Agrobacterium-mediated transformation and genome editing by CRISPR/Cas9 for hexaploid wheat (*Triticum aestivum* L.)

Adapted from Hayta et al. 2019

Introduction

In the early 1990's the first transgenic wheat was produced by direct DNA transfer using particle bombardment of embryogenic callus tissue [1]. The first successful *Agrobacterium*-mediated wheat transformation followed in 1997 [2]. However, despite this first successful report, particle bombardment was the method of choice for some time, as *Agrobacterium*-mediated wheat transformation remained difficult and inefficient [3]. Reports in the literature of *Agrobacterium*-mediated wheat transformation generally describe low transformation efficiencies of around 5%. Efficient transformation of wheat via *in planta Agrobacterium*-mediated inoculation was reported by Risacher, et al. [4] however, this patented method required specialist skills and has not been widely adopted. Another efficient patented transformation system is available through licence from Japan Tobacco Inc (www.jti.co.jp), licenced as two systems, the basic PureIntro™ and the more advanced PureUpgrade™. Ishida, et al. [5], described the process, and reported efficiencies of 40-90% however, our lab and others (personnel communication) have not been able to replicate the process based on the published information. It appears that the specialist training provided to laboratories licensing the technology is a prerequisite to successful reproduction of the method and/or specialist vectors are required. Therefore, a robust, reproducible and transferrable wheat transformation system that is widely available to the research community is still needed.

We have developed and optimised a reproducible *Agrobacterium*-mediated transformation system for the spring wheat cv 'Fielder' that yields transformation efficiencies of up to 25%. This system has been widely used to introduce genes of interest as well as for CRISPR / Cas9 based genome editing [6].

Agrobacterium-Mediated Transformation

Materials

Plant material and growth conditions

1. Seeds of the spring wheat (*Triticum aestivum* L.) genotype 'Fielder' are sown at weekly intervals in a peat and sand mix (85% fine peat, 15% grit, 2.7 kg m^{-3} Osmocote 3-4 months, 4 kg m^{-3} maglime, 1 kg m^{-3} PG Mix 14-16-18 + Te 0.02% and wetting agent).

2. They are initially sown in 5 cm diameter pots and after approximately 30 days the germinated plants are transferred into 13 cm diameter round pots containing John Innes Cereal Mix (40% medium grade peat, 40% sterilised soil (loam), 20% horticultural grit, 1.3 kg m^{-3} PG mix 14-16-18 + Te base fertiliser, 1kg m^{-3} Osmocote mini 16-8-11 2mg + Te 0.02%, 3kg m^{-3} maglime and wetting agent) for continued development.

3. Plants are grown in controlled growth chambers (Conviron Europe Ltd) at 20 ±1°C day and 15 ±1°C night temperatures, 70% humidity with light levels of 800 $\mu mol.m^{-2}s^{-1}$ provided by fluorescent tubes and tungsten lighting.

4. Plants are not sprayed with fungicides or insecticides at any stage of growth.

See "How to grow wheat" for an introduction into growing wheat.

Agrobacterium strains and constructs

1. The hypervirulent *Agrobacterium tumefaciens* strain AGL1 [7] is used in all plant transformation experiments.

2. Vectors are electroporated into *Agrobacterium* AGL1 competent cells as previously described [8], when pGreenII [9] derivatives are used i.e. pBRACT [10] or pGGG they are co-electroporated with the helper plasmid pSoup [9] or its derivative pAL155 with an additional *Vir*G gene.

Bacterial culture medium

1. LB Medium: 5 gL^{-1} Yeast Extract (Duchefa Y1333), 10 g L^{-1} NaCl, 10 g L^{-1} Tryptone and 1 g L^{-1}Bactoagar (Difco).

2. The basic bacterial culture medium is MG/L which contains: 5.0 g L^{-1} tryptone, 2.5 g L^{-1} yeast, 100 mg L^{-1} NaCl, 5 g L^{-1} mannitol, 1 g L^{-1} Glutamic acid, 250 mg L^{-1} KH_2PO_4, 100 mg L^{-1} $MgSO_4$, 1 µg L^{-1} Biotin. pH=7.

