Chapter 13

Rice, Japonica (*Oryza sativa* L.)

Marcy Main, Bronwyn Frame, and Kan Wang

Abstract

The importance of rice, as a food crop, is reflected in the extensive global research being conducted in an effort to improve and better understand this particular agronomic plant. In regard to biotechnology, this has led to the development of numerous genetic transformation protocols. Over the years, many of these methods have become increasingly straightforward, rapid, and efficient, thereby making rice valuable as a model crop for scientific research and functional genomics. The focus of this chapter is on one such protocol that uses *Agrobacterium*-mediated transformation of *Oryza sativa* L. ssp. Japonica cv. Nipponbare with an emphasis on tissue desiccation. The explants consist of callus derived from mature seeds which are cocultivated on filter paper postinfection. Hygromycin selection is used for the recovery of subsequent genetically engineered events.

Key words *Agrobacterium*, Callus, Desiccation, Transformation, Genetic engineering, *Oryza sativa*, Rice, Tissue culture, Transgenic

1 Introduction

Agrobacterium-mediated transformation of rice was conclusively achieved in the mid-1990s [1]. Originally popular for genetic engineering because of its sequenced and small genome, the widespread use of rice for transformation purposes persists: both for primary crop improvement research and also as a model plant, due to high-yielding clone recovery rates and the ease of regeneration/germination of transgenic plantlets [2–4]. Nipponbare has a relatively short turnaround time in tissue culture (Fig. 1) which is practical for timely research results and also beneficial in the avoidance of somaclonal variation.

The following described method, incorporating the influences of tissue desiccation, has been successful in rice transformation and the regeneration of transgenic plantlets from nearly 100 different customer/service constructs processed by the Plant Transformation Facility at Iowa State University (ISU). These constructs consist of varying strains and plasmids, which can have variable impacts on

Kan Wang (ed.), *Agrobacterium Protocols: Volume 1*, Methods in Molecular Biology, vol. 1223,
DOI 10.1007/978-1-4939-1695-5_13,

