

Agrobacterium-Mediated Transformation of *Indica* Rice: A Non-Tissue Culture Approach

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Abstract - Gramineous crop plants including important cereals like rice (*Oryza sativa* L.) were classified as recalcitrant to transformation because of their both low morphogenic potential and transformation efficiency. An efficient *in planta* transformation method, via physical wounding of the embryo region was conducted in two rice landraces (*O. sativa* ssp. *Indica*) where the seeds were soaked in water for 48 hours and pierced with an *Agrobacterium*-coated needle. Two strains of *A. tumefaciens* (EHA105 and LBA4404) harbouring pCAMBIA1105.1r vector were used. A three step method in cell culture was applied for *vir* gene induction in *Agrobacterium*. Integration and presence of the transgene into the genome of putative transgenic rice plants was confirmed using polymerase chain reaction, resistance of leaf tissues to hygromycin and histochemical GUS assay. In a comparative study, EHA105 strain in the presence of 100 μ M acetosyringone on 'Hashemi' cultivar had a significantly higher transformation efficiency (~9.0%) compared to the other treatments. Ninety T₀ seedlings were selected randomly and analysed further at T₁ generation, which ~28% confirmed to be positive.

Keywords - Acetosyringone, *In Planta* Transformation, *Oryza Sativa*, Transformation Efficiency, Virulence Genes Induction.

I. INTRODUCTION

Plant transformation technology has become a useful technique for cultivar improvement as well as for studying gene function in plants. Different systems have been developed for the production of transgenic crops. *Agrobacterium*-mediated transformation technique was developed for cereals and currently, it has become a dominant technology for the production of transgenic crop plants (Nishimura *et al.*, 2006). *Agrobacterium*-mediated transformation is a favourable approach for gene transfer because it is driven by biological processes and results in a high frequency of single locus insertion events without rearrangements of the transferred DNA [12]. In other words, foreign DNA can be transferred with defined ends, higher efficiency, low copy number, and proper integration into the host genome [16]. *Agrobacterium*-mediated transformation can affect the transfer of very large fragments (150–200 kb) into plant genomes [13].

Most of the dicotyledonous crop plants that have been studied so far are successfully transformed by *Agrobacterium tumefaciens*. Monocotyledonous crop plants, on the other hand, are naturally out of the bacteria host range and were considered as recalcitrant to this technology.

In most *Agrobacterium*-mediated transformation methods of *japonica* and *indica* rice cultivars morphogenic calli, mature and/or immature embryos are routinely used as explants *in vitro*. However, most cereal crops,

particularly *indica* rice cultivars used in the present experiment, are characterized by a low morphogenic potential that significantly limits their application for genetic transformation [14]. Plant transformation using *in vitro* methods, in addition, requires sterile conditions, laboratory skills, expensive facilities, and especially, there is a chance of somaclonal variation. Therefore, the next challenge is to develop technology that minimizes or eliminates the tissue culture steps, and provides predictable transgene expression [15]. These methods are called *in planta* transformation since transgenes are generally delivered into intact plants in the form of naked DNA or by the use of *Agrobacterium*.

Techniques of non-tissue culture known as "*in planta*" transformation methods are currently applied in a wide range crop plants including alfalfa [32], wheat [36], maize [7], hybrid aspen (Takata and Eriksson, 2012), sugarcane [21] and rice [20] [27] [23].

In this experiment, we measured the overall efficiency of rice *in planta* transformation; indirect effects of different concentrations of acetosyringone on virulence gene induction; effects of 2 strains of *Agrobacterium* and 2 *indica* rice cultivars on the *in plant* transformation efficiency (TE). The study was conducted in T₀ and T₁ generations.

