Indian J. Genet., 80(2) 147-153 (2020)
DOI: 10.31742/IJGPB.80.2.4

Optimization of genetic transformation method for an *indica* rice (*Oryza sativa* L.) variety Ratnagiri-711

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(Received: December 2019; Revised: April 2020; Accepted: April 2020)

Abstract

A method of *Agrobacterium*-mediated genetic transformation for insect resistance was optimized for an *indica* rice variety Ratnagiri-711. Three different *cry* genes *viz*., *cry1Aabc*, *cry1Fa1* and *cry2Aa* with a vector system consisting of the disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA-105 harbouring *pBinAR* or *BinBt3* were used. The respective genes were linked to CaMV35S promoter and *nptII* gene under control of *nos* promoter and terminator. Scutellum-derived callus bits and embryonic shoot apical meristem of germinating seeds were used as target tissues for callus-mediated and *in planta* transformation, respectively. Kanamycin screening and PCR analysis was employed for confirmation of presence of transgene. Among five methods of colonization and co-cultivation tried with three *cry* genes, a callus-mediated transformation method consisting of 20 minutes colonization and 3 days co-cultivation with *cry2Aa* gene recorded highest transformation frequency (13.79%) but minimum survival (5.27%). On the contrary, considerable transformation frequency (6.35%) with highest survival (79.42%) was observed in an *in planta* method employing mild injury to embryonic shoot apical meristem of germinating seeds followed by injection of *Agrobacterium* having *cry2Aa* gene followed by 15 minutes colonization and then directly sowing in pots. Among three *cry* genes used, the gene *cry2Aa* was found most effective showing more transformation frequency.

Key words: Colonization, co-cultivation, survival, transformation frequency

Introduction

Rice (*Oryza sativa* L.) is the staple food for more than one third of world's population (Anonymous 2016). Therefore, it is of utmost importance to augment the productivity of rice to cope with the increased threat of population boom. The production and productivity of rice suffers significantly because of biotic and abiotic stresses. A large number of insects are known to ravage the rice fields, but the most important pests are rice stem borer species under the Order Lepidoptera. The rice stem borers cause injury to the crop at every phase of crop development. The extent of borer damage induced yield losses is estimated to be 13.07 to 70 % in Asia. Yellow stem borer (*Scirpophaga incertulas*) damage can lead to about 20% yield loss in early planted rice crops and 80% in late-planted crops (Anonymous 2014). The conventional methods of pest control have some drawbacks such as environmental pollution and residual effects of insecticides. More than 39,000 varieties of rice have been screened for resistance against *S. incertulas* at the International Rice Research Institute in Laguna, Philippines (Khan et al. 1991) and many national agricultural research laboratories also screened hundreds of cultivars and breeding lines. Though the resistance in a number of varieties or lines has been detected, high levels of resistance are not found in cultivated rice. Biotechnological approaches are widely used for crop improvement, especially incorporating resistance against insect pest and other stresses. The recent development of transformation techniques has provided the technology for incorporating the bacterial gene lethal to stem borer larvae into rice genome. It has opened a new dimension to the development of pest resistant varieties. Genetic engineering of rice with toxins from *Bacillus thuringiensis* is being pursued by numerous research groups, and *S. incertulas* is a major target of this work (Bottrell et al. 1992). Several methods such as microprojectile bombardment, electroporation,

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Published by the Indian Society of Genetics & Plant Breeding, A-Block, F2, First Floor, NASC Complex, IARI P.O., Pusa Campus, New Delhi 110 012; Online management by www.isgpb.org; indianjournals.com

sonication and *Agrobacterium*- mediated transformation are available for genetic transformation of plants. The *Agrobacterium-mediated* genetic transformation is more efficient and results in integration of potentially low copy number, high co-expression of the introduced genes and preferential integration into actively transcriable regions (Birch 1997). Hence, the present investigation was carried out to efficiently transform a highly regenerable indica rice variety Ratnagiri-711 via *Agrobacterium*-mediated method either through callus or by *in planta* technique imparting resistance to primarily Lepidopteran insects like yellow stem borer.