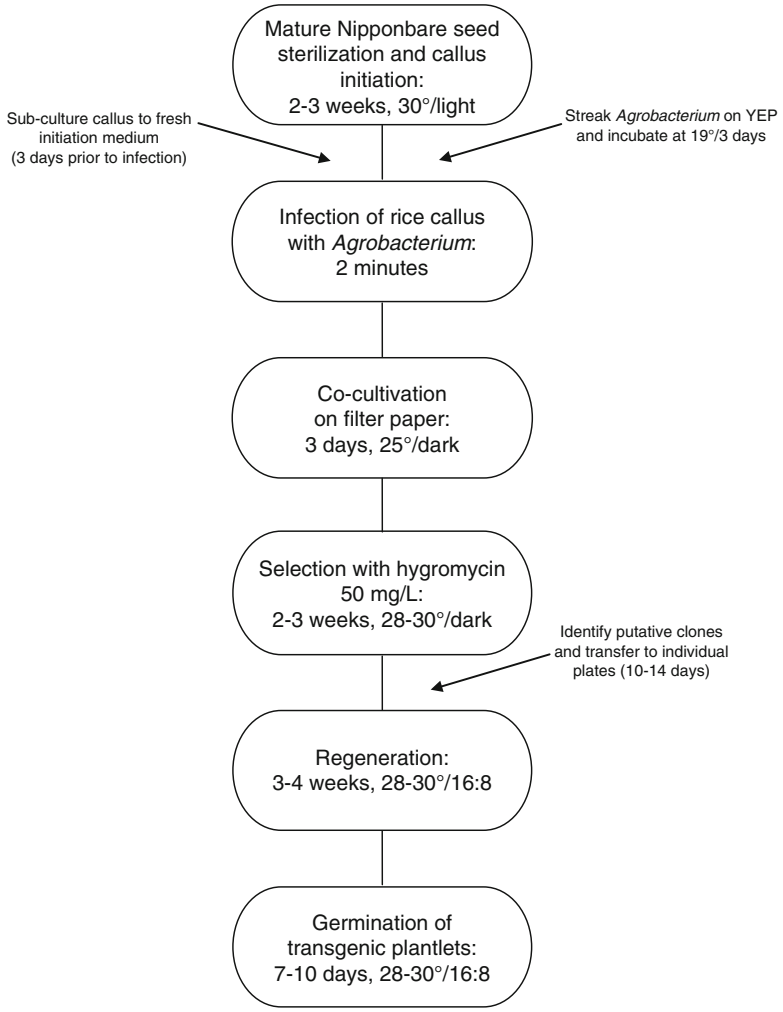

Fig. 1 Rice transformation and regeneration flowchart

the final transformation results [5–7]. Nonetheless, this procedure continually provides both a reliable (average transformation efficiency of 36.4 % and plantlet regeneration rate of 74 %) and rapid system (generally 8–11 weeks from postinfection to transgenic plantlet). This protocol has been successfully utilized with other Japonica cultivars (Kitaake, Zhonghua) and bialaphos selection, as well. It is also adaptable in terms of media composition, environmental factors, and experimental conditions. These flexible aspects of the procedure and the reproducible nature of the protocol make efficient rice transformation relatively easy to implement in a variety of laboratories and research settings.

2 Materials

2.1 Plant Material

Mature Nipponbare seeds currently being used for this protocol were obtained from plants brought to maturity in growth chambers at Iowa State University's Department of Agronomy by the Plant Transformation Facility. Seeds are stored long term in cold storage at 5 °C and 40.0 % relative humidity. It is imperative to start with material that will produce high-quality callus tissue; seeds should be visibly healthy and free of any contaminants. Rice seeds can also be acquired from other sources, such as the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/npgs/orders.html).

2.2 Agrobacterium Strains and Plasmids

Agrobacterium tumefaciens strains EHA101 [8], EHA105 [9], AGL-1 [10], and LBA4404 [11], containing a variety of standard binary vectors (plasmids), are used. The selectable marker cassette of choice for this particular protocol contains the hygromycin phosphotransferase (*hpt*) gene, also using a wide range of different promoters and terminators. Once established, *Agrobacterium* strains/vectors (constructs) are maintained at −80 °C as glycerol stocks.

2.3 Media Preparation

2.3.1 Stock Solutions

1. N6 vitamin stock (1,000×): 2.0 g/L glycine, 1.0 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, and 0.5 g/L nicotinic acid are dissolved in deionized water and filter sterilized.
2. MS vitamin stock (1,000×), modified [12]: 2.0 g/L glycine, 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, and 0.05 g/L nicotinic acid are dissolved in deionized water and filter sterilized.
3. 2,4-Dichlorophenoxyacetic acid (2, 4-D): 1 mg/mL, dissolve in 1 N KOH over low heat and bring up to volume with deionized water.
4. Kinetin: 1 mg/mL, dissolve in 1 N KOH and bring to volume with deionized water.
5. Naphthalene acetic acid (NAA): 0.25 mg/mL, dissolve in 1 N NaOH (or KOH) and bring up to volume with deionized water, filter sterilize.
6. Carbenicillin: 100 mg/mL, dissolve 1 g of carbenicillin into 10 mL of deionized water, sterilize with 0.22-μm filter.
7. Cefotaxime (Claforan, Sanofi-Aventis, sterile 1-g vial): 200 mg/L, dissolve 1 g of cefotaxime in 5 mL of sterile water. Aliquot and freeze.
8. Vancomycin (NovaPlus, sterile 1-g vial): 200 mg/L, dissolve 1 g in 5 mL sterile water. Aliquot and freeze.
9. Hygromycin: 50 mg/mL, sterile solution in phosphate buffered saline (PBS), from PhytoTechnology Laboratories, #H370-20 mL.

10. Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone, AS): 100 mM, dissolve in dimethyl sulfoxide (DMSO) and deionized water 1:1. Filter sterilize prior to freezing.

