Chapter 2-2

Agrobacterium-mediated transformation system using immature embryos can effectively generate transgenic rice plants with a single copy of the transgene

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Abstract

Agrobacterium-mediated transformation in rice has been widely used for both basic and applied research. Previous reports on *Agrobacterium*-mediated transformation in rice have used mature seed-derived callus or immature embryos as starting materials and have briefly mentioned that the transgenic plants generated by these methods contained a low copy number of the transgene, which offers less labor to fix transgene genetically, to evaluate position effects, and to determine insertion site of transgene, particularly in the transgenic plants with one copy of transgene. However, most of them did not include quantitative observations regarding copy numbers of the transgene; the relationship between transgene copy numbers and starting materials, seed-derived callus, or immature embryos, has also not been elucidated. In this study, we generated transgenic rice plants from mature seed-derived callus and immature embryos of the rice variety Nipponbare and then compared the copy numbers of the transgene in transgenic plants. Transgenic plants from immature embryos had a significantly lower copy number of the transgene than those obtained from mature seed-derived callus, and the percentage of transgenic plants with one copy of the transgene was also significantly different: 25.0% from mature seed-derived callus and 46.7% from immature embryos, respectively. We generated transgenic plants in other five rice varieties—Koshihikari, NERICA1, NERICA4, Curinga, and Kasalath—by transformation using immature embryos. Transgenic plants with one copy of the transgene were generated at a high frequency in all varieties examined; this frequency ranged from 37.6 to 87.0%. We conclude that *Agrobacterium*-mediated transformation using immature embryos effectively generates transgenic rice plants with one copy of the transgene—a desirable trait in molecular breeding and in basic plant research.

Keywords: Copy number, Immature embryos, Rice, Transformation

Introduction

Since Hiei et al. (1994) established a system for genetic transformation of rice varieties mediated by *Agrobacterium*, this procedure has become routine. This technique has been widely used for both basic — i.e., functional analyses of genes—and applied research aiming to introduce desirable traits into rice. The first generation of transgenic plants (T_0) with a single copy of the transgene is desirable for molecular breeding and sometimes for basic research as well, because genetic fixation from T_0 with a single copy of the transgene is more straightforward than that from T0 carrying multiple transgene copies. The evaluation of position effects, that is, qualitative or quantitative variations in transgene expression due to different insertion sites (Matzke and Matzke 1998; Bhat and Srinivasan 2002; Schubert et al. 2004), becomes easier once selected T_0 with a single copy of the transgene. For commercialization of transgenic rice, site-specific sequence data for the entire inserted DNA along with adjacent genomic sequences near the insertion site are required to submit to regulatory agencies (Bradford et al. 2005). T_0 with a single copy of transgene is also ideal in this context.

In studies on *Agrobacterium*-mediated transformation in rice, callus derived from mature seeds (Hiei et al. 1994, Toki 1997, Mohanty et al. 1999, Rachmawati et al. 2004, Lin and Zhang 2005, Toki et al. 2006) or immature embryos (Hiei and Komari 2008, Ishizaki and Kumashiro 2008) have been used as starting materials. The advantage of the methods using mature seed-derived callus is the ease of preparation of materials. On the other hand, the methods using immature embryos have advantages regarding the range of applicable host varieties and transformation efficiencies. These reports briefly mentioned that transgenic rice plants generated by *Agrobacterium*-mediated methods contained a low copy number of the transgene; however, most of the papers did not include quantitative observations.

In this study, we compared transgene copy numbers in transgenic rice plants generated from mature seed-derived callus and immature embryos in Nipponbare variety. We also transformed several rice varieties by *Agrobacterium*-mediated transformation systems using immature embryos and investigated the transgene copy numbers obtained.

