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Differential transformation efficiency of *Japonica* rice varieties developed in northern China

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Abstract – *The production of japonica rice in northern China plays an important role in food security for the world. Agrobacterium-mediated gene transfer is becoming a powerful approach to generate germplasm and develop varieties. However, the transgenic efficiency of the japonica rice varieties in northern China has been completely unknown, which obstructs the development of transgenic breeding and the exploration of gene functions. In this study, the transgenic efficiencies of six japonica rice varieties developed in northern China are evaluated. The rates of primary and secondary callus induction of the varieties are similar. However, transgenic efficiency and the regeneration ability of the varieties are greatly different. The results have established a platform for transformation of the rice varieties and proposed a suitable variety, SN9816, for gene transfer. SN9816 can be applied as an elite germplasm for transgenic breeding and basic research of molecular biology in northern China or an area in the same latitude.*

Key words: *Agrobacterium-mediated transformation, transformation efficiency, japonica rice in northern China, Shen Nong 9816.*

INTRODUCTION

Rice (*Oryza sativa* L.), which is cultivated in more than 115 countries and feeds over one third of the population worldwide, is one of the world's most important crops (Khush 1997, Raimondi et al. 2014). More than 90% of the world's rice is grown and consumed in Asia, where 60% of the earth's people live. Rice is also one of the major staple foods in China, a center of origin of Asian cultivated rice (Khush 1997, Londo et al. 2006). The production of *japonica* rice in northern China is famous for its higher yield and good quality and accounts for 50% of the total production. Enhancing the yield and improving the quality of rice through breeding are effective ways of responding to increasing food demands. Molecular breeding, including marker assistant selection (MAS), transgenic breeding, and genome-wide association study (GWAS) provides breeders with new challenges and opportunities to increase the efficiency of selection, shorten the breeding period, and generate new germplasm with improved traits, which are difficult to improve by conventional methods (Jung et al. 2008).

Improvement of rice varieties through transferring genes or cis-acting elements from rice, plants, or other organisms to rice to confer new agronomic traits in rice, termed transgenic breeding, is an effective way to create new germplasm and breed new varieties (McLaren 2005). *Agrobacterium*-mediated transformation is a general method for genetic modification in many plant species because it allows efficient insertion of stable, unrearranged, single-copy sequences into plant genomes (Nishimura et al. 2006). Efficient methods for *Agrobacterium*-mediated transformation of *japonica* rice have been developed (Hiei et al. 1994, Hiei et al. 1997, Hiei and Komari 2008). Two *japonica* varieties, Nipponbare and Kitaake (developed in Japan), are good genotypes for *Agrobacterium*-mediated transformation. Based on this method, *Agrobacterium*-mediated transformation methods have been improved, with minor modification for many rice varieties, not only *japonica* but also *indica* and *tropical japonica* (Nishimura et al. 2006, Hiei and Komari 2008, Saika and Toki 2010).

It has been demonstrated that transformation efficiencies are genotype dependent (Hiei et al. 1997, Hiei and Komari 2008). Although efficient procedures for *Agrobacterium*-mediated transformation of either *japonica* or *indica* rice have been developed, the transgenic efficiency of the rice varieties developed in northern China have been completely

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unknown (Hiei and Komari 2008, Saika and Toki 2010). Additionally, because of the higher latitude in northeast China and the sensitivity of rice heading to day length, Nipponpare could not successfully develop seed in the field under normal conditions. Even though Kitaake could be used to replace Nipponbare in northern Asia, it was hard for breeders to use Kitaake as breeding germplasm because the plant size of Kitaake is smaller than desired and its agronomic traits are not as good as expected. These factors restrict the development of transgenic breeding and the performance of molecular biological study in northeast China. In this study, five varieties which are popularly cultivated in northern China are used as explants to compare their response to *Argobacterium*-mediated transformation by using Nipponbare as control. Our studies have established a platform for *Agrobacterium*-mediated transformation of the *japonica* rice varieties developed in northern China and proposed a suitable variety, Shen Nong 9816, for *Agrobacterium*-mediated gene transfer with a transgenic efficiency and regeneration rate similar to Nipponbare.