2.3.2 Media

1. YEP: 5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, 15 g/L Bacto agar. Adjust pH to 6.8 with 1 N NaOH. After autoclaving, add the appropriate antibiotics for the given strain/vector and pour into 100 × 15 mm Petri plates.
2. Initiation medium: 4 g/L N6 salts [13], 2 mg/L 2,4-D, 300 mg/L casamino acids, 2.8 g/L L-proline, 30 g/L sucrose, and 4 g/L Gelrite (pH 5.8). Add 1 mL/L N6 vitamin stock (filter sterilized) after autoclaving (100 × 15 mm plates).
3. Liquid infection medium: 4 g/L N6 salts, 1 mL/L N6 vitamin stock, 1.5 mg/L 2,4-D, 68.4 g/L sucrose, 36.0 g/L glucose, 0.7 g/L L-proline (pH 5.2) [1, 14]. Filter sterilize and store at 4 °C. Acetosyringone (AS) from 100 mM sterile stock is added to a final concentration of 100 μM just prior to use for infection.
4. Selection medium: 4 g/L N6 salts, 2 mg/L 2, 4-D, 300 mg/L casamino acids, 2.8 g/L L-proline, 30 g/L sucrose, and 4 g/L Gelrite (pH 5.8). Sterile stocks of 1 mL/L N6 vitamin stock, 50 mg/L hygromycin, and 250–500 mg/L carbenicillin (*see* **Note 1**) are added after autoclaving (100 × 15 mm plates).
5. Regeneration medium I: 4.3 g/L MS salts [15], 2 mg/L kinetin, 2 g/L casamino acids, 30 g/L sucrose, 30 g/L sorbitol, and 8 g/L agar (pH 5.8). Sterile stocks of 1 mL/L MS (modified) vitamin stock, 0.02 mg/L NAA, 100 mg/L cefotaxime, 100 mg/L vancomycin, 50 mg/L hygromycin are added after autoclaving (100 × 25 mm plates).
6. Regeneration medium II: 4.3 g/L MS salts, 1 mL/L MS (modified) vitamin stock, 100 mg/L myoinositol, 30 g/L sucrose, 3 g/L Gelrite, pH 5.8 (100 × 25 mm plates).

3 Methods

The following procedures are carried out in a laminar flow hood using aseptic technique and sterile equipment (i.e., forceps, scalpels, Petri plates, conicals, etc.).

3.1 Mature Seed Sterilization

1. Place 15–20 dehusked Nipponbare seeds (Fig. 2a) into a sterile 50-mL conical tube containing 15 mL of 70 % ethanol; allow 1 min of shaking.
2. Rinse the seeds several times with sterile, deionized water.
3. Add 15 mL of 50 % bleach solution (sodium hypochlorite) to the seeds and place the tube on a shaker for 15 min (*see* **Note 2**).