Materials and methods

Plant materials

We used six rice varieties. Nipponbare is a “model variety” of rice for which there is solid genome sequence information (International Rice Genome Sequencing Project.2005, Kawahara et al. 2013). Koshihikari has been the most popular variety in Japan for over 30 years (Nitta 2010). NERICA1 and NERICA4 are popular varieties of upland new rice in Africa (Kaneda 2007). Curinga is an elite upland rice

cultivar in South America (de Morais et al. 2005). Kasalath carries several beneficial traits such as tolerance to phosphorus deficiency (Chin et al. 2011, Gamuyao et al. 2012) and has been used for the development of a series of genetic and genomic resources (Ebitani et al. 2005, Kanamori et al. 2013). Nipponbare and Koshihikari are temperate japonica varieties; NERICA1 and NERICA4 derive from an interspecific hybrid of *Oryza sativa* L. and *Oryza glaberrima* Steud., and, together with Curinga, are tropical japonica varieties. Kasalath is an indica variety.

Production of transgenic plants

Agrobacterium tumefaciens strain LBA4404 harboring pBIG-ubi::GUS, which contained the hygromycin phosphotransferase gene (*hpt*) and the ß-glucuronidase gene (*gusA*) in the T-DNA region (Ishizaki and Kumashiro 2008), was used throughout the experiments. The transformation of Nipponbare rice from mature seed-derived callus was performed according to a method previously described (Ishizaki and Kumashiro 2008), with the modification of hygromycin concentration (50 mg/L). Transformation from immature embryos was carried out with a method previously established for NERICA varieties (Ishizaki and Kumashiro 2008), with some modifications depending on the target variety: no modifications for NERICA1, NERICA4, and Curinga; hygromycin concentration was modified from 20 to 50 mg/L for Nipponbare and Kasalath varieties. DKN medium (Daigen et al. 2000) was used as a basal medium for callus induction, selection, and regeneration for Koshihikari variety. Transformation protocols timelines are indicated in **Fig. 1**. The culture media used are listed in **Table 1**.