Efficient protocols for *Agrobacterium*-mediated transformation were reported for Japonica rice (Hiei et al. 1994), and subsequently for Javanica (Dong et al. 1996) and Indica (Rashid et al. 1996) rice. A key point in the various protocols is the use of tissue that consists of actively dividing, embryonic cells, such as calli induced from scutella. Such cells are co-cultivated with *Agrobacterium* in the presence of acetosyringone, a potent inducer of the *Agrobacterium* genes that are involved in the transfer of DNA. Several such tissue culture based callus mediated transformation events are reported. However, they require sterile conditions and are time-consuming. Also, somaclonal mutation or variation frequently occurs in plant cells during *in vitro* culture and some plants are recalcitrant to regeneration (Hiei and Komari, 2008). On the other hand, *in planta* transformation excludes *in vitro* culture and chances of somaclonal variation induced through *in vitro* culture (Supartana et al. 2005). The *in planta* rice transformation was firstly established by Supartana et al. (2005) for japonica varieties by inoculating the embryonic apical meristems of soaked seeds with *A. tumefaciens* injection.

Materials and methods

Agrobacterium-mediated genetic transformation experiments were carried out in the rice variety Ratnagiri-711 which had shown better regenerability (Sawant et al. 2018). Three different strains of *Agrobacterium tumefaciens* having different genes *viz*. *cry1Aabc*, *cry1Fa1* and *cry2Aa* collected from the National Research Centre for Plant Biotechnology, New Delhi were used for genetic transformation experiments (Figs. 1, 2 and 3). The disarmed hyper virulent *Agrobacterium tumefaciens* strain EHA-105 harbouring *pBinAR* or BinBt3 was used as vector system. It contains the respective gene linked to cauliflower mosaic virus (CaMV) 35S promoter and neomycin