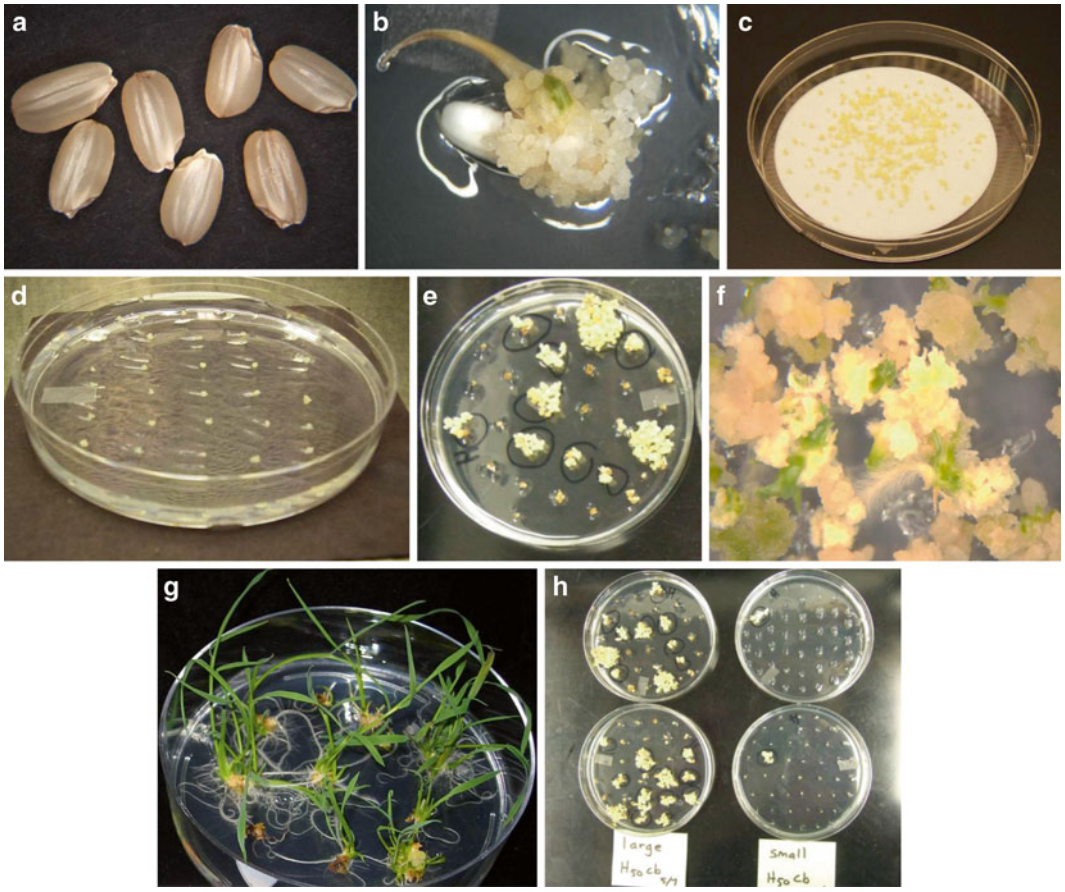

Fig. 2 *Agrobacterium*-mediated rice transformation. (**a**) Nipponbare rice seeds prior to sterilization. (**b**) Callus induction from scutellum of mature Nipponbare seed. (**c**) Rice cocultivation step using filter paper. (**d**) Rice calli on selection medium. (**e**) Putative rice events emerging from 50 mg/L hygromycin selection, prior to be transferred to individual clone plates. (**f**) Regeneration of transgenic Nipponbare event. (**g**) Transgenic plantlets on germination medium. (**h**) Hygromycin selection plates comparing callus size at the time of infection. Subsequent transformation rates are greatly diminished with the smaller-sized calli (<2 mm)

4. Pour off the bleach solution and rinse several times with sterile, deionized water. With a small portion (<5 mL) of the final rinse, pour the seeds into a Petri plate containing sterilized filter paper and allow them to dry.

3.2 Explant Preparation

1. Using aseptic technique, transfer up to ten seeds onto the initiation medium (10 × 15 mm plate) and wrap with Parafilm.
2. Incubate Petri plates under continuous light (80–100 μmol/m^2/s) at 30 °C for approximately 2 weeks [16] (*see* **Note 3**). After the first week of callus induction, it may be useful to remove any unwanted coleoptiles in order to ensure adequate contact between the scutellum and the surface of the medium to encourage maximum callus production.

3. As soon as developing (embryogenic) callus (Fig. 2b) is visible on the scutellum of the mature seed, subculture the tissue to fresh initiation medium and allow the callus to proliferate for another 5–7 days (*see* **Note 4**).
4. Three days prior to a scheduled infection, cut actively proliferating callus tissue into 2–3 mm pieces (*see* **Note 5**). Again transfer the tissue to a new plate of initiation medium, but this time, wrap the plate with vent tape to provide maximum "ventilation" prior to infection (*see* **Note 6**).

