDA-Germplasm Resources Information Network (GRIN) facility (http://www.ars-grin.gov/npgs/). Crocus is a high-quality hard red Canadian spring germplasm line *(7)* and Chinese Spring is commonly used in genetic studies (*see* **Note 2**).

2.2. Growth Facilities

1. A controlled temperature greenhouse or walk-in growth chamber.
2. Standard potting mix such as Fafard #2 (Knoxville Seed and Greenhouse).
3. Osmocote 14-14-14 (Hummert International).
4. Six inch plastic pots.

2.3. Agrobacterium Growth

1. A glycerol stock, derived from a single colony of AGL1 or C58C1 *Agrobacterium* transformed with pBECKSred *(8, 9)* by a standard electroporation protocol (*see* **Note 3**).
2. LB broth (Fisher Biotech) with 50 µg/ml of spectinomycin and 50 µg/ml of rifampicin.

3. LB agar plates (LB broth with 12 g/l of bacterial agar (Fisher)) with 50 µg/ml of spectinomycin and 50 µg/ml of rifampicin.
4. YEP broth (20 g/l of peptone, 10 g/l of yeast) with 50 µg/ml of spectinomycin, 50 µg/ml of rifampicin, and 200 µM of acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone). The acetosyringone is dissolved in a couple of drops of dimethyl sulfoxide (DMSO).

2.4. Infiltration Medium and Agrobacterium Treatment

1. Half-strength Murashige and Skoog (MS) *(10)* medium (Fisher Biotech) pH 5.8, 5% w/v sucrose, 0.04% v/v Silwet L-77, 0.5 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES), and 200 µM of acetosyringone (*see* **Note 4**) *(11, 12)*.
2. Small plastic bottles that will hold 250 ml of liquid and can be autoclaved afterwards.
3. Small clear, plastic bags and glassine pollination bags.
4. Small scissors and tweezers.

2.5. Screening

1. Dissecting stereoscope.
2. Commercial bleach (30%); optiona l- cefotaxime 1,000 ppm (Fisher) *(13)*.
3. A 2% (w/v) of paromomycin spray (Fisher) mixed with 0.2% (v/v) of Tween 20 to act as a surfactant *(14)*.
4. *NPTII* ELISA (Enzyme Linked ImmunoSorbent Assay) kit (Agdia, Inc, Indiana) and a microplate reader at 540 nm wavelength.

2.6. Copy Number Southern Gel Blots to Show Integration of the T-DNA

1. *See* Chapter 13.

3. Methods

3.1. Growth of the Wheat Plants

1. The temperature of the growth facility should never exceed 25°C. Ideal daytime temperatures are 21–25°C with a nighttime temperature of 16°C. A light cycle of 16 h is desirable.
2. Six-inch pots are filled with Fafart #2 potting medium and mixed with a standard analysis fertilizer such as Osmocote 14-14-14. The wheat seeds are planted one per pot. Additional nutrients are applied if required.
3. Light quality and/or quantity has not been monitored but never drops below 400–500 µE.
4. Water the plants well before floral transformation and then do not water until at least 2 days after the *Agrobacterium* treatment.

3.2. Growth of Agrobacterium and Induction of the Vir Genes

1. The glycerol stock of *Agrobacterium* harboring the plasmid is used to inoculate 5 ml of LB broth with the appropriate antibiotics and grown overnight at 22 ± 4°C with shaking at 2 × *g*.
2. The next day, 2.5 ml of this culture is used to inoculate 250 ml of YEP broth containing the antibiotics and 200 μM of acetosyringone, and grown at 22 ± 4°C with shaking at 2 × *g* until the optical density (OD_{600}) = 1.0.

3.3. Infiltration Media

The YEP/*Agrobacterium* culture is centrifuged at 6,400 × *g* for 15 min at room temperature and the supernatant is discarded. All reagents except the Silwet L-77 are mixed and used to gently re-suspend the cells to a final cell density of OD_{600} = 1.0. The Silwet L-77 is added to the infiltration medium just prior to treating the plant.