II. MATERIALS AND METHODS

Plant Material and Growth Condition

Two landrace cultivars, Hashemi (HA) and Hasan Saraei (HS; *O. sativa* ssp. *Indica*) with a wide genetic diversity within the genome were obtained from the 'Rice Research Institute', Rasht, Iran (<http://berenj.arei.org>). Rice plants were grown in walk-in growth rooms with day temperatures at 28 \pm 2°C, a 16-h light cycle and at night temperatures were 16 \pm 2°C with an 8-h dark cycle. Intensity of the lighting never fell below 400 IE.m⁻¹.s⁻¹.

Agrobacterium Strains and Plasmid

Plants were transformed with the binary vector pCAMBIA1105.1r (GeneBank accession number AF354045) obtained from the CAMBIA, Canberra, Australia. The plasmid has hygromycin resistance (*hpt*) and β -glucuronidase (*gus*) genes with castor bean catalase gene intron near the 5' end of the *gus* gene to improve the overall expression of the GUSPlus protein. GUSPlus gene was driven by the CaMV 35S promoter. The plasmid contains the spectinomycin/streptomycin resistance (*aadA1*) gene, which used to select transformed bacteria (Fig. 1).

The construct was mobilized into two *A. tumefaciens* strains EHA105 and LBA4404 through electroporation method by a GenePulser (Bio-Rad).

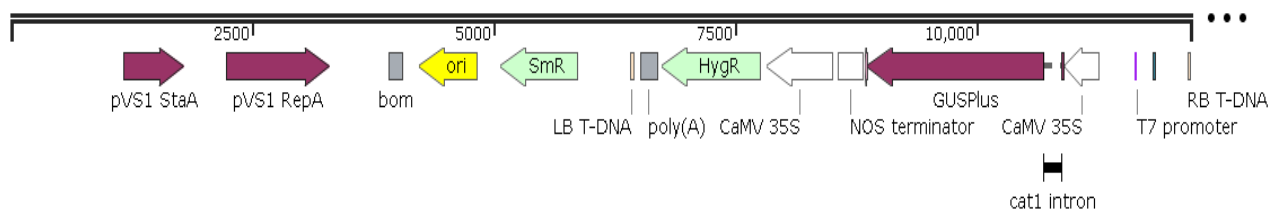


Fig.1. Schematic diagram of pCambia1105.1R plasmid (Genbank accession number: AF354045). LB, left border for T-DNA repeat; RB, right border for T-DNA repeat; HygR: hygromycin resistance gene; GUSPlus: β -glucuronidase gene (includes catalase intron; cat1); CaMV 35S: cauliflower mosaic virus 35S promoter (The map of the vector has drawn by SnapGene[®]).

Agrobacterium Culture and vir Gene Induction

A 'minimal medium' specially designed for *vir* genes induction was used in this study [11]. *A. tumefaciens* strains were grown at 28°C (shake at 225–250 rpm) overnight in 5 ml YEP medium [(per liter): 10 g of peptone, 10 g of yeast extract, 5 g of NaCl, and 15 g of Bacto Agar if the medium was to be solidified in Petri plates] containing rifampicin (100 μ g/ml) and streptomycin (100 μ g/ml). Approximately, 0.1 ml of the culture medium was diluted into 10 ml of AB-sucrose minimal medium [50 ml sterile 20X AB buffer: 60 g/L of K_2HPO_4 , 20 g/L of NaH_2PO_4 , adjust pH to 7.0 and 50 ml sterile 20X AB salts (20X AB salts: 20 g/L of NH_4Cl , 6 g/L of $MgSO_4 \cdot 7H_2O$, 3 g/L of KCl, 0.2 g of $CaCl_2$, 50 mg of $FeSO_4 \cdot 7H_2O$) with 900 ml sterile sucrose-water (0.5%) containing the above antibiotics overnight until the bacteria were in the late log phase ($\sim A_{600} = 0.8$). Bacteria were pelleted by centrifugation (5 min at $\sim 9000g$) and resuspended in 2 vol of the induction medium [1X AB salts, 2mM phosphate buffer (pH 5.6), 50 mM 2-(4-morpholin)-ethan sulfonic acid (MES), 0.5% glucose] containing certain levels of acetosyringone. Bacteria were shaken very gently (approx. 50 rpm) for 14–24 h at room temperature. Bacteria were pelleted and resuspended in 1/2 MS plant tissue culture medium to inoculate the soaked seeds. Acetosyringone (AS; 3,5-methoxy-4-hydroxyacetophenone) was used in 3 different concentrations (0, 50, and 100 μ M) in the induction medium in order to induce *vir* genes. All antibiotics and hormones in the experiment were purchased from Sigma-Aldrich (CA, USA) and chemicals were obtained from Merck (Germany) companies.