3.3 Agrobacterium Infection

1. *Agrobacterium* strains are typically taken from glycerol stock and grown on YEP medium amended with appropriate antibiotics at 19 °C for 3 days prior to an experiment.
2. A small amount of *Agrobacterium* culture is removed from the YEP plate using a 1-μL (0.9-mm diameter) sterile inoculating loop and added to a sterile 50-mL conical tube containing 15 mL of the liquid infection medium with AS (100 μM).
3. Once the *Agrobacterium* is uniformly suspended or after 10 min on a rotary shaker (gentle agitation), adjust the optical density of the mixture to 0.03–0.05 (OD_{600}) prior to use.
4. Transfer previously prepared callus into a 50-mL conical tube containing 5 mL of liquid infection medium with 100 μM of AS (*see* **Note 7**). Remove this liquid immediately after all of the calli have been transferred over from the initiation medium plate and replace it with the *Agrobacterium* suspension.
5. Place the conical tube on a vortex for 2 min of gentle shaking. Transfer callus with the use of a sterile 25-mL wide-bore pipette and pipette-aid onto a stack of sterile filter paper (Whatman #4, 70 mm) in a 100 × 15 plate. Allow the tissue to air-dry in the laminar flow hood (*see* **Note 8**).
6. Add 1.5 mL (*see* **Note 9**) of the liquid infection medium (with 100 μM AS) to two pieces of sterile filter paper (Whatman #4, 70 mm) in a 100 × 15 Petri plate [6, 17–19].
7. Transfer callus with forceps to the premoistened filter paper (Fig. 2c). Seal the plate securely with Parafilm (*see* **Note 10**).
8. Incubate in the dark at 25 °C for 3 days.

3.4 Selection of Transformed Embryogenic Tissues

1. Transfer calli, about 35 individual pieces, from the cocultivation plate to the surface of selection medium; wrap with Parafilm (Fig. 2d) (*see* **Note 11**). The tissue will be much lighter and of a softer consistency after cocultivation due to dehydration. Some callus pieces may need to be gently teased apart from one another prior to being taken to selection in order to ensure their uniqueness as "independent events".
2. Plates are incubated in the dark at 30 °C for 2–3 weeks, based on growth (*see* **Note 12**). Rapidly growing putative events

(Fig. 2e) are taken to their own selection plates ("clone plates"), assigned an identification number/name, and allowed to "bulk up" prior to being transferred to the regeneration medium. This step should be done with the aid of a microscope in order to look for any evidence of *Agrobacterium* overgrowth. This is a particularly important step when working with constructs that contain the *bar* gene (*see* **Note 13**).

3.5 Regeneration of Transformed Plantlets

1. Once the clone is approximately 1 cm in diameter ("dime-sized"), again use the microscope to transfer actively growing, embryogenic calli to regeneration medium I; wrap with vent tape. Tissue typically begins to turn green after 10 days of incubation (16:8 photoperiod, 80–100 μmol/m²/s, 28 °C). Small shoots (Fig. 2f) should be evident after an additional 7–14 days of incubation (*see* **Notes 14** and **15**).
2. Transfer green shoots to regeneration medium II for the completion of germination. Transgenic plants (Fig. 2g) will be ready to go to soil in 7–10 days.

3.6 Transplanting and T1 Seed Production

1. Nipponbare plants are grown in a chamber with a photoperiod setting of 10:14 (day/night) and temperatures of 28 °C day/25 °C night. The light intensity of the chamber is approximately 350 μE/m²/s.
2. Prepare soilless mix (Sunshine Universal Mix SB300 or Metro Mix 900; Sun Gro Horticulture) in 3½ in. square pots with 1 tsp of 17-2-12 Osmocote (B&T Grower Supply #FZ17712) and ¼ tsp of Sprint 330 chelated iron (Hummert #07-1511-1).
3. Rice plantlets are ready to be transferred from the Petri plate (germination medium) into the pre-wetted soilless mix when the leaves have reached 4–6 cm in length. Place pots in a flat (Hummert #11-3050-1), cover with Humi-dome (Hummert #14-3850-1), and add ambient-temperature water (pH of 5.5–6.0), as needed.
4. Remove Humi-dome after plants have hardened off and are well established (or just touching the top of the Humi-dome). Keep the flat filled ½–¾ full with water to maintain constant water availability until the dry down stage, 3–4 months (*see* **Note 16**). See also the "Rice Growth Chamber Care" protocol section listed at http://agron-www.agron.iastate.edu/ptf/.