3.4. Preparation of the Wheat Spikes and Agrobacterium Treatment

1. Pre-anthesis wheat spikes at the early, mid to late uninucleate microspore stage yield transformants. This is the early boot stage when the spike, still enclosed in the sheath, is 6–7 cm long (**Fig. 1**, *see* **Note 5**).
2. The sheath is opened and the developing spike is carefully removed. The terminal florets and the inner florets can be removed, as they are frequently sterile, or they can be left

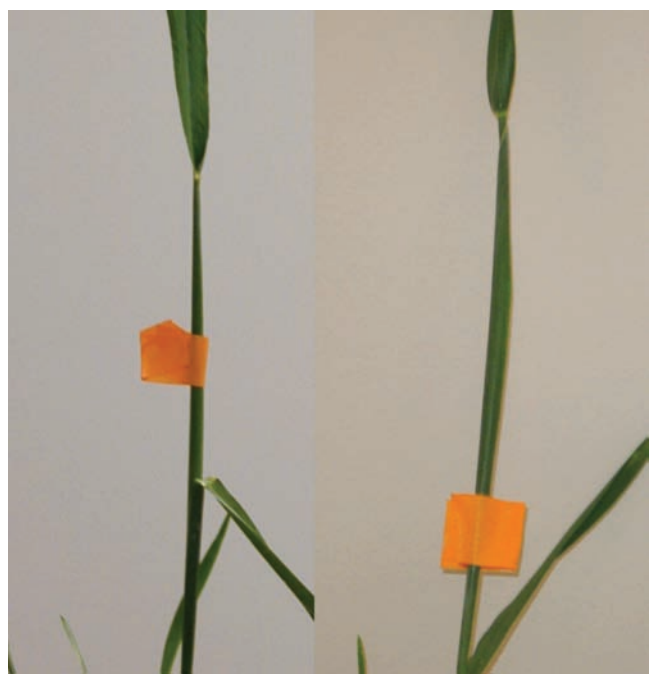

Fig. 1. The early boot stages of wheat. The early uninucleate stage (*left*) and the mid to late uninucleate stage (*right*) are the correct stages for floral transformation. The spike, still enclosed in the sheath, is gently removed from the sheath and the florets are clipped before treatment with *Agrobacterium* infiltration medium. Floral transformation at an earlier stage will produce fewer seeds.

intact. The remaining florets are clipped to slightly below the end of the glumes.

3. Shake the *Agrobacterium* infiltration medium before immersing the spike in it, and keep the spike immersed for 1–2 min (*see* **Note 6**).
4. Cover the spike in small, clear plastic bags for 2 days to ensure high humidity.
5. Remove the plastic bags and allow the spikes to recover from the stress. Allow the spikes to dry after the *Agrobacterium* treatment, and as they near anthesis, cover them in glassine bags to prevent cross pollination and let the T_1 seeds set naturally (*see* **Note 7**).

3.5. Screening

A series of facile screens are conducted consecutively before performing the time-consuming and labor-intensive Southern blots for determining copy number, and reverse transcriptase polymerase chain reaction (RT-PCR) or northern blots for studying gene expression.

3.6. Visual Inspection of the Seed

1. The TI seeds are examined for red embryos and compared with several wild-type seeds under a stereoscope (**Fig. 2**, *see* **Note 8**).
2. Putatively transformed seeds are washed for 30 min with shaking in 30% commercial bleach solution to kill residual *Agrobacterium*, and rinsed three times with water at 5 min per wash before planting in potting mix.