Seed Transformation

To prepare the seeds, they were surface sterilized by soaking for 1 min in 70% (v/v) ethanol followed by 20 min in 10% (v/v) sodium hypochlorite with gentle shaking. Seeds were rinsed at least 3 times with sterilized water and soaked in water at 20°C for 48 h in darkness during, which water was changed once.

The embryonic apical meristem in the embryo region of the soaked seeds were pierced (at the depth of ~ 1 mm) by an *Agrobacterium* coated needle (\varnothing 0.7 mm, Fig. 2A). Inoculated seeds were placed on top of a filter paper on wet vermiculite inside a jar and incubated in the dark at 23°C. Nine days later, the germinated seeds were immersed into sterilized distilled water containing 1000 mg/ml cefotaxime for 1 h and seedlings were transferred

to Yoshida medium (1.4 mM of NH_4NO_3 , 0.32 mM of NaH_2PO_4 , 0.51 mM of K_2SO_4 , 1.0 mM of $CaCl_2$, 1.7 mM of $MgSO_4$, 12 mM of $MnCl_2$, 0.075 of mM (NH_4) $6Mo_7O_{24}$, 19 mM of H_3BO_3 , 0.15 mM of $ZnSO_4$, 0.15 mM of $CuSO_4$, 36 mM of $FeCl_3$, and 78 mM of citric acid; [34]. The medium was made by mixing 800-times concentrated stock solutions and adjusted to pH 5.0 just before the renewal of the medium every 2 days. Seedlings after being kept in a growth chamber at 28°C were transferred to potting soil when strong rooting system was obtained.

DNA Extraction and PCR Analysis of T0 and T1 Plants

Genomic DNA was extracted from the upper leaf of putative transgenic plants because rice embryos contain various organs and primordium and only the shoot apices could produce the germ cells [19]. A pair of primers amplifying spectinomycin-resistance gene (*aadA1*) was used to analyse contamination of the transformed plants with *Agrobacterium* in T_0 generation. PCR reactions were performed using the SPEC-F: 5'-ATT TGC CGA CTA CCT TGG TG-3' and SPEC-R: 5'-GAA CAT AGC GTT GCC TTG GT-3' as left and right primers, respectively.

To screen for foreign DNA insert into the genome, a ~ 450 bp fragment of the CaMV 35S promoter was amplified using forward CaMV-F: 5'- GAA CTC GCC GTG AAG ACT GG -3' and reverse CaMV-R: 5'- GTC TTG CGA AGG ATA GTG G -3' primers. Some of PCR products, with the correct size were isolated from the gel and sequenced. Similarly, to check the integration of T-DNA in the rice genome a 480-bp fragment of *hpt* was amplified with HYGF: 5'-GAT GTT GGC GAC CTC GTA TT-3' and HYGR: 5'-GTG CTT GAC ATT GGG GAG TT-3', gene specific primers. The PCR conditions for both reactions include 95°C for 5 min followed by 34 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.

Hygromycin Screening for Transformed Plants

To assay resistance of putative transformants to Hygromycin, Flag leaf (2 cm) was excised from each plant and immersed into the selective media composed of 1.0 mg/L 6-benzylaminopurine (6-BA) and 50 mg/L hygromycin. The assays were kept at $\sim 24^\circ C$ under 16h/8h light/dark period for 7 days (Wang and Waterhouse, 1997).