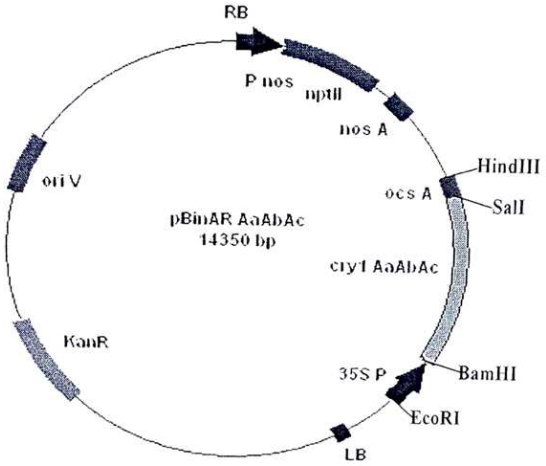

Fig. 1. Map of the *cry1Aabc* gene construct in Ti plasmid

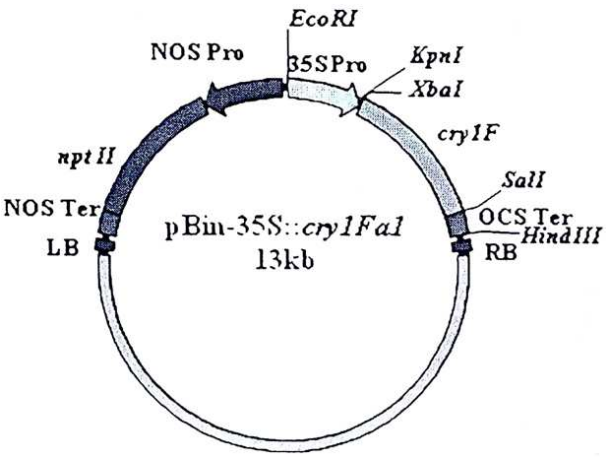

Fig. 2. Map of the *cry1Fa1* gene construct in Ti plasmid

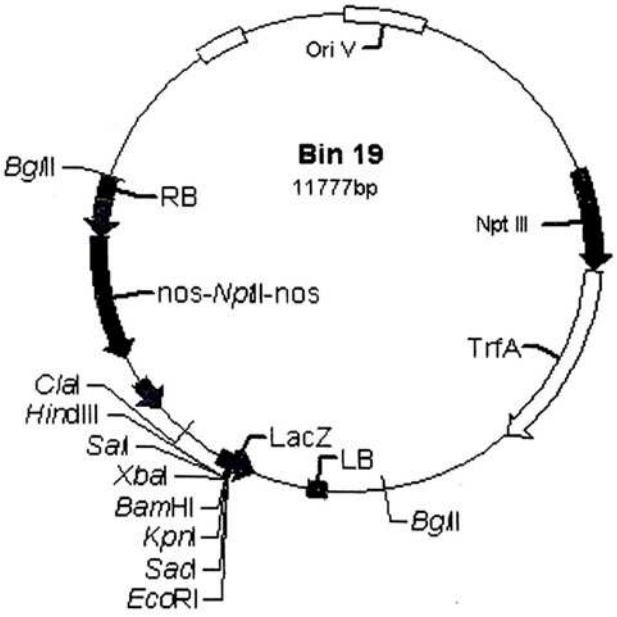

Fig. 3. Map of the *cry2Aa* gene construct in Ti plasmid

phosphotransferase (nptII) gene under control of the nopaline synthase (nos) promoter and terminator. The *Agrobacterium* strains were maintained in solid YEMA (Yeast Extract Mannitol Agar) medium of HiMedia Laboratories. Sub culturing was done every fortnight in the fresh YEMA medium. A single *Agrobacterium* colony was taken from the plate with the help of sterilized loop and inoculated into 25 ml YEM liquid medium containing 250 µl kanamycin (10 mg/ml) and was incubated on shaker for 48 hrs at 26°C temperature for Optical Density (OD_{600}) reaching 0.4-0.6 and fresh culture (OD_{600} = 0.4-0.6) was used for transformation. Acetosyringone at a concentration of 350 µM was added in suspension culture 45 minutes prior to infection and co-cultivation. The different five methods of colonization and co-cultivation were tried of which three were callus-mediated transformation methods and two were *in planta* transformation methods (Table 1). Scutellum-derived callus bits of 0.7-1 cm diameter

Table 1. Different methods of colonization

Method	Details
	Callus mediated transformation
MT_1	Callus bits colonized for 15 minutes without injury and 2 days co-cultivation
MT_2	Callus bits colonized for 20 minutes without injury and 2 days co-cultivation
MT_3	Callus bits colonized for 20 minutes without injury and 3 days co-cultivation
	***In planta* transformation**
MT_4	Seeds were just germinated and *Agrobacterium* suspension culture was injected at the apical tip of emerging plumule i.e. embryonic apical meristem using a fine syringe and directly sown in pots
MT_5	Seeds were just germinated, mild injury to plumule tip and then injection of *Agrobacterium* suspension culture at the apical tip of emerging plumule i.e. embryonic apical meristem using a fine syringe followed by 15 minutes colonization and then sown in pots