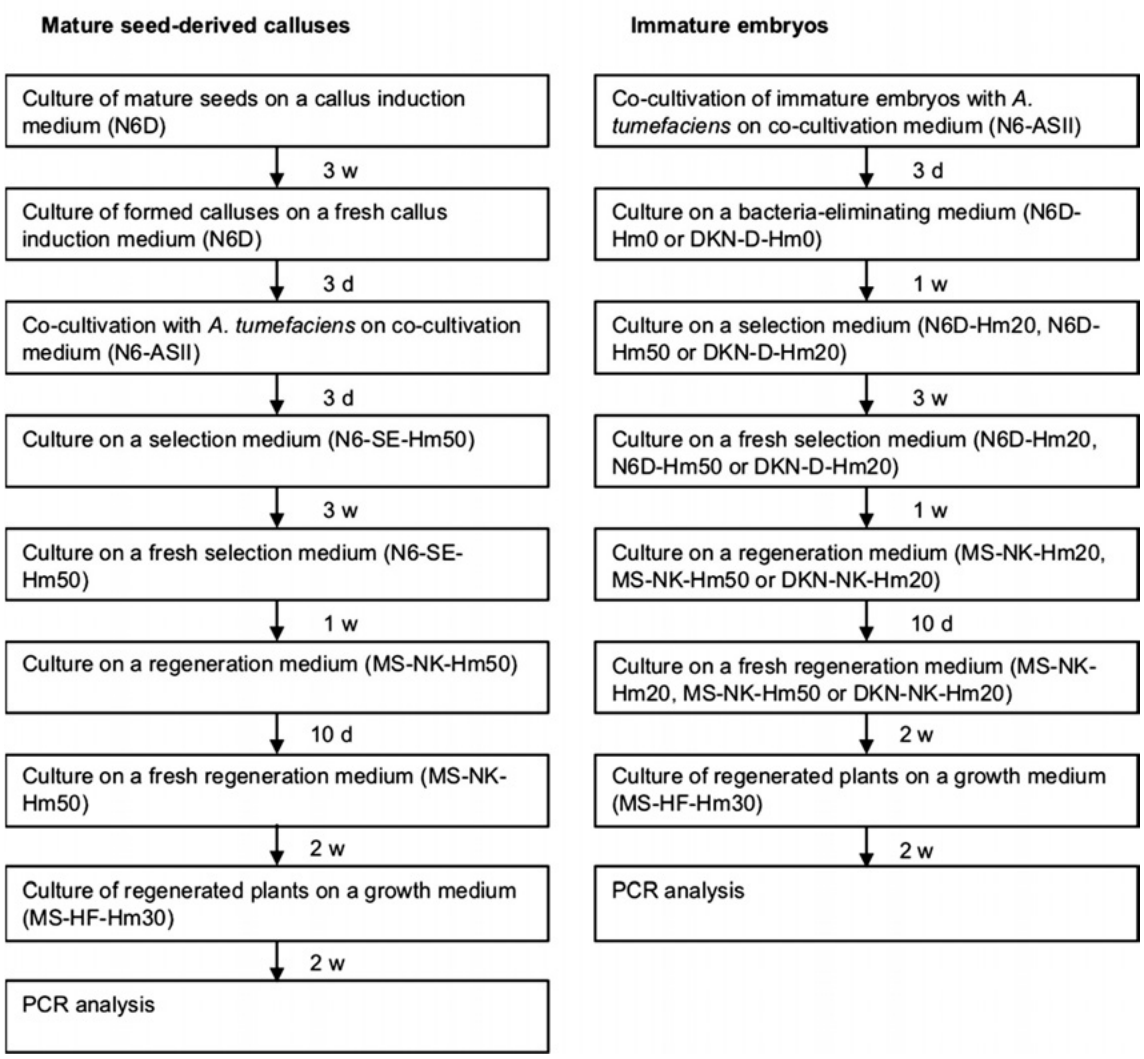

Fig. 1. Transformation protocols timelines from mature seed-derived callus and immature embryos in rice. The composition of media is indicated in **Table 1**.

Table 1. List of media used for transformation of rice varieties

Culture medium	Ingredients
DKN-D-Hm20	DKN salts and vitamins (Daigen et al. 2000), 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan (Sigma, St. Louis, MO, USA), 500 mg/L cefotaxime, 20 mg/L hygromycin, pH5.8
DKN-NK-Hm20	DKN salts and vitamins, 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 20 mg/L hygromycin, pH 5.8
MS-NK-Hm20	MS salts and vitamins (Murashige and Skoog 1962), 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 20 mg/L hygromycin, pH 5.8
MS-NK-Hm50	MS salts and vitamins, 20 g/L sucrose, 30 g/L sorbitol, 2 g/L casamino acids, 0.25 mg/L NAA, 2.5 mg/L kinetin, 4 g/L Gelzan, 250 mg/L cefotaxime, 50 mg/L hygromycin, pH 5.8
N6-ASII	N6 salts and vitamins (Chu 1978), 30 g/L sucrose, 10 g/L glucose, 0.3 g/L casamino acids, 2 mg/L 2,4-D, 50 μM acetosyringone, 10 g/L Type-II agarose (Sigma), pH 5.2
N6-SE-Hm50	N6 salts and vitamins, 30 g/L sucrose, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 50 mg/L hygromycin, pH5.8,
N6D	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, pH5.8
N6D-Hm0	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, pH5.8
N6D-Hm20	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 20 mg/L hygromycin, pH5.8
N6D-Hm50	N6 salts and vitamins, 30 g/L sucrose, 0.3 g/L casamino acids, 25 mM proline, 2 mg/L 2,4-D, 4 g/L Gelzan, 500 mg/L cefotaxime, 50 mg/L hygromycin, pH5.8
MS-HF-Hm30	MS salts and vitamines, 30 g/L sucrose, 4 g/L Gelzan, 30 mg/L hygromycin, pH5.8