MATERIAL AND METHODS

Plant materials

Six varieties of *japonica*-type rice were used in the study, including Shen Nong 9816 (SN9816), Shen Nong 265 (SN265), M31, Liao Xing 1 (LX1), Toyonishiki (Toyo, from Japan), and Nipponbare (Nipp, from Japan, sequenced). Among them, SN9816, SN265, M31, LX1, and Toyonishiki are widely cultivated in northern China, which is the major region for *japonica* rice production in China. Nipponbare was used as control, the seeds of which were produced in the Experimental Station of the Chinese Academy of Agricultural Science, Beijing (lat 39° 92' N, long 116° 46' E). The mature seeds of the other five varieties used for callus induction were produced in the Experimental Station of the Rice Research Institute at Shenyang Agricultural University (lat 41° 80' N, long 123 °38' E) in northeast China.

Binary vector and *Agrobacterium*-mediated transformation

The pCambia 1301 binary vector was applied, harboring the expression cassettes of the *35S:intron-GUSA* gene (tagged by hexaHis), the *35S:aadA* gene (conferring kanamycin resistant to bacterium), and the *35S:hptII* gene (conferring hygromycin resistance to callus or plantlets) (Figure 1). The binary vector was transformed into the *Agrobacterium* strain EHA 105 through the Freeze-Thaw method.

A procedural representation of *Agrobacterium*-mediated transformation using secondary callus is shown in Figure 2. Dehulled mature seeds, which were sterilized with 70% ethanol for 1 minute and then 2.5% sodium hypochlorite for 20 minutes (Figure 2A), were inoculated in callus induction N6D medium (Figure 2B), containing 3.99 g L^{-1} Chu N6 Basal Medium with Vitamins (Phyto Technology Laboratories, Product No. C167), 0.1 g L^{-1} myo-inositol, 0.3 g L^{-1} casamino acids, 2.878 g L^{-1} proline, 2 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D), 30 g L^{-1} sucrose, and 5 g L^{-1} agar, pH = 5.8. Three-week-old secondary calli (Figure 2D) derived from 7-day-old primary calli (Figure 2C) were subcultured in fresh N6D medium for 3 days before co-cultivation with *Agrobacterium* (Figure

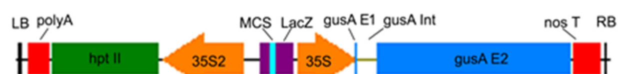

Figure 1. A map of pCambia 1301 binary vector between the left border and right border. LB: left border repeat; polyA: CaMV 3' UTR polyA signal; hpt II: hygromycin resistance gene; 35S2: CaMV 35S promoter duplicated; MCS: multiple cloning site; Lac Z: Lac Z promoter and Lac Z alpha fragment; 35S: CaMV 35S promoter; gusA E1: gusA first exon; gusA Int: gusA intron; gusA E2: gusA second exon; nos T: nopaline synthase 3'UTR polyA signal; RB: right border repeat.

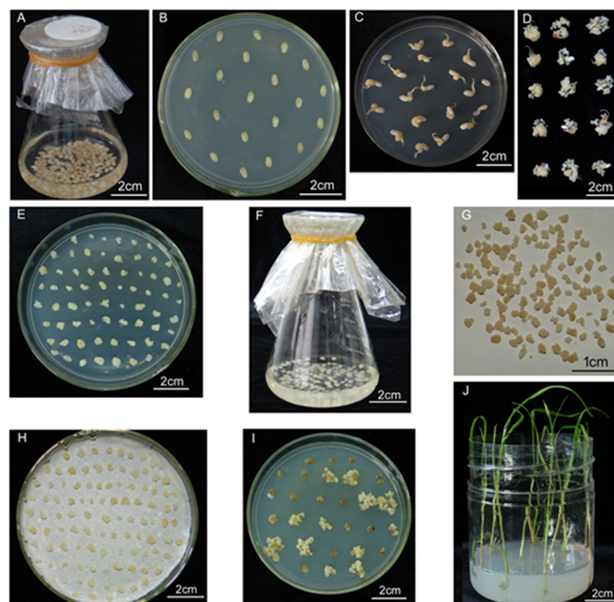

Figure 2. Procedures of *Agrobacterium*-mediated transformation for *japonica* rice varieties in northern China. (A) Sterilization of Seeds. (B) Inoculation of seeds in N6D medium. (C) Formation of primary calli in N6D medium. (D) Induction of secondary calli in N6D medium. (E) Preculture of the secondary calli in N6D medium three days before transformation. (F) Infection of the preculture secondary calli with the *Agrobacterium* harboring pCambia 1301 vector. (G) Drying of the infected calli before co-cultivation. (H) Co-cultivation with the *Agrobacterium* for three days in the dark. (I) Proliferation of Hyg-tolerant calli on the secondary selection medium, N6D-S medium. (J) Regeneration of transgenic shoot in root generation medium, MS-HF. The white or black line is the scale bar, representing 2cm or 1cm as indicated.