Plant culture medium

1. Wheat inoculation medium (WIM): 0.44 g L^{-1} Murashige and Skoog (MS) (Murashige and Skoog, 1962) plant salt base (Duchefa M0222), 10 g L^{-1} glucose, 0.5 g L^{-1} 2-(Nmorpholino) ethanesulfonic acid (MES).

2. Wheat co-cultivation medium: WIM with 100 µM AS, 5 µM $AgNO_3$, 1.25 mg L^{-1} $CuSO_4 \cdot 5H_2O$ and 8 g L^{-1} agarose.

3. Wheat callus induction (WCI): 4.4 g L^{-1} Murashige and Skoog plant salt base (Duchefa M0221), 30 g L^{-1} maltose, 1.0 g L^{-1} casein hydrolysate, 10 mL of BCI vitamin stock, 2 mg L^{1} Picloram (Sigma-P5575), 0.5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 1.25 mg L^{-1} $CuSO_4 \cdot 5H_2O$ and 5 g L^{-1} agarose.

4. Wheat regeneration medium (WRM): 4.4 g L^{-1} MS (Duchefa M0222), 20 g L^{1} sucrose, 0.5 g L^{-1} MES supplemented with 0.5 mg L^{-1} Zeatin, 160 mg L^{-1} Timentin and 20 mg L^{-1} Hygromycin, 3 g L^{-1} Gelzan (Sigma-Aldrich).

All the media is adjusted to pH 5.8.

Media stocks

1. 100x vitamin stock for callus induction (WCI) medium: 100 mg L^{-1} thiamine HCl, 35 g L^{-1} myo-inositol, and 69 g L^{-1} proline. This stock should be filter sterilized and stored at 4 °C.

2. $CuSO_4$ stock: 125 mg of $CuSO_4 \cdot 5H_2O$ dissolved in a total volume of 100 mL water, filter sterilized, and stored frozen.

3. $AgNO_3$: 0.85 mg L^{-1} stock made up in water, divided into 1 mL aliquots, and stored frozen.

4. Acetosyringone (AS): 100 mM stock made up in DMSO and divided into 1 mL aliquots and stored frozen.

5. Hygromycin: Purchased as a sterile 50 mg L^{-1} stock, divided into 1 mL aliquots, and stored frozen.

6. Timentin: 160 mg L^{-1} stock made up in water, divided into 1 mL aliquots, and stored frozen.

7. Picloram: 2 mg L^{-1} stock, dissolved 100 mg in NaOH and then made up to 50 mL with water, filter sterilized, divided into 1 mL aliquots, and stored frozen.

8. 2,4-D: 2.5 mg L^{-1} stock made up in 100% ethanol and stored at −20 °C.

9. Zeatin: 0.5 mg L^{-1} stock, dissolved in a few drops of 1N NaOH and made up to volume to 50 m L^{-1}.

Methods

Tissue culture media preparation

1. All solid media components used throughout the tissue culture process, except for gelling agent, are made as a double-concentrate and filter-sterilised. The gelling agents are made up as a double concentrate (in H_2O) and autoclaved.

2. After autoclaving the gelling agents (2x) are maintained at 60°C and the filter-sterilised media components (2x) are warmed to 60°C prior to mixing both and pouring. The phytohormones and antibiotics are added as filter sterilised stocks just before pouring.

   All media and stocks made up using water from an Elga water purifier.

Collection and sterilisation of immature seeds

1. Wheat spikes are collected approximately 14 days post anthesis (dpa), when the immature embryos are 1-1.5 mm in diameter (**Error! Reference source not found.C, D**) and early milk stage GS73 [11]. See "Wheat Development" for details on the growth stages (GS) of wheat.

2. Kernels from floret 1 and 2 on central spikelet (**Error! Reference source not found.A, B**) are used for transformation. The awns are cut off the ears approximately 3-5 mm from the grain. The seed coat can be removed but this is not essential unless contamination problems are encountered.