2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; BA, benzyladenine

DNA analyses

The presence of the introduced gene in 2-week old T0 plants was confirmed by polymerase chain reaction (PCR). The sequences of the primers used for the detection of *hpt* were 5′-TCGTGCTTTCAGCTTCGATG-3′ and 5′-TCCATCACAGTTTGCCAGTG-3′, and for the detection of *gusA* were 5′-CTGGTATCAGCGCGAAGTCT-3′ and 5′-CGATGGATTCCGGCATAGTT-3′. After confirming the presence of the transgene by PCR, transgenic plants were transferred to a 4-L pot with soil and placed in a greenhouse. Total DNA from the leaves of transgenic plants growing in the greenhouse was extracted and then subjected to Southern blot analyses, as previously described (Ishizaki and Kumashiro 2011), to determine the copy number of the transgene.

Statistical analysis

The data were analyzed by Mann-Whitney's U test and the Chi-square test of independence. Multiple comparisons were performed by Tukey's test or Steel-Dwass' test ($P < 0.05$).

Results and discussion

Transgene copy numbers of transgenic plants obtained from mature seed-derived callus and immature embryos

To compare the transgene copy numbers in transgenic plants developed from different starting materials, mature seed-derived callus and immature embryos, we generated transgenic rice plants from these materials in Nipponbare variety. Both protocols allowed the transformation of this rice variety: 190 transgenic plants were generated from 592 mature seed-derived callus and 67 transgenic plants were generated from 94 immature embryos, respectively, in total of repeated experiments. Then 40 transgenic plants from mature seed-derived callus and 45 transgenic plants from immature embryos, respectively, were subjected to Southern blot analyses to determine the copy number of the transgene in each individual. Transgenic plants from immature embryos had significantly lower copy numbers of the transgene than those obtained from mature seed-derived callus (**Fig. 2**). From both materials, transgenic plants carrying one copy of the transgene were generated at a relatively high frequency. However, only 25.0% of transgenic plants possessed one copy of the transgene when mature seed-derived callus were used as starting materials, while this ratio was significantly enhanced when immature embryos were used, viz., 46.7% of transgenic plants had one copy of the transgene. These results indicate that the transformation system with immature embryos can generate transgenic rice plants with one copy of the transgene more effectively than the system based on the use of mature seed-derived callus in Nipponbare cultivar. The mechanisms underlying the phenomenon is unclear.