2E). *Agrobacterium* co-cultivation followed the modified protocol described in (Toki et al. 2006). The infected and air-dried secondary calli (Figures 2F, G) were co-cultivated with *Agrobacterium* for 3 days under constant darkness in 2N6-AS medium (Figure 2H), containing 3.99 g L^{-1} Chu N6 Basal Medium with Vitamins, 0.1 g L^{-1} Myo-inositol, 0.3 g L^{-1} casamino acids, 2 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D), 30 g L^{-1} sucrose, 10 g L^{-1} glucose, 5 g L^{-1} agar, and 1 ml of 100 mM AS, pH = 5.2. The calli were then washed with water and cephalothin solution, and cultured in the N6D medium containing 50 mg L^{-1} hygromycin B (Hyg) (Roche Diagnostics, Basel, Switzerland, Product No. 10843555001) and 450 mg L^{-1} cephalothin for 10 days (N6D-S medium). After 10 days of selection, calli were transferred to fresh N6D-S medium for another 10 days (Figure 2I). For regeneration, calli growing vigorously on Hyg were transferred to MS-NK medium, containing 4.43 g L^{-1} MS Basal Medium (Phyto Technology Laboratories, Product No. M519), 0.1 g L^{-1} Myo-inositol, 2 g L^{-1} casamino acids, 0.20 mg L^{-1} alpha-naphthalene acetic acid (NAA), 2 mg L^{-1} kinetin, 30 g L^{-1} sucrose, 30 g L^{-1} sorbitol, 30 mg L^{-1} Hyg, and 450 mg L^{-1} cephalothin, pH = 5.8, and cultured for more than 4 weeks. Shoots arising from callus in MS-NK medium were transferred to MS-HF medium, containing 4.43 g L^{-1} MS Basal Medium, 0.1 g L^{-1} Myo-inositol, and 30 g L^{-1} sucrose, pH = 5.8 with 25 mg L^{-1} Hyg, and 450 mg L^{-1} cephalothin to allow vigorous root growth (Figure 2J).

GUS staining approach

Transient expression of *35S:intron-GUS* was assayed after 3 days of co-cultivation through detection of histochemical *β*-glucuronidase (GUS) reporter activity with 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc, Thermol Scientific Cat# R0851) as a substrate (Jefferson et al. 1987).

Methods used in statistical analysis

Three independent biological replicates were performed. The data were analyzed using the GraphPad Prism 5 software. The Tukey multiple comparison test of one-way analysis of variance (ANOVA) was used to detect the significance of differences.

Genomic DNA extraction and PCR analysis

For extraction of genomic DNA, rice seedlings were harvested, immediately frozen in liquid N_2, and stored at -80℃. Genomic DNA was extracted through the 2×CTAB method. PCR analysis to detect the *hpt* gene was performed by using rTaq DNA polymerase (Takara Biotechnology, Cat# R500Z) with the primer pair of forward primer, HygF CTTCTGCGGGCGATTTGT, and reverse primer, HygR CAGCGTCTCCGACCTGAT.

RESULTS AND DISCUSSION

Callus formation abilities are similar in the varieties

It is known that some rice varieties are tissue-culture sensitive, especially the *indica* rice varieties. To evaluate the ability for callus formation, sterilized mature seeds of the varieties were inoculated in N6D medium and grown for 7 days or 3 weeks. The status of primary callus growth, the rate of primary or secondary callus induction, and the rate of secondary callus growth were observed (Figure 3).

