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RESEARCH NOTE

Heat and hydrolytic enzymes treatment improved the *Agrobacterium*-mediated transformation of recalcitrant indica rice (*Oryza sativa* L.)

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Abstract Reported *Agrobacterium* transformation efficiency of indica rice shoot apices varied from 0.4 to 13.8 %. In order to improve the transformation efficiency, modification of transformation protocols through heat and hydrolytic enzyme treatments on rice shoot apices were carried out. Transient expression study using reporter genes revealed that shoot apices heat treated for 3 min at 42 °C during bacterial immersion showed improved GFP (63.0 %) and GUS (42.5 %) expressions per plant as compared to standard protocol (34.0 % GFP and 36.25 % GUS). Shoot apices pre-treated with hydrolytic enzymes containing macerase, pectinase and cellulase at concentration ratio of 1:1:1 (w/v) also demonstrated high percentage of transient GFP (40.0 %) and GUS (35.0 %) expressions per plant. PCR analyses further confirmed the presence of GFP and GUS genes in the transformants. Stable expressions of GFP and GUS were also obtained in multiple shoots of regenerated shoot apices after 4 weeks of culturing in shoot proliferation media without hygromycin. In conclusion, the transformation efficiencies were improved significantly when heat (15.83 %) and hydrolytic enzymes (16.67 %) were applied as individual treatments as compared to the standard transformation method which only accounted for 5.83 %.

Keywords *Agrobacterium* · Heat · Hydrolytic enzymes · Rice · Shoot apices

Introduction

Genetic transformation of various economically important plants has led to the production of transgenic crops with improved agronomic traits (Tee and Maziah 2005; Yong et al. 2009). Besides that, transgenic crops have also been utilized as biofactories for recombinant protein production (Lai and Yusoff 2013; Lai et al. 2013). Rice, which is the staple food for more than half of the world's population, is one of the major cereal crops that is widely utilized as a monocot model in transgenic studies. Hence, improving transformation efficiency is crucial so as to hasten the production of transgenic rice with superior traits that could feed the growing population (Yookongkaew et al. 2007) and also, to be used as biofactories to mass produce economically important and pharmaceutical proteins (Wang et al. 2013). Modifications made on the conventional *Agrobacterium* transformation protocols such as heat and hydrolytic enzyme treatments on target tissues have demonstrated improved transformation efficiency. Target tissues such as immature embryos of sorghum (Gurel et al. 2009), seedlings of switchgrass (Chen et al. 2010) and

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embryogenic calli of ryegrass and rice (Patel et al. 2013), which were heat treated at temperatures from 42 to 47 °C, showed improved transformation efficiency when compared to untreated tissues. It was postulated that certain proteins induced during the heat treatment could be responsible for the *vir* induction and T-DNA transfer mechanism during *Agrobacterium*-host infection (Tsai et al. 2012). Similarly, tissues pre-treated with hydrolytic enzymes such as cellulase, macerase and pectinase was found to be advantageous in enhancing transformation efficiency. The application of these enzymes was reported to be less disruptive than mechanical wounding which could reduce the recovery and regeneration rates of transformed cells (Weber et al. 2003). By increasing cell wall porosity without compromising cell viability, the penetration of *Agrobacterium* into plant cells was made easier (Cantu et al. 2008). Several reports have shown improved transformation efficiency by treating explants from sunflower (Weber et al. 2003) and barley (Karakas 2011) with hydrolytic enzymes prior to *Agrobacterium* immersion. Additionally, the monosaccharides released from the enzymatic digestion of the plant cell wall were suggested to be *vir* inducers (Brencic and Winans 2005).