were used as target tissue in the case of callus mediated *in vitro* transformation method, while for *in planta* transformation, embryonic apical meristem of germinating seeds were used. In the callus mediated transformation method, *Agrobacterium* infected calli were co-cultivated on same callus induction medium containing MS (Murashige and Skoog medium) supplemented with 1.5 mg/L 2,4-D (2,4-Dichlorophenoxy Acetic Acid) and 0.5 mg/L BAP (6-Benzyl Amino Purine) in dark for 2 or 3 days. After co-cultivation, callus bits were washed with 600 mg/L cefotaxime and transferred to regeneration medium MS supplemented with BAP (3 mg/L) and IAA (1 mg/L) containing 300 mg/L cefotaxime which eliminated *Agrobacterium* overgrowth completely. Observation of plant survival rate was recorded. Regenerated shoots were transferred to selection medium containing 100 mg/L kanamycin (found to be optimum turning non-transformed plantlets to albino while retaining transformed ones as green due to the presence of antibiotic resistance gene *nptII* as selectable marker). Recovery through kanamycin screening of putative transgenic plants was recorded. Recovered plants were subjected to Polymerase Chain Reaction (PCR) analysis to confirm the presence of transgene using gene specific primers (which are having sequences as 1) *cry1Aabc* primer sequence: Forward primer: 5' CCC AGA AGT TGA AGT ACT TGG TGG 3', Reverse Primer: 5' CCG ATA TTG AAG GGT CTT CTG TAC 3'; 2) *cry1Fa1* primer sequence: Forward primer: 5' GGA GTG GGA GTG GCG TTT GGC CT 3', Reverse Primer: 5' CCA GTT TGT TGG AAG GCA ACT CCC 3' and 3) *cry2Aa* primer sequence: Forward primer: 5' GGG CAC TGT GTC CTC CTT CCT CCTC 3', Reverse primer: 5' GGG GAG ATG GTG AAG CCG GTG TAG 3') and transformation frequency was calculated.

In two *in planta* methods, the infected seeds were directly sown to pots and obtained seedlings were analyzed through PCR assay to confirm the transgenic status. Survival of plants and transformation frequencies for all the methods using three *cry* genes were compared.

Results and discussion

Transformation efficiency mostly depends on the efficient transformation method, infection period and co-cultivation time, which affect the survivability and regeneration of plants. In the present investigation, five methods of colonization and co-cultivation were tried with three different *cry* genes *viz*. *cry1Aabc*, *cry1Fa1* and *cry2Aa* genes. Their effect was first assessed in terms of establishment of plants.

Optimization of colonization and co-cultivation method

Among the callus mediated transformation methods,

the method (MT_1) employing calli colonization for 15 minutes without injury and 2 days co-cultivation with the *cry1Aabc* gene was found with the maximum survival percentage (22.22%) whereas the minimum survival percentage (5.27%) was found in the method (MT_3) in which callus bits colonized for 20 minutes without injury and co-cultivation for 3 days with *cry2Aa* gene.

Among the *in planta* methods, the method MT_4 recorded the highest survival percentage (89.60%) in which seeds were just germinated and *Agrobacterium* suspension culture, having *cry1Aabc* gene was injected at the apical tip of emerging plumule or embryonic apical meristem using a fine syringe and directly sown in pots (Table 2). Whereas the lowest but considerable survival (79.42%) was also observed in method MT_5 in which germinated seeds were mildly injured to plumule tip and injected with *Agrobacterium* suspension culture having *cry2Aa* gene at the apical tip of emerging plumule or embryonic apical meristem using a fine syringe followed by 15 min. colonization and then sown in pots. The survival percentages observed with *in planta* methods were found much higher than those with callus mediated transformation methods.

Similarly, several researchers also reported such a differential survival percentages for different colonization and co-cultivation periods. Puhan et al. (2012) observed 100% survival with colonization period of 35 min (OD_{600}- 0.3-0.6) and co-cultivation for 2 days. Whereas in cotton, Keshamma et al. (2008) reported 60% plant survival with colonization period of 60 min and then directly transfer to the soilrite instead of co-cultivation. On the contrary, Solanki et al. (2011) observed 73.61% survival in cotton with co-cultivation period of 3 days after *Agrobacterium* injection excluding colonization.

Among the three *cry* genes used in the present study, the *cry1Aabc* gene was found with more survival percentage, the *cry2Aa* gene with least survival percentage and *cry1Fa1* gene was found intermediate between them.