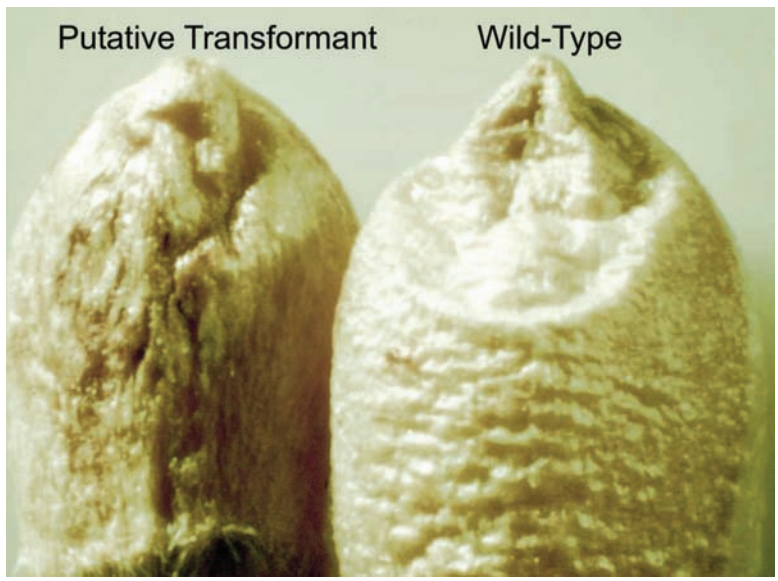

Fig. 2. Seeds of a putative transformant (*left*) versus wild type (*right*). This putative transformant was produced after treatment with *Agrobacterium* harbouring pBECKSred which carries *Lc/C1*, and the embryo/endosperm is a deeper red than wild type.

3. Optionally, a cefotaxime treatment (1,000 ppm; in addition to **step 1** – **Section 3.6**) can also be applied to germinating seedlings for 2 h at room temperature to kill residual *Agrobacterium (13)*.

3.7. Screening for Resistance to 2% Paromomycin at the Whole Plant Level

1. Putative transformed seedlings and several wild-type control plants are sprayed at the 3–4 leaf stage with the 2% (w/v) paromomycin spray containing 0.2% (v/v) Tween 20 to act as a surfactant *(14)*.
2. The plants are scored as resistant or sensitive 5–7 days later (*see* **Note 9**). Save the resistant plants as putative transformants.

3.8. Screening with NPTII ELISAs

1. Follow the protocol for the *NPTII* ELISA (Agdia Inc.).
2. It is important to solubilize the *NPTII* standards provided with the kit in the protein extracts of the wild-type control plants.
3. Include more than one wild-type control plant, as one may produce a false positive (*see* **Note 10**).
4. Samples of healthy plant tissue of the putative transformants are selected and extracted in microfuge tubes at 4°C to produce concentrated protein samples (1:5 tissue weight:buffer volume) using the Agdia extraction buffer. A 1-ml pipette tip works well to macerate the tissue in place of a pestle. The samples are centrifuged for 5–10 min at 7,500 × *g* and the supernatant is saved.
5. If possible, triplicate wells for each sample and standard should be assayed in the ELISA.
6. At the completion of the assay, stop the reaction with 3 M H_2SO_4 and use a microplate reader at 450 nm to quantify the results. Select the plants with ELISA readings higher than those of wild type as putative transformants.

3.9. Copy Number Southern Blots to Show Integration of the T-DNA

Genomic DNA of putative transformants, wild-type controls, and plasmid is extracted, digested to completion, fractionated according to size in agarose gel electrophoresis, blotted on to a membrane, and hybridized with a probe complementary to the gene of interest. *See* Chapter 13.

4. Notes

1. Patent Application "In Planta Transformation of Cereals" U.S. Serial No. 11/112,393, filed April 22, 2005 – Patent Pending.
2. Crocus is subject to post harvest grain dormancy. To speed up germination, incubate the seeds on moistened filter

paper at 4°C. Crocus and Chinese Spring both carry the double recessive alleles for high crossability with rye *(7, 15)*. It is not yet known if this transformation method is genotype dependent.