To the best of our knowledge, there were no available reports investigating the effect of heating and hydrolytic enzymes on the *Agrobacterium* transformation efficiency of indica rice shoot apices. Current literature have reported varied rice transformation efficiencies ranged from 5.6 to 8 % (Padua et al. 2001) and 0.4 to 13.8 % (Arockiasamy and Ignacimuthu 2007) by utilizing the standard transformation procedures. Recently, the transformation efficiency of shoot apices derived from recalcitrant Malaysian indica rice cultivars was reported to be 5.83 % (Fook et al. 2015). Thus, by determining and comparing the transformation efficiency of standard and modified transformation protocols, this study will serve as a good starting point to accelerate the genetic engineering process and also, to utilize shoot apex as the preferred target tissue as compared to embryogenic calli (Bairu et al. 2010). Utilizing shoot apex as explant for genetic transformation presents several advantages. Shoot apex culture is a reproducible and economically feasible method for producing plants that are free from pathogens (Badoni and Chauhan 2009) since meristems are often devoid of differentiated conducting tissues (Alam et al. 2013). In addition, Bairu et al. (2010) revealed that shoot apex did not undergo dedifferentiation stage as in callus cultures. Hence, the chances of obtaining somaclonal variant or genetic mutations were low. Generally, meristematic tissue in the shoot apex region develops and regenerates directly into shoot and whole plant which significantly reduces lengthy subculturing and regeneration steps when compared to embryogenic calli (Gamborg 2002).

Therefore, this study then sought to improve the transformation efficiency of recalcitrant Malaysian indica rice cultivar MR219 by imposing shoot apices through various heating duration during bacterial immersion as well as pre-treating the explants with hydrolytic enzymes prior to bacterial immersion.

Materials and methods

Plant materials

Dehusked mature seeds of MR219 were cleaned with household detergent by shaking vigorously for a minute followed by rinsing. Using 95 % ethanol, the seeds were surface sterilized for 2 min and then, rinsed with sterile distilled water for 3 times. Later, the seeds were immersed in 5 % NaOCl for 30 min followed by agitation at 100 rpm. Finally, the seeds were rinsed with sterile distilled water for 5 times and transferred aseptically to solidified Murashige and Skoog's (MS) medium (Murashige and Skoog 1962). After 4 days of germination, the in vitro shoots were separated aseptically from the roots and endosperm. The shoots were carefully excised leaving behind approximately 3 mm of thick basal portion which exposed the shoot apex tissue region.

Agrobacterium-mediated transformation

A colony of the EHA 101 strain harbouring pCambia 1304 (Supplementary Fig. 1) was cultured overnight (in LB broth with 50 mg/l of kanamycin) in an incubator shaker at 80 rpm, 30 °C. Next, the suspension culture was spun down at 5000 rpm for 5 min, 4 °C. After discarding the supernatant, the bacterial pellet was resuspended in modified liquid MS medium, pH 5.4 (*vir* pre-induction medium) supplemented with 10 g/l glucose and 100 µM acetosyringone. The bacterial suspension was incubated on an incubator shaker (100 rpm, 30 °C) for 3 h before the shoot apices were immersed for 30 min. The explants were air-dried on a piece of sterile filter paper and then transferred aseptically to co-cultivation medium (100 µM of acetosyringone and 4 mg/L kinetin). Co-cultivation was carried out at 25 °C for 3 days in a dark growth chamber.

Heat and hydrolytic treatments on shoot apices

Explants, immersed in bacterial suspension culture, were incubated in a 42 °C water bath at different incubation periods (1, 3, 5, 7 and 10 min). The explants were immersed for another 30 min after each heat treatment. Non-heat treated explants served as a control in this study. Pre-treatment of explants with 1 % (w/v) of filter sterilized

hydrolytic enzymes dissolved in sterile deionized water (macerase Onozuka R10, cellulase Onozuka RS, pectinase from Sigma Aldrich and a combination of three enzymes in a concentration ratio of 1:1:1) was carried out. Enzyme-treated plants that gave the highest percentage of GFP and GUS expression were determined. By using the optimal enzymatic treatment, the explants were immersed in filter-sterilized enzyme solution for different incubation periods (30, 60, 90 and 120 min) at 100 rpm, in dark. Then, the explants were washed thoroughly with sterile deionized water to remove excessive enzyme, blot dry and then immersed in bacterial suspension for an additional of 30 min. Non-enzyme treated explants served as a control.