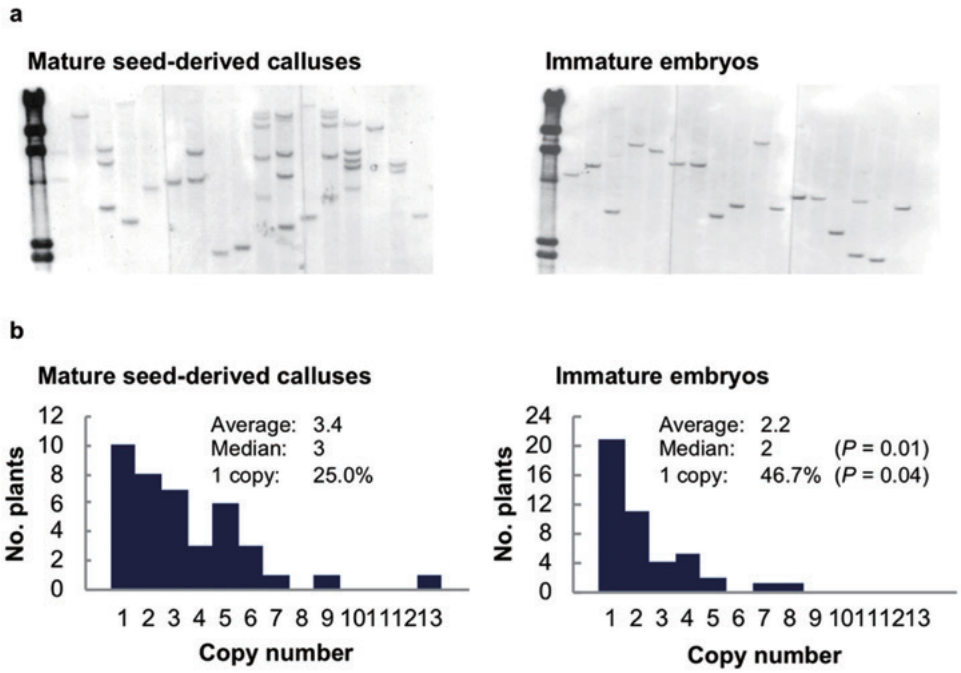

Fig. 2. Comparison of transgene copy numbers in transgenic plants obtained from mature seed-derived callus vs. immature embryos in Nipponbare variety.

(a) Examples of Southern blot analyses. The number of bands in each lane represents the transgene copy number in each individual. The leftmost lane in each blot shows lambda DNA digested with *Hin*dIII (used as a molecular marker); (b) Histograms of transgene copy numbers. Bars represent the number of plants having each transgene copy number. *P* values shown in the graph at the right side indicate significance levels of the differences between mature seed-derived callus and immature embryos. *P* value for the median was calculated by Mann-Whitney's U test. *P* value for the ratio of transgenic plants with 1 transgene copy was calculated by Chi-square test of independence. Since normality test revealed that both data sets had non-normal distribution ($P < 0.01$), statistical analysis for the differences in transgene copy number averages was not carried out.

Transgene copy numbers of transgenic plants generated from immature embryos of other rice varieties

To assess if transformation using as starting material immature embryos generates transgenic plants with one copy of the transgene at high frequency also in other rice varieties, we generated transgenic plants by several repeated experiments in five rice varieties: Koshihikari, NERICA1, NERICA4, Curinga, and Kasalath. The numbers of transgenic plants subjected to Southern blot analyses in each variety were as follows: Koshihikari, 82; NERICA1, 142; NERICA4, 92; Curinga, 57; and Kasalath, 23. Transgenic plants with one copy of the transgene were generated at a high frequency in all varieties we examined: 37.6% in Koshihikari, 42.3% in NERICA1, 42.4% in NERICA4, 40.4% in Curinga, and 87.0% in Kasalath (**Fig. 3**). Transgenic plants in Kasalath had significantly lower copy numbers of the transgene than those obtained from the other varieties examined, and the ratio of transgenic plants carrying one copy of the transgene in Kasalath was significantly higher. Overall, these results suggest that the transformation system with immature embryos can effectively generate transgenic rice plants with one copy of the transgene in several rice varieties, particularly in Kasalath.