Yellow, compacted primary callus can be induced from all six varieties, but the emergence of primary calli from Liao Xing 1 (LX1), M31, and Shen Nong 265 (SN265) was later and slower than that of Nipponbare (Nipp), Shen Nong 9816 (SN9816), and Toyonishiki (Toyo) (Figure 3A). The rates of primary or secondary callus induction of the varieties were similar (Figures 3B, C). No significant difference in the rate of primary or secondary callus induction of the varieties was analyzed. However, a highly significant difference in the fresh weight of 100 callus cluster of the secondary calli (FWSC) after 15 days of subculture was observed among

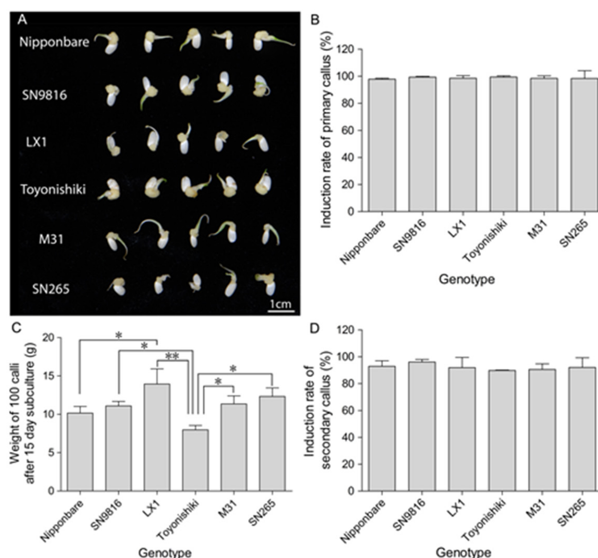

Figure 3. Callus formation of *japonica* rice varieties in northern China. (A) The 7-day-old primary calli of the varieties. (B) The rate of primary callus induction of the varieties. (C) The rate of secondary callus induction of the varieties. (D) The fresh weight of 100 callus cluster of secondary calli after 15 days of subculture. The white line is the scale bar, representing 1cm. The values are the mean and standard deviation (SD) from three independent biological experiments. SN9816: Shen Nong 9816; LX1: Liao Xing 1; SN265: Shen Nong 265. One asterisk (*) represents significant difference ($P<0.05$). Two asterisks (**) represent highly significant difference ($P<0.01$).

the varieties (Figure 3D). The FWSC of LX1 (13.94g) was the highest, and that of Toyo (7.97g) was the lowest. The FWSC of LX1 was significantly higher than that of Nipp (10.16g). The FWSC of LX1 (13.94g), SN265 (12.33g), M31 (11.36g), or SN9816 (11.09g) was significantly higher than that of Toyo. However, no significant difference of the FWSC was detected between Nipp and SN9816, between Nipp and Toyo, and among SN9816, LX1, M31, and SN265. These results indicated that the secondary calli induced from the five varieties developed in northern China are of good status for *Agrobacterium*-mediated transformation compared to Nipp. Thus, we conclude that it is easier to induce calli from the mature seeds of the varieties, including SN9816, LX1, Toyo, M31, and SN256.

Transformation efficiencies of the six varieties are vary

To investigate the transformation rates of the *japonica* rice varieties, 3-week-old secondary calli were infected with the *Agrobacterium* strain EHA105 harboring the pCambia 1301 vector. After 3 days of co-cultivation with *Agrobacterium*, transient expression efficiency of GUS was determined (Figure 4A). *Intron-GUS* is a convenient reporter gene since this gene is strongly expressed in rice cells but not in *Agrobacterium* cells that attach to the tissues (Chan et al. 1993). The rates of the GUS stained calli were analyzed (Figure 4B). The results showed that the GUS staining rate of SN9816 (59.17%) was the highest, which was higher than that of Nipp (48.78%). However, the rates of LX1 (20%), Toyo (18.89%), M31 (17.50%), and SN265 (25.54%) were lower than that of Nipp (48.78%).

After 3-week selection in the N6D medium with 50 mg L^{-1} Hyg (N6D-S), the proliferation of Hyg-resistant calli was observed from all the varieties (Figure 4C). However, the rates of Hyg-tolerant calli or transgenic efficiencies of the varieties were different (Figure 4D), which is consistent with the previous results that transgenic efficiency is genotype dependent (Hiei et al. 1997, Hiei and Komari 2008, Saika and Toki 2010). The highest rate of Hyg-resistant calli was obtained from Toyo (92.69%) and the lowest rate from SN265 (36.61%). Toyo (92.69%), SN9816 (91.60%), and Nipp (82.96%) had much more significant rates than LX1 (33.62%), M31 (34.52%), and SN265 (36.61%) (Figure 4D). There was no significant difference among LX1, M31, and SN265. These results indicated that, in addition to Nipp, both SN9816 and Toyo were genotypes tolerant to *Agrobacterium*-mediated gene transfer, which gave rise to higher transformation efficiency after secondary selection on Hyg containing N6D-S medium (Figure 4D).