3. The immature grains are separated from the ear and placed in a 150 mL Sterilin jar. Under aseptic conditions within a laminar airflow cabinet the grains are surface sterilised using 70% ethanol (v/v) for 1 min, given 1 rinse with sterile distilled water, followed by 7 min in 10% (v/v) sodium hypochlorite (Fluka 71696). The grains are then washed 3 times with sterile distilled water.

Isolation of immature embryos

1. All subsequent operations are performed in a laminar flow hood under sterile conditions. Embryos are isolated from the immature grains using fine forceps under a dissecting microscope.

2. Approximately 100 embryos are put into two 1.7 mL Eppendorf tubes (**Error! Reference source not found.E**) containing 1 mL wheat inoculation medium (WIM).

Preparation of *Agrobacterium* for transformation

1. Single colonies of *Agrobacterium* AGL1, containing the desired vector, are inoculated into 10 mL of LB [12] liquid medium containing appropriate antibiotics and incubated at 28°C, shaken at 200 rpm for ~65 hours.

2. A modified method of Tingay, et al. [13] is used to prepare *Agrobacterium* standard inoculums for transformation as previously described by Bartlett, et al. [8].

3. Equal quantities of the *Agrobacterium* culture and 30% sterile glycerol are mixed and made into 400 ul aliquots in 0.5 mL Eppendorf tubes. The standard inoculum aliquots are frozen at -80 °C and stored until required.

4. The day before wheat transformation a single 400 µL standard inoculum is used to inoculate 10 mL of MG/L [14] (5.0 g L^{-1} tryptone, 2.5g L^{-1} yeast, 100 mg L^{-1} NaCl, 5 g L^{-1} mannitol, 1 g L^{-1} Glutamic acid, 250 mg L^{-1} KH_2PO_4, 100 mg L^{-1} $MgSO_4$, 1 µg L^{-1} Biotin. pH=7) liquid medium without antibiotics and incubated at 28°C shaken at 200 rpm overnight (~16h).

5. On the day of transformation, the bacteria are pelleted by centrifugation in a 50 ml Falcon tube at 3 100 rpm for 10 min at 24 °C. The supernatant is discarded,

and the cells resuspended gently in 10 mL wheat inoculation medium (WIM) to an optical density of 0.5 OD (600 nm) and then, 0.05% Silwet L-77 and 100 µM Acetosyringone (AS) is added fresh just before use.

6. The culture is incubated for 4-6 hours at room temperature with gentle agitation (80 rpm) in the dark.

Inoculation with *Agrobacterium* and co-cultivation

1. The isolated embryos are placed into fresh WIM medium prior to centrifugation at 14 000 rpm at 4 °C for 10 min [15].

2. WIM is removed with a pipette and after 1 mL *Agrobacterium* solution added, the tubes are inverted frequently for 30 sec and incubated at room temperature for at least 20 mins.

3. After the incubation period, the *Agrobacterium* suspension is poured with the embryos into a 50-mm diameter Petri plate and the *Agrobacterium* suspension is removed with a pipette.

4. The embryos are transferred, scutellum side up, to the co-cultivation medium consisting of WIM supplemented with 100 µM AS, 5 µM $AgNO_3$, 1.25 mg L^{-1} $CuSO_4 \cdot 5H_2O$ and 8 g L^{-1} agarose [5].

5. Twenty-five embryos are placed in each 90 mm single vent Petri plate (Thermo Scientific No 101R20) and incubated at 24 ± 1°C in the dark for 3 days co-cultivation (**Error! Reference source not found.F**).

Resting period, callus induction and selection of transformed material

1. After three days' co-cultivation, the embryogenic axes are excised from the embryos using forceps (**Error! Reference source not found.G**).