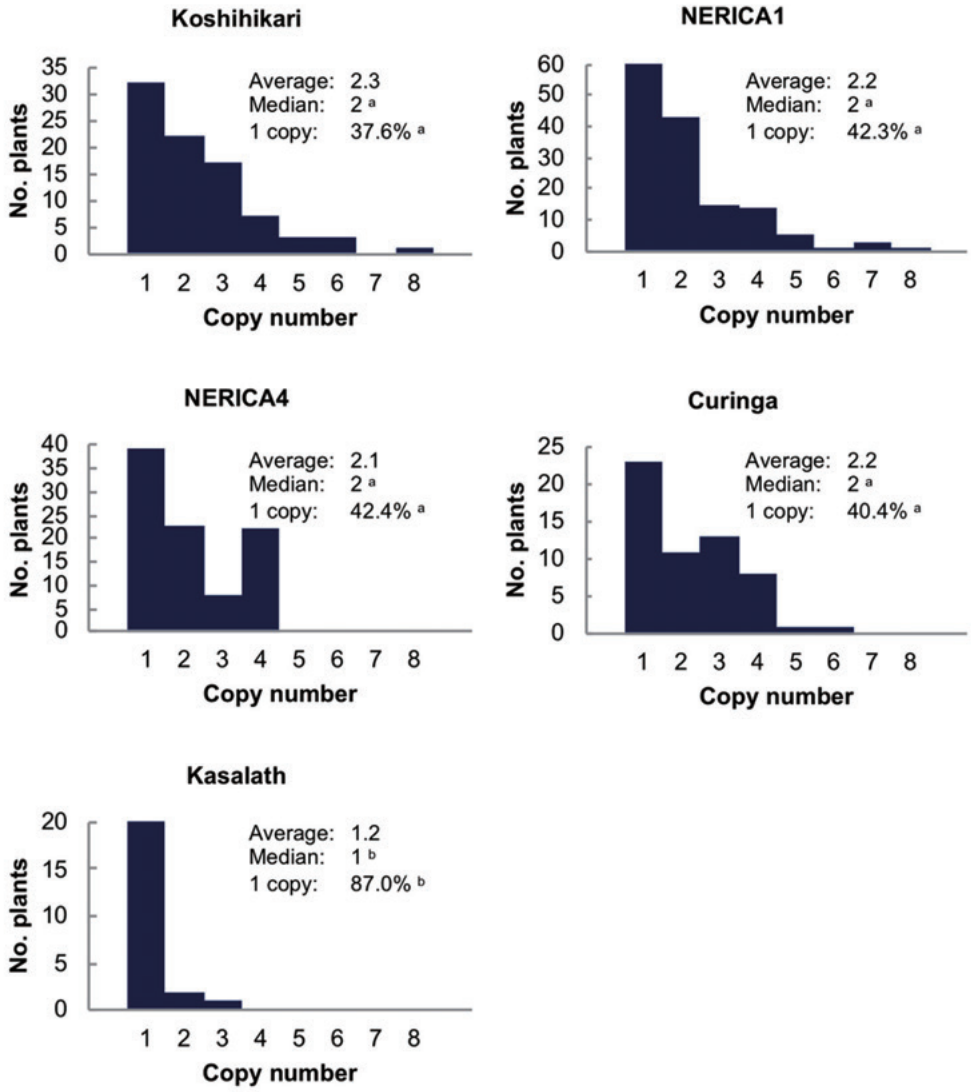

Fig. 3. Histograms showing transgene copy numbers in transgenic plants from immature embryos, in different rice varieties.

Bars represent the number of plants having each transgene copy number. Different letters denote significant differences at $P < 0.05$, as determined by Steel-Dwass' test for the median and by Tukey's test for the ratio of the transgenic plants with 1 transgene copy.

Conclusion

Higher transformation efficiencies and broader ranges of transformable varieties have been considered advantages of transformation protocols using immature embryos as starting materials, instead of mature seed-derived callus (Hiei and Komari 2008). Our results indicate that a higher frequency of transgenic plants with one copy of the transgene is also an advantage of transformation using immature embryos. Transgenic plants with a single copy of the transgene are desirable to fix transgenes genetically, to evaluate the position effects of them, and to determine their insertion sites. In target-mutagenesis technologies by

genome editing, like CRISPR/Cas9, removability of transgenes by segregation is a benefit in the context of molecular breeding (Bortesi and Fischer 2015, Hartung and Schiemann 2014). Additionally, the importance of transgene elimination by segregation to ensure stable inheritance of mutations and to avoid chimerism in later generations was also suggested (Ishizaki 2016). In this context, transgenic plants with a single copy of the transgene are ideal, since the efficiency of transgene elimination by segregation is determined by the number of loci where a transgene insertion is present: 25% (1/4) for insertion at one locus, 6.25% (1/16) for insertion at two loci, and so on. To sum up, the transformation protocol using immature embryos described in this study can be used as a reliable tool to generate transgenic rice plants with one transgene copy. Transgenic plants carrying a single transgene copy are desired in molecular breeding and in plant basic research relying on transgenic approaches and also on genome editing.

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