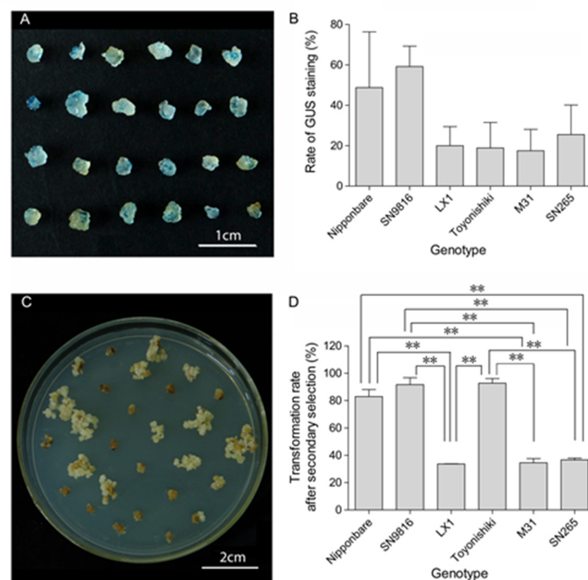

Figure 4. *Agrobacterium*-mediated transformation efficiency of the varieties in northern China. (A) Transient expression of *35S:intron-GUS* after 3 days of co-cultivation with *Agrobacterium*. (B) The rate of GUS stained calli of the varieties. (C) The proliferation of the Hyg-tolerant calli in secondary selection medium, N6D-S medium. (D) Transgenic efficiencies of the varieties in northern China after secondary selection. Three biological replicates were performed. Similar results were obtained. The results from two or three replicates are shown. The values are the mean and standard deviations from three independent biological experiments. One asterisk (*) represents significant difference ($P<0.05$). Two asterisks (**) represent highly significant difference ($P<0.01$). SN9816: Shen Nong 9816; LX1: Liao Xing 1; SN265: Shen Nong 265. White lines are the scale bar, representing 1cm or 2cm as indicated.

The regeneration ability of the varieties differ from each other

To explore the regeneration capability of the varieties, the secondary calli were transferred directly to the MS-NK regeneration medium. The regeneration rates without transformation of the varieties on an inoculated callus basis were counted. The results revealed that the calli derived from all the varieties had the ability to regenerate (Figure 5A), but the rates of regeneration of the varieties differed. The regeneration rate of Toyo (73.99%) was the highest, and that of M31 (22.93%) was the lowest (Figure 5C). The regeneration rates of Toyo (73.99%), Nipp (66.12%), SN9816 (64.66%), and SN265 (52.50%) were significantly higher than LX1 (35.55%) and M31 (22.93%) (Figure 5C). The regeneration rate of SN265 was significantly higher than that of M31 (Figure 5C). No significant difference was detected among Toyo, Nipp, SN9816, and SN265, and between LX1 and SN265 (Figure 5C). These results suggest that there is a greater possibility of growing plants from the callus for Toyo, Nipp, SN9816, and SN265. However, the question remains as to whether the regeneration ability of