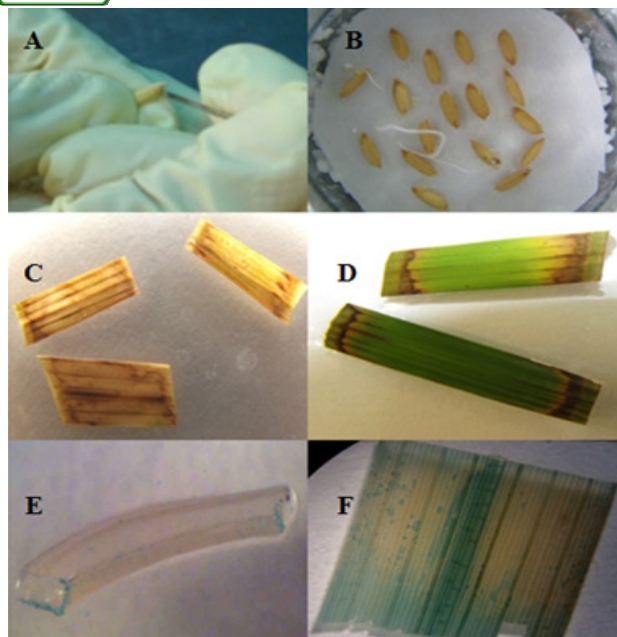


Fig.2. Procedure of Agrobacterium-mediated transformation of rice seed by piercing. (A) Inoculation of soaked seeds with a ϕ 0.7 mm needle, (B) Transfer of inoculated seeds on top of a filter paper located on surface of wet vermiculite and kept in dark condition at 23°C, (C) Development of necrosis and dark brown strips symptoms on non-transgenic control rice leaves 7 days after incubation on 50 mg/L hygromycin selection buffer, (D) Transformed plant remains green 7 days after incubation on hygromycin selection buffer, (E) Development of blue patches along cut surfaces of the stem fragment of a transformed rice genotype, (F) Histochemical localization of GUS expression of transformed plant leaves of a rice plant.

Histochemical GUS Assay

To assess expression of the GUSPlus gene in putative T_0 transformants, leaf and stem fragments were incubated at 37°C for 2–3 h by vacuum infiltration in 0.05 M phosphate buffer (pH 7.0) contain 0.5 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-gluc; Jefferson, 1987). To remove chlorophyll, leaves were incubated overnight in 70% ethanol until they become bleached.

Statistical Analysis

A completely randomized design based factorial experiment was carried out with 3 biological replications each contains 150 seeds. Data were subjected to analysis of variance using SAS (version 9.1) statistical software. Means were compared by Tukey's test ($P < 0.05$).

III. RESULTS

PCR Analysis

As a result of PCR analysis of CaMV35S promoter and Hygromycin gene, in positive samples, a ~450 bp and ~480bp PCR products obtained, respectively (Fig. 3). Five PCR samples from each amplified target were randomly chosen and sequenced for further confirmation of foreign DNA integration.

Hygromycin Resistance Assay

Hygromycin resistance assay was performed on flag leaf of T_0 plants. Control non-transgenic plants began to develop necrosis and dark brown strips symptoms after 2 days and become completely uniform necrosis 7 days later (Fig. 2C). Leaf tips of all transgenic rice plants remained almost green and healthy (with little symptom of necrosis) 7 days after incubation in selection medium (Fig. 2D).

Histochemical GUS Assay

In order to confirm the presence of the transgene in putative transformants, the histochemical GUS assay was carried out. As a result of staining blue patches developed in positive stem and leaf samples (Fig. 2E and 2F, respectively).

Statistical Analysis

The *in planta* TE was analysed statistically where plants positively succeed in PCR tests, Hygromycin and GUS assays were considered as transgenic.