4 Notes

1. If the *Agrobacterium* strain AGL-1 is being used, an alternative antibiotic, such as cefotaxime and/or vancomycin, should be added to the selection medium instead of carbenicillin; AGL-1 has chromosomal resistance to carbenicillin [20].

2. A variety of bleach concentrations and durations have been used effectively in the lab; longer sterilization times are appropriate when using field seed.
3. In our experience, prolific callus production can occur under various environmental conditions. For example, continuous light, darkness, 16:8 photoperiod, and temperatures ranging from 25 to 32 °C have all proven to be suitable for the induction and growth of embryogenic callus ([1, 16, 21], *M. Main*, *unpublished*).
4. It is desirable to minimize the length of the entire tissue culture/transformation/selection process as much as possible. Thus, callus induction should be closely monitored and subcultures performed as soon as possible for use in *Agrobacterium-mediated* transformation experiments. In the case of transformation of variety Kitaake, an additional 7–10 days of initiation time (compared to Nipponbare) may be necessary in order to produce sufficient amounts of responsive callus tissue to be used for large-scale transformation experiments.
5. Callus size, at the time of infection, appears to have an influence on transformation rates (%TF). In particular, we found that the use of very small pieces (<2 mm at the time of infection) with 50 mg/L hygromycin selection results in a decreased %TF. Photographed in Fig. 2h are "side-by-side" experiment plates in which callus size was the only treatment being evaluated. In this particular experiment, the transformation frequencies for small versus larger callus (with all other parameters being held constant) were strikingly different. Conversely, in a similar experiment conducted using 2 mg/L bialaphos selection, this correlation between small callus size and low %TF was not observed (results not shown).
6. Tissue desiccation at the preinfection stage can be favorable to the transformation process. This can be achieved by using vent tape to wrap explant plates prior to infection, increasing the concentration of gelling agents/types in the initiation medium or air-drying the prepared callus plate in the laminar flow hood just before inoculation [6, 22, 23].
7. Successful transformation of rice can be achieved without the use of AS ([24–26], *M. Main*, *unpublished*). In fact, the absence of acetosyringone has been reported to decrease the amount of detrimental *Agrobacterium* overgrowth [24].
8. The duration allowed for air-drying is dependent on the amount of liquid infection media used to transfer over callus from the conical and the quantity and absorbency of the filter paper. Our protocol typically uses a 10-min air-drying period after infection. Desiccation is widely reported to increase transformation frequencies in rice as well as other cereals during the pre- and postinfection and cocultivation steps [6, 17, 19, 22, 23].