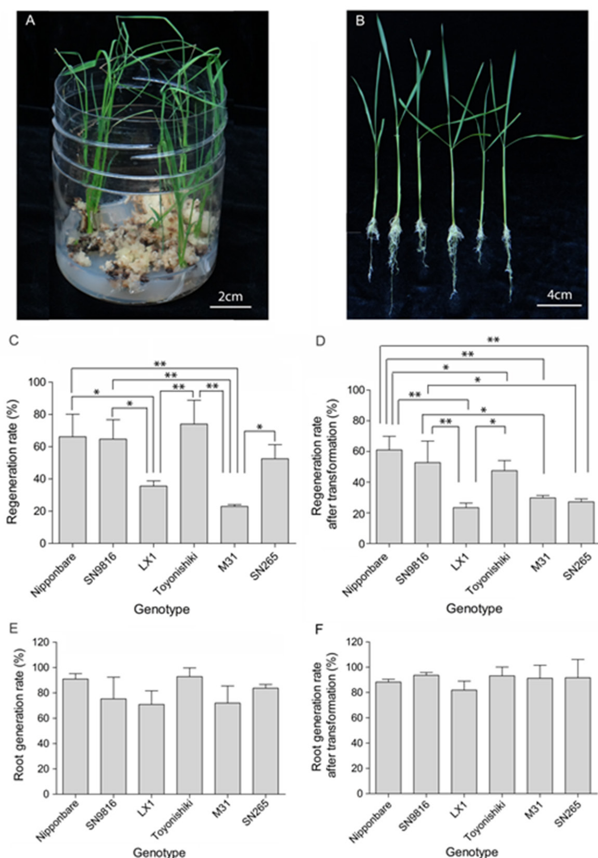

Figure 5. Regeneration ability of the varieties in northern China. (A) Regenerated shoots from the Hyg-tolerant tissues. (B) Plantlets with roots derived from *Agrobacterium*-mediated transformation. (C) Regeneration rates without *Agrobacterium*-mediated transformation of the varieties in northern China. (D) Regeneration rates with *Agrobacterium*-mediated transformation of the varieties in northern China. (E) The rate of root generation from the regenerated shoots without transformation. (F) The rate of root generation from the regenerated shoots with *Agrobacterium*-mediated transformation. Three biological replicates were performed. Similar results were obtained. The results from two or three replicates are shown. The values are the mean and standard deviationsof two or three independent biological experiments. One asterisk (*) represents significant difference (P<0.05). Two asterisks (**) represent highly significant difference (P<0.01). SN9816: Shen Nong 9816; LX1: Liao Xing 1; SN265: Shen Nong 265. White lines are the scale bar, representing 2cm or 4cm as indicated.

the varieties may be influenced by *Agrobacterium*-mediated transformation.

Regeneration of the varieties is transgenic sensitive

To investigate the response of regeneration to *Agrobacterium*-mediated transformation, the Hyg-tolerant calli of the varieties in N6D-S medium were transferred to the MS-NK regeneration medium. Regeneration ability was evaluated. The results showed that the regeneration rates after transformation of all the varieties were lower than the rate of calli subcultured in the regeneration medium without transformation (Figures 5C, D). The highest regeneration efficiency after *Agrobacterium*-mediated transformation was observed from the Nipp variety (60.98%), and the lowest was from LX1 (23.33%) (Figure 5D). The regeneration rates after *Agrobacterium*-mediated transformation of Nipp (60.98%) and SN9816 (52.75%) were significantly higher than those of LX1 (23.33%), SN265 (27.14%), and M31 (29.74%) (Figure 5D). The rates of Nipp (60.98%) and Toyo (47.43%) were significantly higher than the rates of Toyo (47.43%) and LX1 (23.33%), respectively (Figure 5D). There was no significant difference between Nipp and SN9816 (Figure 5D). These results revealed that the regeneration of all six varieties was sensitive to *Agrobacterium*-mediated transformation, especially the Toyo variety (Figures 5C, D). *Agrobacterium*-mediated transformation reduced regeneration ability. However, the regeneration rates of Nipp and SN9816 were similar, at the level of 50% to 60%, based on the number of Hyg-tolerant calli (Figure 5D).

The rates of root growth of the regenerated shoots were similar among the varieties

After regeneration, the regenerated shoots were transplanted to the root growth medium (MS-HF) to allow vigorous root growth (Figure 5B). The root-generating ability among the varieties was similar. No significant difference of root-emergence rates was observed among the varieties without or after *Agrobacterium*-mediated transformation (Figures 5E, F). We conclude that the regenerated shoots from the calli transformed or non-transformed varieties had