Analysis of treatments of HA cultivar demonstrated that the highest TE obtained with EHA105 strain at 100 μ M concentration of AS (9%). The TE at this entry was significantly higher than the treatments where concentrations of AS were 0 or 50 μ M (0% and 4.1%, respectively; Fig. 4A). The transformation of the same cultivar (HA) with LBA4404 strain at 100 μ M AS resulted in 4% transformation, that was significantly higher than the treatments that concentrations of AS were 0 or 50 μ M (0.19% and 3.2%, respectively; $P < 0.05$; Fig. 4A).

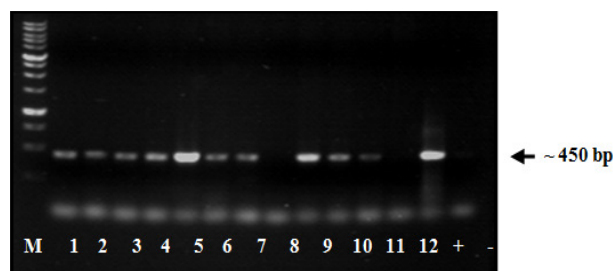


Fig.3. PCR amplification of putative transformants at T_1 generation using CaMV 35S specific primers producing an amplicon at 450-bp. M: 100 bp ladder, Lane 1-12: putative transformants. +: pCAMBIA1105.1r plasmid as positive control, -: negative control.

According to the results of experiments with HS cultivar, the highest percentage of transformation (5.1%) observed with EHA105 strain at 100 μ M AS. Transformation at this entry was significantly higher than the treatments that concentrations of AS were 0 or 50 μ M (0% and 3.8%, respectively; Fig. 4B). Transformation of the HS with LBA4404 strain at 100 μ M AS resulted in 2.3% of transformation, that was not significantly higher than the entry where concentration of AS was 50 μ M ($P < 0.05$; Fig. 4B).

Vir Gene Induction

Concerning the use of AS, increasing concentration from 0 to 100 μ M in EHA105 strain had a significantly positive effect on the TE of HA and HS cultivars.

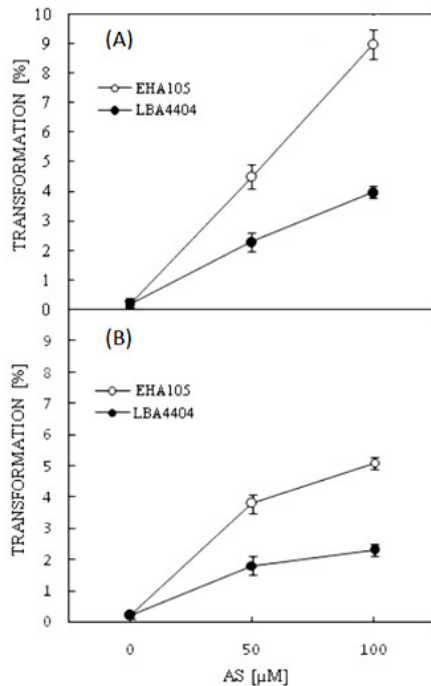


Fig.4. The interaction effects of cultivar × bacteria × AS on *in planta* transformation efficiency. (A) HA and (B) HS cultivars. Asterisks indicate significant differences between treatments ($P \leq 0.05$). Vertical bars represent standard error of 3 replications.

Analysis of T_1 Plants

Analysis of T_0 transformants may not give a solid data regarding T-DNA insertion. The tissues at T_0 are likely to be chimeras or could even be transiently transformed without authentic T-DNA integration in the genome. Therefore, at this study ninety T_0 putative transformed seedlings, selected randomly, grown to maturity and allowed to self pollination in order to set seed (T_1) in pots under greenhouse condition. Examination of transformation of T_1 generation by PCR analysis, hygromycin resistance and GUS assays proved that 28% of plants were transgenic.

