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Agrobacterium-mediated transformation of leaf base derived callus tissues of popular *indica* rice (*Oryza sativa* L. sub sp. *indica* cv. ADT 43)

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ABSTRACT

A simple and efficient protocol for the *Agrobacterium*-mediated transformation of an agronomically useful abiotic sensitive popular *indica* rice cv. ADT 43 has been developed. Initiation of calli were best achieved from the leaf bases of 4 days old rice seedlings on LS medium supplemented with 2.5 mg/L 2,4-D and 1.0 mg/L thiamine–HCl. Rice calli immersed in *Agrobacterium* suspension (strain EHA 105, OD_{600} = 0.8) were co-cultured on LS30-AsPC medium for 2 days at 25 ± 2 °C in the dark. Based on GUS expression analysis, 10 min co-cultivation time with 100 μM acetosyringone was found optimum for the delivery of *gus* gene. Calli were proved to be very sensitive to *Agrobacterium* infection and we found that the level of necrotic response can be minimized after co-cultivation with 30% LS, 10 g/L PVP, 10% coconut water and 250 mg/L timentin which improved the final transformation efficiency to 9.33%. Molecular and genetic analysis of transgenic plants reveals the integration, expression and inheritance of transgene in the progeny (T_1) of these plants. The copy number of transgenes has been found to vary from 1 to 2 in transgenic plants (T_0 and T_1).

1. Introduction

Rice is the world's most important cereal food crop belonging to Poaceae family and a primary food crop for more than a third of the world's population, mainly in tropics [1]. To meet the growing demand for rice, biotechnological intervention for its improvement using genetic engineering is becoming increasingly important. Such interventions hinge on the development of efficient and reproducible transformation protocols for agronomically superior and popular rice varieties grown in rice-consuming countries like India. Among the three subspecies (*Indica*, *Javanica* and *Japonica*), the *indica*-type rice provides the staple food for more than half of the world population and proved to be less tissue culture responsive. To satisfy the growing demand of the ever-increasing population, more sustained production of *indica*-type rice is needed. Thus, it is important to establish parameters for the transformation of popular *indica* rice varieties [2]. Introduction of foreign genes into plant cells can be achieved routinely by either direct DNA transfer or using biological vector such as *Agrobacterium*. During the last few years, introduction of foreign DNA into rice cells, with subsequent recovery of transgenic plants and progeny expressing the introduced trait(s) stably over a number of generations, has been described by many laboratories worldwide. Reports on the recovery of the first transgenic rice plants were published in 1988 by three groups, utilizing protoplasts and electroporation or PEG fusion [3–5]. Hiei et al. [6] recovered transgenic rice plants using *Agrobacterium*. Subsequently, many reports describing recovery of transgenic rice appeared in the literature. The range of transformed genotypes and transformation efficiencies has expanded and improved significantly since these original reports [6].

The advances in the *Agrobacterium*-mediated transformation indicate that we are now in a position to engineer rice using either direct DNA transfer or *Agrobacterium*-mediated transformation [6–9]. The interaction between *Agrobacterium* and plants involves a complex series of chemical signals allowing communication between the pathogen and the host. These signals include neutral and acidic sugars, phenolic compounds, opines, virulence proteins, and the transfer DNA (T-DNA) that is ultimately transferred from the bacterium to the plant cells [10]. The genes in *Agrobacterium* responsible for its attachment to wounded plant cells or tissues, the induction of virulence genes, and T-DNA processing and transfer from bacterium to plant cells have been extensively elucidated and characterized [11–13]. The factors evaluated include choosing *Agrobacterium* strains, engineering multiple copies of *virG* or constitutively active *virG* into binary vectors [6,14–16], and inducing virulence in low phosphate AB basal medium with addition of phenolic compounds and sugars under acidic conditions [17,18].

Manipulation of plant tissues and cells *in vitro* has been employed to increase the T-DNA delivery and thus enable *Agrobacterium* to transform a wide range of recalcitrant plants. These

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factors include selection of competent genotypes [19,20], desirable explants [21,22], appropriate wounding methods [23,24], and a wide range of inoculation and co-culture conditions, such as low concentration of salts in inoculation medium [25], surfactants in inoculation [26,27], cysteine in pre-culture or co-culture medium [28–30], feeder layers for co-culture [31–34], and co-culture temperature [35,36]. For optimizing the efficiency of *Agrobacterium*-mediated rice transformation, many researchers have tried different culture mediums and conditions for the inoculation and co-cultivation of *Agrobacterium*. Some researchers have reported that tissue necrosis (browning) was decreased by using a solid co-cultivation medium containing reductants such as ascorbic acid, or L-cysteine and that the frequency of transformation increased under these conditions [28,29]. The browning is believed to be caused by excess proliferation of *Agrobacterium*, which decreases the frequency of transformation. Therefore, if the proliferation of bacteria can be controlled to maximize bacterial infection and integration of T-DNA into the plant genome and to minimize tissue necrosis, it should be possible to considerably increase the frequency of transformation during the co-cultivation step. In this paper we discuss several factors that influence transformation efficiency, including the sensitivity of rice explants to *Agrobacterium* infection, inoculation methods, co-cultivation media and type of explant. We found that the addition of coconut water and PVP to the co-cultivation medium significantly improved the survival rate of explants and embryogenesis. We also evaluated the use of reduced level of nutrient media were critical to minimize the *Agrobacterium* infection and tissue necrosis.