2. The embryos are transferred to the fresh callus induction plates (WCI) based on the media described in [16] but containing 2 mg L^{-1} Picloram (Sigma-P5575), 0.5 mg L^{-1} 2,4 dichlorophenoxyacetic acid (2,4-D), 160 mg L^{-1} Timentin and 5 g L^{-1} agarose and incubated at 24 ±1°C in the dark for 5 days. Timentin is added to control *Agrobacterium* during the resting period.

3. The embryos are transferred, scutellum side up, to fresh WCI plates as above with 15 mg L^{-1} Hygromycin and incubated at 24 ±1°C in the dark for two weeks. This transfer is referred to as Selection 1.

4. The calli are split at the next transfer into clumps of approximately 4 mm^{-2}, callus pieces derived from each single embryo are labelled to keep track of their origin. The calli are transferred to fresh selection plates (WCI) as above, but with 30 mg L^{-1} Hygromycin (Selection 2) and incubated at 24 ±1 °C in the dark for 2 weeks (**Error! Reference source not found.H**). The number of explants per plate are reduced by approximately half at Selection 2.

5. After two weeks the calli are transferred to a lit culture room under fluorescent lights (100 µmol m^{-2} s^{-1}) at 24 ±1 °C with a 16-hour photoperiod and covered with a single layer of paper towel for a further week. During this period putative transformed lines should start to green and produce small shoots (**Error! Reference source not found.I**).

Regeneration of transgenic plants

1. After the three weeks on selection 2 medium, the calli are transfer one final time to wheat regeneration medium (WRM) containing 4.4 g L^{-1} MS (Duchefa M0222), 20 g L^{-1} sucrose, 0.5 g L^{-1} MES supplemented with 0.5 mg L^{-1} Zeatin, 160 mg L^{-1} Timentin and 20 mg L^{-1} Hygromycin, 3 g L^{-1} Gelzan (Sigma-Aldrich) in deep Petri dishes (tissue culture dish, 90 mm diameter x 20 mm, Falcon 353003).

2. All regenerating callus derived from a single embryo is labelled to track its origin. The paper covering is removed and the calli are cultured under fluorescent lights (100 µmol m^{2} s^{-1}) at 24 ±1 °C with a 16-hour photoperiod.

Rooting

1. Regenerated shoots which are 1-2 cm in length with visible roots (**Error! Reference source not found.J**) are transferred to "De Wit" culture tubes (Duchefa, W1607) containing 8 mL of WCI without growth regulators, solidified with 3 g L^{-1} Gelzan and supplemented with 160 mg L^{-1} Timentin and 15 mg L^{-1} Hygromycin. Putative transformed plants developed a strong root system with root hairs (**Error! Reference source not found.K**).

Acclimatisation

1. Regenerated plantlets with strong root systems (**Error! Reference source not found.L**) are gently removed from the tubes using long forceps and the roots gently washed with cool running water to remove any remaining tissue culture medium.

2. The plants are planted in a peat and sand mix in 5 cm square cell trays and covered with a clear plastic propagator lid. The plants remained covered with the propagator lids for approximately 1 week to maintain high humidity around them while they became established in soil.

3. The plants are grown in a controlled environment room at 18±1 °C day (16 h) and 15±1 °C night temperatures, 65% relative humidity with 400-600 µmol m^{-2} s^{-1} light intensity provided by metal halide lamps (HQI) supplemented with tungsten bulbs at 16 h photoperiod.

4. Once plants are established in soil, leaf samples can be collected for further analysis to confirm the presence of the introduced genes.