3. pBECKSred carries the *NPTII* selectable marker driven by the nos promoter, and the *Lc/CI* anthocyanin regulators can act as cell autonomous indicators of a transformation event by turning the embryo tissue red *(8, 16)*. While several transformants have been produced with this vector, the pCambia vectors are preferred since they are small, stable in *Agrobacterium*, and have been sequenced *(17)*. In addition to *C1*, *B-Peru* allele is another useful reporter derived from a maize transcription factor that regulates the anthocyanin pathway *(18)*. The 35S:*adhI* intron:*B-Peru* and the 35S:*adh1* intron:*C1* reporters have recently been cloned into pCambia2200 and are available on request. pCambia2200 also contains the *NPTII* selectable marker with the double enhancer version of the 35S promoter for better gene expression. The *NPTII* genes in pBECKSred and pCambia do not have introns, therefore, it is possible that the 35S promoter and the nos promoter maintain a low level of functionality in *Agrobacteria (19)*. The pCambia vectors also contain a multiple cloning site for a gene of interest. Both C58C1 and AGL1 *Agrobacterium* strains have been used to produce wheat transformants.
4. Transformants have been produced with acetosyringone concentrations ranging from 200 μM to 1 mM with no additional detriment to seed set.
5. Spike development varies in different environmental conditions. If in doubt about the stage of microspore development, anthers can be fixed in formaldehyde:acetic acid: alcohol (1:1:3) for 24 h and then stored in 70% ethanol. Before examination, stain for 1 h in 1% acetocarmine stain and examine under a light microscope *(20)*. Florets in the middle of the spike mature sooner than the terminal florets. *Agrobacterium* treatment before the early uninucleate microspore stage decreases seed set considerably and after the late uninucleate stage decreases the chance of producing transformants. The spike is fragile at this stage and care must be taken not to break it off.
6. In some environments (e.g. increased heat stress), it may be necessary to decrease the dipping time and/or decrease the Silwet L-77 concentration in order to maximize seed set. Seed set at these recommendations is generally 22–23% of a fully fertile spike.
7. Out-crossing rates for some spring wheat cultivars can be relatively high *(21)*, and it is necessary to prevent cross

pollination. However, covering the spikes in cellophane or glassine bags before the spike has dried after the *Agrobacterium* treatment will increase humidity and fungal growth.

8. Putative T_1 transformants will have red embryos if transformed with a construct containing *Lc* and/or *C1*. It should be kept in mind that severely stressed hard red wheat seeds may also turn a darker red; therefore, one must determine whether the color change in the embryo of the T_1 seed is caused by the stress of the *Agrobacterium* treatment or a transformation event. Additional controls in these experiments could be included, in which wheat spikes are treated with infiltration media alone or with *Agrobacterium* infiltration media without the Silwet, although these controls have been less than helpful in distinguishing true transformation events.
9. Paromomycin is a geneticin G418 analog and useful as a spray at the whole plant level *(14)*. Resistance to paromomycin indicates the presence of the *NPTII* gene. If the *NPTII* gene is present, minimal bleaching and flecking will occur in comparison to wild-type control plants. The strength of the promoter must be considered when scoring the plants; transformed plants carrying the *NPTII* gene driven by a weak promoter (e.g. nos) may bleach minimally in comparison to wild type.
10. False positives may be due to cellular debris in the extract that have not been removed by centrifugation.
11. The anthocyanin transcriptional regulators are toxic to white wheat *(9)*. T_1 seeds may be produced and germinate, but may die before anthesis (J. Zale, unpublished observations).
12. The female gametophyte appears to be the recipient of the T-DNA in *Arabidopsis (22)*. At present, it is not known whether the target of T-DNA transfer is the female or male gametophyte in wheat.
13. PCR is never conducted on putative T_1 transformants because it will produce false positives due to *Agrobacterium* contamination.

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