9. Amount of infection liquid added to the sterile filter paper can be altered to suit the quantity of callus infected and degree of desiccation desired [18].
10. Gelled cocultivation medium wrapped with vent tape can also be used in place of the "moistened filter paper treatment" for the cocultivation step. This medium consists of 4 g/L N6 salts, 2 mg/L 2, 4-D, 30 g/L sucrose, 10 g/L glucose, 300 mg/L casamino acids, and 4 g/L Gelrite (pH 5.2). After autoclaving, 1 mL/L N6 vitamin stock (filter sterilized) and 1 mL/L acetosyringone (AS) from 100 mM sterile stock are added (100 × 15 mm plates). However, lower transformation frequencies have been observed with the use of gelled cocultivation medium ([18], *M. Main*, *unpublished*).
11. To help control *Agrobacterium* overgrowth, should it arise after cocultivation, it may be useful to wash the callus tissue several times with 5 mL of the liquid infection medium (no AS) supplemented with carbenicillin (250–500 mg/L) and vancomycin (100 mg/L) or other antibiotics suitable for the *Agrobacterium* strain in use. Blot the callus on sterile filter paper and allow for sufficient air-drying prior to transferring the tissue to the selection medium. Other steps to consider when working with constructs that may be prone to overgrowth include adjusting the *Agrobacterium* suspension to a lower OD for calli inoculation, omitting AS in the liquid infection/cocultivation media, optimizing tissue desiccation, and using a two-day cocultivation period.
12. Similar to callus production, successful selection can also take place in the same variety of environmental conditions as indicated previously in **Note 3**.
13. If *bar* gene constructs with bialaphos selection (Bialaphos 2 mg/L; Gold BioTechnology Inc.) are being used instead of *hpt* gene constructs (hygromycin), a longer duration in selection with additional transfers and a subsequent lower transformation frequency should be expected ([7, 18], *M. Main*, *unpublished*). This aspect of selection has been particularly noted in indica rice [27].
14. Although 100 % regeneration for a few select constructs has been achieved using the protocol reported here, our average regeneration frequency is 74 %. As such, numerous variations in regeneration medium have been tested, largely based on indica protocols, in an effort to more consistently improve plant recovery rates. Treatments to increase overall regeneration averages have included desiccation techniques, either physically [28–31] or osmotically, with the use of increased concentrations of agar(ose) and/or the inclusion of sorbitol/mannitol in the media [3, 5, 31–33]. Additionally, a pre-dark incubation prior to 16:8 exposure [3, 34, 35], using maltose as

the primary carbohydrate source [32, 36], and adding various auxin to cytokinin ratio combinations and classes [3, 5] have all been shown to have positive effects on rice regeneration rates.

15. The recalcitrance of certain events to regeneration as well as the presence of albino plants in some regenerated cultures should be considered in regard to final plant production requirements. While occasionally, one or two albino plants are noted among several healthy, green plants for any particular regenerated event, an individual event rarely produces entirely albino plants, rate of 1.6 % at PTF. Even non-transgenic callus taken through the tissue culture process (but without *Agrobacterium* or selection) can occasionally produce a few albino plants.
16. Under PTF growth chamber conditions, post-transplant progression is as follows: flowering at 6 weeks, milk stage at 10 weeks, and seed maturation at 14 weeks. As filled seeds begin to harden and turn brown, watering is decreased and eventually stopped. Plants are allowed to dry down and then seeds are harvested approximately 16 weeks after the transplanting date [37]. Seed set can vary widely between constructs with an average of over 70 seeds per plant for customer constructs in Nipponbare.

Acknowledgments

The authors thank Amber Kabala for technical assistance in the lab and Katey Warnberg for the care of transgenic rice plants. This work was supported by the USDA National Institute of Food and Agriculture, Hatch project number # IOW05162, and also by the Department of Agronomy, the Plant Sciences Institute, and the Office of Biotechnology of Iowa State University.

References

1. Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282
2. Tyagi AK, Mohanty A (2000) Rice transformation for crop improvement and functional genomics. Plant Sci 158:1–18
3. Sahoo KK, Tripathi AK, Pareek A, Sopory SK, Singla-Pareek SL (2011) An improved protocol for efficient transformation and regeneration of diverse indica rice cultivars. Plant Methods 7:49–59
4. Izawa T, Shimamoto K (1996) Becoming a model plant: the importance of rice to plant science. Trends Plant Sci 1:95–99
5. Lee S, Jeon J-S, Jung K-H, An G (1999) Binary vectors for efficient transformation of rice. J Plant Biol 42(4):310–316
6. Cheng M, Lowe BA, Spencer TM, Ye X, Armstrong CL (2004) Invited review: factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol Plant 40:31–45
7. Hiei Y, Komari T (2008) *Agrobacterium-mediated* transformation of rice using immature