Recently, several approaches have been adopted to improve *Agrobacterium*-mediated transformation of rice in order to generate a large number of transformants needed for the generation of T-DNA insertion and FOX libraries as well as for gene targeting studies [37,38]. It has been suggested that the development of a highly efficient and large-scale transformation system to handle more than 10^3 transformants would be a prerequisite for successful gene targeting [38]. In rice, factors influencing the efficiency of T-DNA delivery have been evaluated including different explants types, cell density of *Agrobacterium* for inoculation, inoculation period, co-culture medium, surfactants in the inoculation medium, and the induction agent, such as acetosyringone, in the inoculation and co-culture media [9]. Tyagi et al. [39] and Khanna and Daggard [40] reported that the use of super binary vectors carrying additional *vir* genes, the addition of 100 to 250 μM acetosyringone to the inoculation and co-cultivation media, and the modification of the polyamine ratio in the regeneration medium greatly improved the final transformation efficiency of 22.2% in rice and 3.9% in wheat respectively [39,40]. During the inoculation and co-cultivation period, acetosyringone appeared to act together with specific temperature requirements and an acidic environment to promote the expression of *Agrobacterium* vir genes. The presence of 200 μM acetosyringone markedly increased T-DNA delivery in rice transformation [38]. However, 400 μM acetosyringone might have a possible harmful effect on T-DNA transfer and it is not an absolute requirement for all genotypes and explants for stable transformation [26,41,42]. Hiei and Komari reported that efficiency of transformation was extremely low when acetosyringone was omitted [43]. In addition, the major constraint to the improvement of *Agrobacterium*-mediated transformation of rice is the ability to regenerate plants successfully from transformed callus. Media composition, especially the concentration of auxin, is one of the major factors influencing embryogenic response and regeneration of the explants after infection by *Agrobacterium*. Two auxins commonly used to induce somatic embryogenesis and allow regeneration from cereal tissues are 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram [44]. Both are synthetic auxins with distinct effects on the induction of cell division, proliferation, and further regeneration. They act by inducing auxin-sensitive non-dividing cells, arrested in G1, to re-enter S phase and mitosis. The timing of this process depends on the auxin type and on the concentration applied [44–46]. 2,4-D is the most commonly used exogenous growth regulator added to the culture medium for cereals, whereas picloram in the induction medium gave rise to more regenerative cultures than 2,4-D in wheat, with the optimal range between 2 mg/L and 6 mg/L [47].

Most of the agronomically superior *indica* rice varieties grown in India do not have reproducible transformation protocols. ADT 43 is a popular, medium slender white, abiotic stress sensitive, dwarf rice of IR 50x improved Ponni parentage yielding 56 Q/Ha in 110 days. In Tamil Nadu, rice is cultivated in 1.52 million hectares of which ADT 43 alone constitutes nearly 21% [48]. In the present study we report the development of reliable and reproducible protocol to optimize conditions for *Agrobacterium*-mediated transformation in one of the salt sensitive popular *indica* rice cultivar ADT 43. High efficiency of T-DNA delivery and good regeneration ability after *Agrobacterium* inoculation remain crucial for the success of *Agrobacterium*-mediated rice transformation. Based on empirical studies through manipulation of culture medium components, such as phenolic inducers and growth regulators, here the successful evidences of transgene integration and its expression in leaf base derived callus cultures of ADT 43 using the binary pCAMBIA 1301 vector system with improved transformation efficiency is reported.

2. Materials and methods

2.1. Rice transformation

Apparent stable rice transformation was carried out and achieved by following the modified rice transformation protocol of Hiei et al. [6]. Rice seeds of *indica* rice (*Oryza sativa* L. *indica* cv. ADT 43) collected from Tamil Nadu Rice Research Station, Aduthurai, Tamil Nadu, India were used as starting material. Healthy and disease free seeds were manually dehusked and washed under running tap water for 20 min. Seeds were sterilized with 70% (v/v) ethanol for 60 s followed by 0.1% mercuric chloride for 4 min and thoroughly rinsed five times in sterile distilled water. For *in vitro* germination, surface sterilized seeds were placed on 20 mL of half strength MS basal medium without any hormones in culture tubes and incubated at 25 ± 2 °C in total darkness for 4 days [49]. Aseptically the plantlets were transferred to Petri dishes containing sterile Whatman No. 1 filter paper and leaf segments (3–4 mm in length) were dissected from leaf base to tip of the seedlings.

To test the totipotency, leaf base segments (3–4 mm in length) were cultured on LS medium supplemented with different concentrations of 2,4-D (0.5–4.5 mg/L), 1.0 mg/L thiamine–HCl and 3.0 g/L gelrite [50]. The pH of the medium was adjusted to 5.8 with 0.1 N NaOH and autoclaved at 121 °C for 20 min. Before placing the leaf bases, the attached coleoptiles were removed and cultured on solid LS at 25 ± 2 °C in total darkness. After 28 days of initiation of calli from the cut ends of leaf base segments were transferred to the same fresh medium for 6 days to facilitate further increase in fresh weight. During the course of sub culture, only compact, nodular embryogenic calli were separated from non-embryogenic (mucilaginous and sticky) portions and subcultured to fresh medium were used for regeneration experiments. Cultures were regularly monitored for callus initiation and further growth. Randomly five calli were selected and growth in terms of fresh weight was monitored. *Agrobacterium tumefaciens* strain EHA 105 harboring the binary plasmid pCAMBIA 1301 plasmid (11.8 kb) contained the selectable marker gene, hygromycin phosphotransferase (*hpt*) and the *uidA* reporter gene GUS (β-glucuronidase) in the T-DNA region driven by the cauliflower mosaic virus 35S promoter