Recovery of putative transgenics and transformation frequency

Sensitivity to the transformation system of different bacterial strains may also affect the transformation frequency. The frequencies of putative transgenics for different five methods using three different *cry* genes were estimated both from the Kanamycin resistance screening as well as PCR analysis using gene specific primers.

Kanamycin screening was carried out only for 3 callus mediated transformation methods after shoot regeneration. The maximum kanamycin resistant plants (17.24%) were obtained from method (MT_3) involving colonization of callus bits for 20 minutes without injury and 3 days co-cultivation with the strain having *cry2Aa* gene (Table 3). Similarly, about 17.5% kanamycin resistant plants were obtained by Hossain et al. (2009) with 25 min colonization period (OD_{600}-0.6) and 3 days of co-cultivation. Islam et al. (2015) whereas recorded the maximum 32% kanamycin resistant plants with the same conditions of 25 min colonization and 3 days co-cultivation.

As per the PCR analysis, the highest transformation frequency among the callus mediated transformation methods (13.79%) was found with the method MT_3 consisting of 20 minutes colonization of callus bits without injury and 3 days co-cultivation with the *cry2Aa* gene (Table 3). Employing the same conditions as 20-25 min colonization followed by 3 days of co-cultivation, maximum transformation frequency (40-46%) was obtained by Sahoo et al. (2011). Whereas, Chakraborty et al. (2016) recorded

Table 2. Effect of co-cultivation of different *cry* genes on survivability and plant establishment

Method	No. of explants used for co-cultivation			No. of plants obtained			Survival (%)		
	cry1Aabc	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*
MT_1	135	145	150	30	27	26	22.22	18.62	17.33
MT_2	250	270	300	45	47	38	18.00	17.40	12.66
MT_3	450	445	550	32	29	21	7.11	6.51	5.27
MT_4	550	500	600	493	436	522	89.6	87.20	87.00
MT_5	900	930	1050	746	752	834	82.80	80.86	79.42

Table 3. Frequency of putative transgenics

Method	No. of plants obtained			No. of Kanamycin resistant plants			PCR positive plants			Transformation frequency (%)		
	cry1Aabc	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*	*cry1Aabc*	*cry1Fa1*	*cry2Aa*
MT_1	30	27	26	0(0.00%)	0(0.00%)	0(0.00%)	0	0	0	0.00	0.00	0.00
MT_2	45	47	38	0(0.00%)	1(2.12%)	1(2.63%)	0	1	1	0.00	2.12	2.63
MT_3	32	29	29	1(3.12%)	2(6.89%)	5(17.24%)	1	1	4	3.12	3.44	13.79
MT_4	493	436	522	—	—	—	2	2	3	0.40	0.46	0.57
MT_5	746	752	834	—	—	—	11	18	53	1.47	2.39	6.35