IV. DISCUSSION

Two landraces of HA and HS with a wide genetic diversity within population were used in the experiment that regarding TE, HA performance was significantly higher than HS (6.5% vs 3.7%, respectively; Table 1). Very little is known about the timing and type of response plants mount to *A. tumefaciens* [2]. To elucidate the nature of plant response to infection and transformation by *A. tumefaciens*, [8] compared the differential gene expression patterns in *Ageratum conyzoides* cell cultures following infection with cell wall attachment-deficient and attachment-proficient *Agrobacterium* strains. This study suggests that an attachment-deficient mutant hyperinduced the plant defense, while the attachment-proficient strain was capable of down regulating plant defenses. This study implies that *Agrobacterium* successfully utilizes the existing host cellular machinery for the genetic

transformation purpose. Alternatively, a combinatorial approach involving subtractive hybridization and macroarrays was applied on tobacco BY-2 cells suspension culture [30]. Based on data generated with *Agrobacterium* transfer-competent (capable of transferring T-DNA and Vir proteins) and transfer-deficient (lack Ti plasmids, cannot transfer T-DNA and Vir proteins) strains, the authors observed plant cell to respond as early as 6 hours postinfection leading to inactivation of the defense-related genes. Interestingly, many defense-related genes were significantly induced during later stages (24-36 hours) of infection with transfer deficient strain but not in cell inoculated with a transfer-competent strain. This suggest that transfer of T-DNA and Vir proteins are defense response during later stages of transformation in other word, general plant defense responses are suppressed during a successful transformation.

Some of the hygromycin-resistance and PCR positive genotypes did not demonstrate any blue patches or escapes. There are numerous reports of transgene silencing in high-copy-number lines, which may vary for different transgenes. Reference [25] quantified reporter gene expression in 132 *Agrobacterium*-mediated *Arabidopsis* transformants and found that reporter gene expression, with more than two copies of a GUS transgene, was silenced by post transcription gene silencing. Escapes might be due to chimeric tissues in a single plant [33]. In almost all studies some degree of chimerism has observed, and frequency of chimerism seemed to be correlated to the method used for transformation. For example more than 70% of GUS-positive shoots obtained in alfalfa seedling meristem transformation showed partial staining of leaves [23].

Table 1: Analysis of variation for percentage of transformation of 'Hashemi' and 'Hasansarai' cultivars

Source of Variation	df.	Means of Squares
Cultivar	1	5.152 [*]
Bacterium	1	0.901 ^{ns}
Acetosyringone	2	3.231 [*]
Cultivar×Bacterium	1	1.521 ^{ns}
Cultivar×AS	2	3.395 [*]
Bacterium×AS	2	2.912 ^{ns}
Cultivar×Bacterium×AS	2	3.269 [*]
Error	24	0.94
Coefficient of variation		%11.9

* indicates significant difference at ($p \leq 0.05$)

The T-DNA can be inserted into the cell apical meristems as well as various organs and primordia of soaked seeds. Therefore, putative transformants at T_0 could be chimeras. Testing an upper leaf of the putative transgenic plants can facilitate identification of transformed plants in T_1 generation.

Developmental stage of the plants are selected for *in planta* transformation is variable but for many species it is around the time of zygote formation. This choice stems from the underlying assumption that at fertilization the egg cell accepts the donation of an entire genome from the sperm cell and it might thus be the appropriate stage to integrate transgenes [4]. In another approach, meristematic

cells located in the apical dome of nodal buds have also been considered ideal recipients for transgenes because growth and development occur in this area. Furthermore, some cell layers of the apex will eventually contribute to the germline and are thus transmitted to the sexual offspring [15]. *In planta* transformation has been carried out at different developmental stages of plant. Among them, germinating seeds would be a better choice for *in planta* Agrobacterium-mediated transformation since year round availability, ease of handling, can perform selection effectively, and the plants obtained from infected seeds will be truly transformed [21] [26].