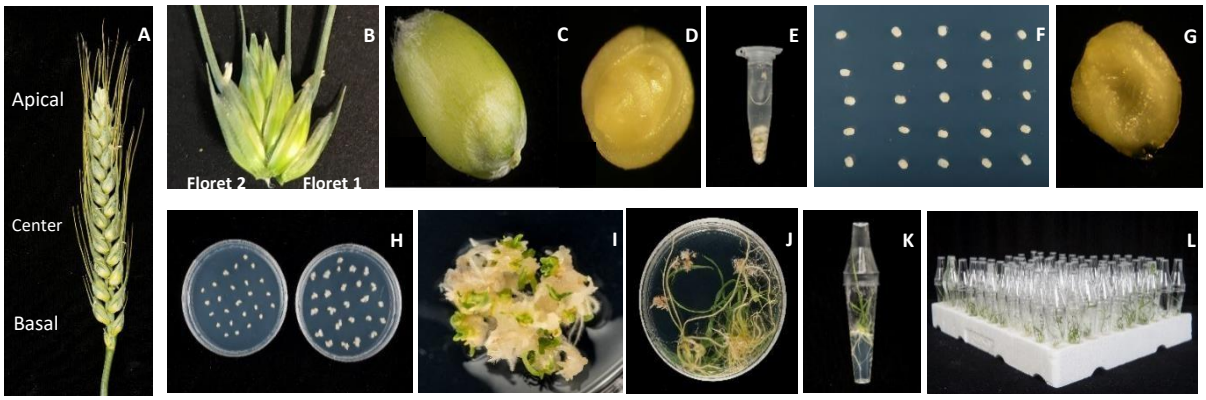

Figure 1. Step-by-step overview of wheat transformation
Selection of wheat spikes and immature embryos at the correct stage (A-C), isolated immature embryo (D), Immature embryos in Eppendorf tube containing 1 mL WIM (E), Immature embryos on co-cultivation medium (F), Immature embryo with the embryonic axis removed before transferring to resting medium (G), Callus induction on Selection 1 and Selection 2 media (H), Transformed callus starting to green and produce small shoots (I), Regenerated shoots with visibly strong roots (J), Transgenic wheat plant transferred to culture tube showing strong root system in hygromycin containing medium (K), Transgenic wheat plants before transferring to soil (L).

CRISPR/Cas genome editing in hexaploid wheat

Selection of target sequences

In hexaploid wheat, genome editing using CRISPR/Cas system usually requires the knocking out or modifying all three homoeolog copies of a gene target within each subgenome (A, B, and D). See "Genome Assemblies" for an introduction to the polyploid wheat genome, and "Gene Models" for details on the most up-to-date wheat gene annotations.

For knockouts, if possible, guides are usually designed close to ATG to cause a frameshift early on in the sequence. In order to design generic guide-RNA's targeting all gene copies the first step is to align the target sequences looking for areas of high homology. These areas of high homology can then be submitted into online guide design tools such as Deskgen.com. The target sequence can be present on either the sense or antisense strand and should conform to the template GN20GG (**Figure 2**). Ideally, paired guides are designed 100-150 bp apart, which will give an easily recognisable deletion, if the guides work simultaneously. To identify possible off-target activity BLASTn search the guide sequence at Ensembl Plants http://plants.ensembl.org/Triticum_aestivum. (see "Using Ensembl Plants")

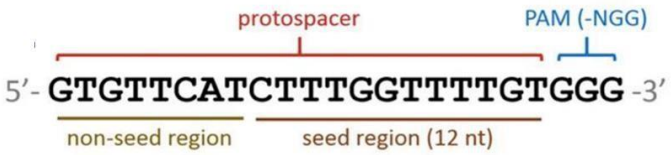

Figure 2. Target sequence template.

Once guides have been identified, PCR primers should be designed and tested using genomic DNA extracted from the transformable wheat cultivar 'Fielder'.

Screening transformed wheat for Cas-induced edits

For screening hexaploid wheat, PCR amplicons need to be identified that span the gene target within each subgenome (A, B, and D) individually.

There are two proven approaches for screening in wheat (**Figure 3**); the first uses a set of generic primers that amplify all target gene copies; This first PCR is then used as an enriched DNA template for amplification of subgenome specific (SGS) target area, using SGS nested primers. The second approach, for screening in wheat, uses SGS primers that amplify the individual target regions directly from 'Fielder' genomic DNA.