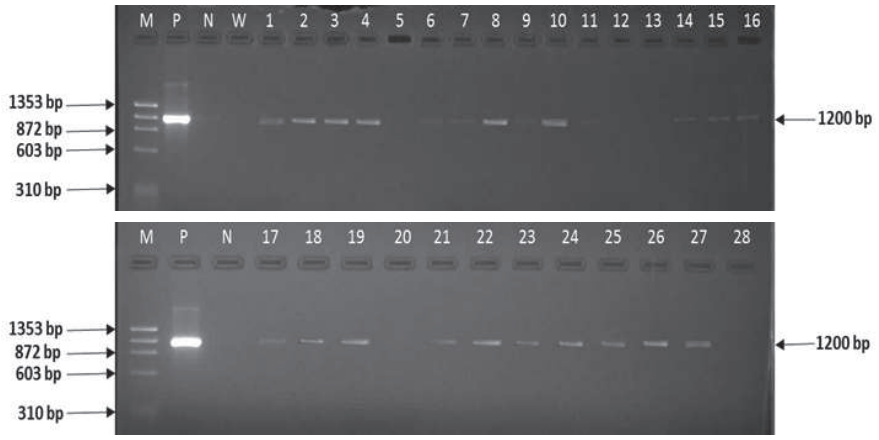

Fig. 4. PCR assay of putative T_0 plants transformed with *cry2Aa* gene. M=Marker (M); P=Plasmid (Positive control); N=Normal plant (Negative control); W=Water (Control); 1-4, 6-11, 14-16, 17-19, 21-27 – Putative transgenic plants; 5, 12, 13, 20, 28 – Non-transgenic plants

3.6% transformation frequency with 15 min of colonization and 2 days co-cultivation. The majority of researchers have reported 3 days of co-cultivation as necessary for getting maximum transformation frequency (Huang et al*.* 2001; Saharan et al*.* 2004; Ignacimuthu and Raveendar, 2011 and Kshitij Kumar et al*.* 2012).

Among the *in planta* transformation methods, the highest transformation frequency (6.35%) was recorded in MT_5 method in which germinated seeds were mildly injured to plumule tip and injected with the *Agrobacterium* suspension culture of *cry2Aa* gene at the apical tip of emerging plumule or embryonic apical meristem using a fine syringe followed by 15 min. colonization and then sown in pots. The lowest transformation frequency (0.23%) was observed in the MT_4 method in which embryonic plumule tip of germinated seeds were injected with *A.tumefaciens* having *cry1Fa1* gene without any colonization and co-cultivation. Keshamma et al*.* (2008) also reported transformation frequency of 8.3% to 19% in cotton by employing the same method with a slight variation in colonization period as 60 min instead of 15 min used in the present investigation. Similar technique giving transformation frequencies of 24% and 9% were also reported in rice by Naseri et al*.* (2012) and Allahi et al*.* (2014) by the same injection method, but without colonization. Several other reports are in close conformity with these results *viz*. Solanki et al*.* (2011) specially assuring for 3 days co-cultivation and also by Sivakumar et al*.* (2014) in cotton.

Among the overall methods with all the three genes, the method MT_3 which is a callus mediated method with the *cry2Aa* gene recorded the highest transformation frequency (13.79%) followed by the method MT_5 which is an *in planta* method using the same gene (6.35%). But as far as the survivability along with the transformation frequency is concerned, the *in planta* method MT_5 proved to be better than MT_3 for its greater survivability (79.42%) in combination with also a considerable transformation frequency (6.35%). Whereas the callus mediated transformation method MT_3, although recorded the highest transformation frequency (13.79%) overall, was found with a low survival percentage due to growth stress while hardening.

Among the three *cry* genes used for transformation, the *cry2Aa* gene was found to be the most effective showing more transformation frequency than other two in all the methods. PCR assay showing amplification of *cry2Aa* gene sequence (1.2 kb) in T_0 generation plants is presented in Fig. 4 which confirmed the integration of the transgene. Thus the *Agrobacterium_tumefaciens* with *cry2Aa* gene was found to be the most effective in transforming rice.

Authors' contribution

Conceptualization of research (SGB, SVS); Designing of the experiments (SGB, SVS); Contribution of experimental materials (SGB, SVS, NBG); Execution of field/lab experiments and data collection (GBS); Analysis of data and interpretation (GBS, SGB, SVS, MMV, NBG, VSD); Preparation of manuscript (GBS).

Declaration

The authors declare no conflict of interest.

AcknowIdgement

The authors express their grateful thanks to Plant Biotechnology Centre and Department of Agricultural Botany, College of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli for providing all the necessary facilities. Further they are also very thankful to Regional Agricultural Research Station (RARS), Karjat, Dist-Raigad (Maharashtra) and Agricultural Research Station (ARS), Shirgaon, Dist-Ratnagiri (Maharashtra) for providing valuable seed material to carry out the research work.

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