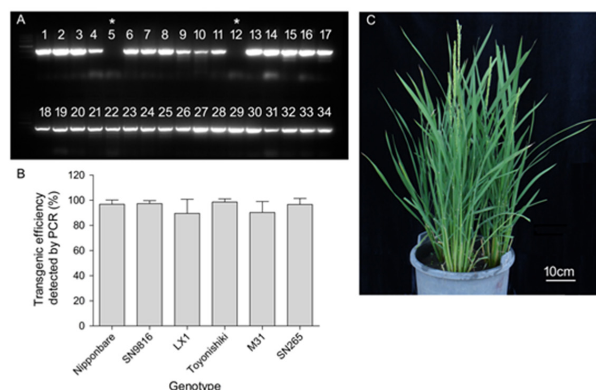

Figure 6. Detection of transgenic plantlets with PCR. (A) A gel image of PCR products with the HygF/HygR primer pair on Hyg-tolerant transgenic plantlets. The number represents the individual transgenic plantlets. The asterisk (*) labeled 5 and 12 represents negative plantlets. (B) Transgenic efficiency detected by PCR based on the number of the rooted regeneration plantlets. (C) Transgenic plants in heading stages. The white line is the scale bar, representing 10cm.

similar ability to generate roots (Figures 5E, F).

To determine the rate of positive transgenic plants, Polymerase Chain Reaction (PCR) was performed on the genomic DNA of the candidate transgenic plants obtained with the primer pair (HygF/HygR) of the Hyg gene in the pCambia 1301 binary vector (Figure 6A). The transgenic rates of the plants from the varieties were measured with the statistical method (Figure 6B). The transgenic rates of the candidates were from 93.75% to 98.58%. No significant difference existed among the varieties. The transgenic plants were then transplanted to the soil to harvest seeds for the subsequent studies (Figure 6C).

To summarize, the transgenic efficiency of five varieties commonly grown in northern China were evaluated in this study and compared to Nipp. Though the rates of the secondary callus growth vary, the proliferation rates of primary and secondary calli of the varieties are similar, indicating that it is easier to induce calli from the mature seeds of the varieties (Figure 3). The transgenic efficiency of the varieties is different after secondary selection in the Hyg-containing N6D-S medium. The transgenic efficiencies of Toyo, Nipp, and SN9816 are similar, but much more highly significant than those of LX1, M31, and SN265 (Figure 4). *Agrobacterium*-mediated transformation suppresses regeneration of the varieties. The regeneration rates of Toyo, Nipp, and SN265 without transformation are higher than those of LX1 and M31 (Figure 5C). The regeneration rates after transformation of Nipp and SN9816 are higher than those of LX1, M31, and SN265 (Figure 5D). The regeneration of Toyo is hypersensitive to *Agrobacterium*-mediated transformation (Figures 5C, D). We may easily conclude that the performance of SN9816 during *Agrobacterium*-mediated gene delivery, including callus formation, transgenic efficiency, regeneration rate, and root generation rate, is similar to Nipp. Our data suggest that SN9816, which is the major cultivated super *japonica* variety with erect panicle, ideal plant architecture, and higher yield, is an elite germplasm for *Agrobacterium*-mediated transformation in northern China and the area at the same latitude. These results lay the sound foundation for us to generate the large number of T-DNA insertion plants needed for functional analysis of the rice genome and for breeding super *japonica* rice varieties with additional agronomic value.

ACKNOWLEDGMENTS

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Eficiência diferencial de transformação de variedades de arroz *japonica* desenvolvidas no norte da China

Resumo – *A produção de arroz japonica no norte da China desempenha um papel importante na segurança alimentar para o mundo. Transferência de genes mediada por Agrobacterium está se tornando uma abordagem poderosa para gerar germoplasma e desenvolver variedades. No entanto, a eficiência transgênica das variedades de arroz japonica no norte da China tem sido completamente desconhecida, o que obstrui o desenvolvimento da criação de transgênicos e a exploração das funções dos genes. Neste estudo, as eficiências da transgenia de seis variedades de arroz japonica desenvolvidas no Norte da China são avaliadas. As taxas de indução de calo primário e secundário das variedades são semelhantes. Contudo, a eficiência transgênica e a capacidade de regeneração das variedades são bem distintas. Foi estabelecida uma plataforma para a transformação das variedades de arroz e proposta uma variedade adequada, SN9816, para transferência de genes. SN9816 pode ser aplicada como um germoplasma-elite para melhoramento transgênico e pesquisa básica de biologia molecular no norte da China ou em uma área na mesma latitude.*

Palavras-chave: *Transformação mediada por Agrobacterium, eficiência de transformação, arroz japonica, no norte da China, Shen Nong 9816.*

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