In the bacterial cell culture protocol, which was adopted from reference [11], three steps of culture (culture in YEP medium, AB medium, and induction medium) were used with the intention of *vir* gene induction of *Agrobacterium*. *Vir* gene induction is maximal at acidic pH (~5.2 – 6.0) and this effect is mediated by VirA [5]. Rich medium e.g. LB, even when adjusted to pH 5.6 also is a poor induction medium. AS also acts as a strong inducer for *vir* gene expression between pH 5.0 and pH 5.5 [35]. Therefore it is useless to add AS to the “rich media” frequently utilized to grow *Agrobacterium* vegetatively. The temperature optimum for *vir* gene induction (~25°C) is generally lower than that optimal for vegetative growth of *Agrobacterium* (28 – 30°C [1]. In addition certain coinducing sugar such as glucose are frequently substituted by sucrose in vegetative growth medium. However, sucrose is not an effective *vir* coinducer.

To increase the delivery of bacteria to the plant tissues, physical wounding was conducted using a needle. The side of the plumage was pierced in order to avoid mechanical damage to the embryo. Epicotyl or plumule (embryonic shoot above the cotyledonary node) lies beneath the husk, give rise to shoot apex and later on germ cells [19]. Wounding eventually led to the inability of certain percentages of seeds to germinate or abnormal seedlings with average mortality rates of ~44% for HA and ~65% for HS cultivars. HA cultivar was more resistant to the damages by needle than HS, and a higher percentage of normal seedling obtained. It might be as a result of fabrication of more substances like reducing agents from this particular genotype, which minimize the effects of oxidizing agents produced by infected plant tissues [9].

Neither the GUS nor the HYG gene had any apparent effect on normal development and morphology of the transgenic rice plants as all plants maintained in the greenhouse showed normal growth and morphology with uniform appearance (data are not shown).

Most plant species show a significantly higher sensitivity to hygromycin B than to kanamycin or geneticin [22]. Hygromycin resistance and phosphinotrycin resistance have been shown to be the most effective for the transformation of rice and successfully used in different experiments [28]. Because only a limited combination of marker genes and rice species has been tested so far [18], there may be a good opportunity to improve the *in planta* transformation of this crop by additional marker gene studies.

When performing histochemical GUS assays, substrate penetration into the target tissue is often limiting, resulting in blue coloration in the vascular tissues and along cut surfaces, where the substrate can more easily penetrate and move [9] (Fig. 2E and Fig. 2F).

Comparative study of the two *A. tumefaciens* strains for their capability of *in planta* transformation verified that average TE of EHA105, a hyper virulent strain, was higher than LBA4404 strain (7% vs. 3.15%, respectively). One of the limitations of *Agrobacterium*-mediated transformation of rice is strict interaction between the genotype and the *Agrobacterium* strains [29].

The final concentration of 100 µM AS has reported to be optimum for rice tissue culture transformation [3]. Similarly, reference [6] reported that the infection and co-cultivation medium supplemented with 100 µM acetosyringone favored the *Agrobacterium* infection in switchgrass and produced 6 % of GUS-positive seedlings, which was similar to our results. It might be useful to test even higher concentration of AS (e.g. 200 µM) for the *in planta* transformation method.

An efficient system for the production of transgenic *indica* rice varieties via *in planta* transformation method was conducted which further confirmed in T₁ generation. Many researchers have developed various methods for *in planta* transformation, one of the simple protocol and widely used to produce transgenic plants. *In planta* transformation of germinating cereal seedlings has been demonstrated after needle inoculation [20] [27], and after shoot excision with no callus phase [37].

V. CONCLUSION

We have developed a slightly modified *indica* rice transformation protocol via *in planta* transformation method. This is a promising methodology for genetic transformation of recalcitrant *indica* rice species and facilitates its improvement via genetic engineering.

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