The SGS target area amplicons are then directly sequenced in both approaches using the relevant forward SGS primer. SGS screening primers should be designed spanning the target area, at least 120-150 bp from the first and last guides. Therefore, producing a 450-500 bp sized PCR amplicon, which facilitates easy identification of large (~100bp) deletions in edited plants using gel electrophoresis and produces a good-sized amplicon for direct sequencing. This initial test PCR should produce a clean single band on a gel and be of good quality capable of being directly sequenced. Most wheat sequences within databases are based on 'Chinese Spring', therefore, the sequences obtained from the 'Fielder' target site should be checked for polymorphisms, primarily within the guide sequence.

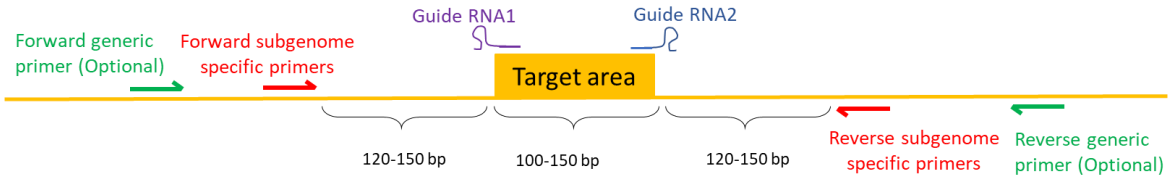

Figure 3. Screening strategies wheat.

Transformation strategy

The strategy is to produce two constructs each containing at least two guide RNAs. The two constructs are assembled using standard Golden Gate assembly [17] with each construct containing the hygromycin resistance gene under the control of a rice Actin1 promoter, Cas9 under the control of the rice ubiquitin promoter and two sgRNAs each under the control of a wheat U6 promoter (**Figure 4**). Once the vectors are completed and fully sequenced, they are electroporated into *Agrobacterium* AGL1 and transformed into wheat as described in the wheat transformation protocol (see above).

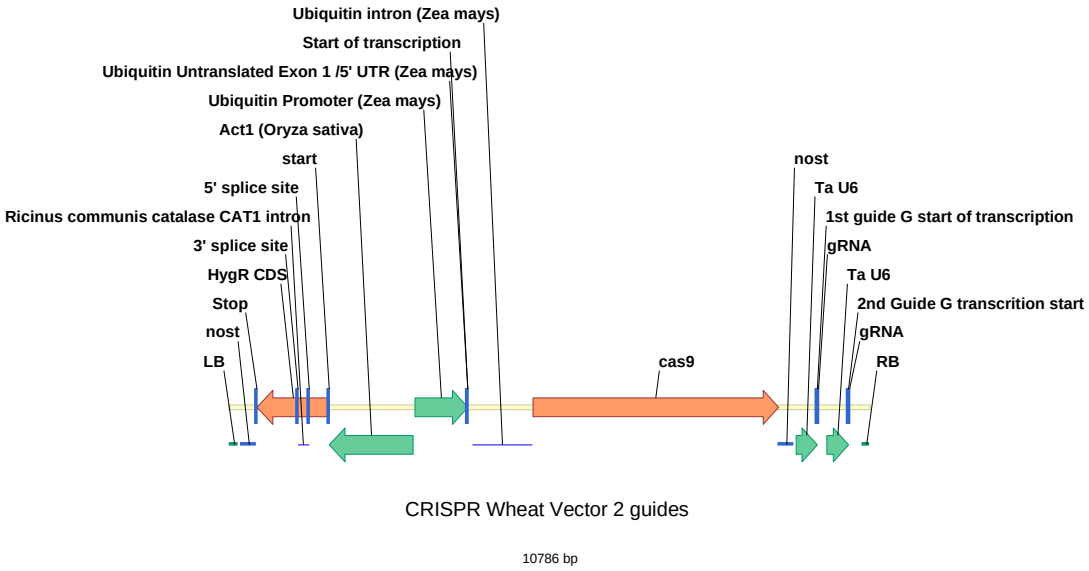

Figure 4. Schematic of a wheat CRISPR/Cas9 T-DNA

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