embryos or calli induced from mature seed. Nat Protocols 3:824–834

8. Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291–1301
9. Hood EE, Gelvin SB, Melchers S, Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants (EHA105). Trans Res 2:208–218
10. Lazo GR, Stein PA, Ludwig RA (1991) A DNA transformation-competent Arabidopsis genomic library in *Agrobacterium*. Nat Biotechnol 9:963–967
11. Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vector strategy based on separation of vir-and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303:179–180
12. Frame BR, McMurray JM, Fonger TM, Main ML, Taylor KW, Torney FJ, Paz MM, Wang K (2006) Improved *Agrobacterium*-mediated transformation of three maize inbred lines using MS salts. Plant Cell Rep 25:1024–1034
13. Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FY (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen source. Sci Sin 18:659–668
14. Zhao Z-Y, Gu W, Cai T, Tagliani L, Hondred D, Bond O, Schroeder S, Rudert M, Pierce D (2001) High throughput genetic transformation mediated by *Agrobacterium tumefaciens* in maize. Mol Breed 8:323–333
15. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
16. Toki S (1997) Rapid and efficient *Agrobacterium*-mediated transformation in rice. Plant Mol Biol Rep 15:16–21
17. Cheng M, Hu T, Layton J, Liu C-N, Fry JE (2003) Desiccation of plant tissues post-*Agrobacterium* infection enhances T-DNA delivery and increases stable transformation in wheat. In Vitro Cell Dev Biol Plant 39: 595–604
18. Ozawa K (2012) In: Dunwell JM, Wetten AC (eds) Transgenic plants-methods and protocols, 2nd edn. Humana Press-Springer Science-Business Media, New York, pp 51–57
19. Vogel J, Hill T (2008) High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. Plant Cell Rep 27:471–478
20. Hellens R, Mullineaux P, Klee H (2000) A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci 5:446–451
21. Cho SK, Chung YS, Park SJ, Shin JS, Kwon HJ, Kang KH (1998) Efficient transformation of Korean rice cultivars (*Oryza sativa* L.) mediated by *Agrobacterium tumefa*ciens. J Plant Biol 41:262–268
22. Urushibara S, Tozawa Y, Kawagishi-Kobayashi M, Wakasa K (2001) Efficient transformation of suspension-cultured rice cells mediated by *Agrobacterium tumefaciens.* Breed Sci 51: 33–38
23. Kumar KK, Maruthasalam S, Loganathan M, Sudhakar D, Balasubramanian P (2005) An improved *Agrobacterium*-mediated transformation protocol for recalcitrant elite indica rice cultivars. Plant Mol Biol Rep 23:67–73
24. Puhan P, Vipparla A, Vemireddy LR, Anuradha G, Siddiq EA (2012) An efficient and universal *Agrobacterium*-mediated transformation protocol in rice. J Plant Biochem Biotechnol 21:252–260
25. Rao MVR, Rao GJN (2007) *Agrobacterium-mediated* transformation of indica rice under Acetosyringone-free conditions. Plant Biotechnol 24:507–511
26. Aananthi N, Anandakumar CR, Ushakumari R, Shanthi P (2010) *Agrobacterium*–mediated transformation of indica rice under Acetosyringone-free conditions. Electron J Plant Breed 1:1244–1248
27. Li Z, Upadhyaya NM, Meena S, Gibbs AJ, Waterhouse PM (1997) Comparison of promoters and selectable marker genes for use in indica rice transformation. Mol Breed 3:1–14
28. Thadavong S, Sripichitt P, Wongyai W, Jompuk P (2002) Callus induction and plant regeneration from mature embryos of glutinous rice (*Oryza sativa* L.) cultivar TDK1. Kasetsart J Nat Sci 36:334–344
29. Rancé IM, Tian W, Mathews H, Kochko AD, Beachy RN, Fauquet C (1994) Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. Plant Cell Rep 13:647–651
30. Tsukahara M, Hirosawa T (1992) Simple dehydration treatment promotes plantlet regeneration of rice (*Oryza sativa* L.) callus. Plant Cell Rep 11:550–553
31. Jain RK, Jain S, Wu R (1996) Stimulatory effect of water stress on plant regeneration in aromatic indica rice varieties. Plant Cell Rep 15:449–454