Transient GFP and GUS expression monitoring

The GUS assay procedure was carried out according to Jefferson et al. (1987) with slight modifications. The explants were incubated in a GUS gene assay buffer at 30 °C for 48 h. GUS expression was observed using a light stereomicroscope (Zeiss, Axio) while GFP expression was observed with a fluorescent microscope (Leica MZFL III) equipped with GFP 2 filter (Excitation filter: 480/40 nm). A Leica DC 200 imaging camera connected to the fluorescent microscope was used for real-time image capturing via the Leica DC Viewer software. Results were expressed as percentage of plant expressing GFP/GUS:

% plant expressing GFP/GUS = (No. of plants expressing GFP or GUS/total number of plants) × 100 %

Hygromycin selection and PCR verification of putative transformants

After 3 days of co-cultivation, shoot apices were transferred aseptically to MS selection medium supplemented with 4 mg/L kinetin, 20 mg/L hygromycin and 200 mg/L of cefotaxime. The explants were subcultured twice for 30 days. These transformed lines were subjected to GUS and GFP expression monitoring and PCR to detect the presence of the transgenes. The plant genomic DNA from the putative transformants was isolated according to Staub et al. (1996). A PCR reaction mixture containing 50 ng DNA (purity: 1.7–2.0), 1 × of DreamTaq™ PCR buffer, 200 µM of dNTP mixture, 0.6 µM of forward and reverse primers, 1.25 units of DreamTaq™ polymerase and sterile ultrapure water (Millipore, Merck) to a final volume of 25 µl was briefly vortex. The specific forward and reverse primers for GFP (GFPF 5′-ATGGTAGATCTGACTAGT AAAGGAG-3′, GFPR 5′-TCAAGAAGGACCATGTGG TC-3′) and GUS (GUSF 5′-TAGAGATAACCTTCACCC GG-3′, GUSR 5′-CGCGAAAACTGTGGAATTGA-3′) were used in this study. The PCR protocol for GFP and GUS genes were as follow: initial denaturation for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and elongation at 72 °C for 2 min, and a final elongation at 72 °C for 7 min. All PCR products were analyzed through 0.8 % gel electrophoresis.

Statistical analysis

All experiments were repeated 3 times. The data obtained from each set of experiment were analyzed by one-way ANOVA in a completely randomized design. Mean values were compared by Duncan's multiple range test at 5 % ($p = 0.05$) significance level, using SPSS software version 20.0 (SPSS Inc. USA).

Results and discussion

The application of heat treatment during the *Agrobacterium* infection of rice shoot apices has significantly improved the percentage transient expression of GFP and GUS after 3 days of co-cultivation. Based on Fig. 1a, a 3-min heat treatment resulted in 63.0 % and 42.5 % of transient GFP and GUS expression followed by 1 min treatment which produced explants with high percentage of expression for GFP (50.0 %) and GUS (37.5 %) genes. Explants without heat treatment (control) showed 34 % GFP and 36.25 % GUS expressions which were lower than those treated in 1 or 3 min of heat. When the heat treatment duration prolonged, the percentage of GFP expression dropped from 53.0, 23.0 to 18.0 % whilst GUS expression decreased from 25.0, 22.5 to 17.5 % at 5, 7 and 10 min respectively. Referring to Fig. 4a–l, the transient expression of GFP and GUS of shoot apices heat treated for 1 and 3 min was evident but as heating time prolongs, the expression both reporter genes gradually faded at 5, 7 and 10 min.

Similar to Gurel et al. (2009), heat pre-treatment of sorghum immature embryos for 3 min at 43 °C showed increased GFP expression from 20.7 to 39.0 % and then a steady reduction was observed at 5, 10 and 30 min. Heat treatment for 3 min at 42 °C during bacterial immersion of embryogenic calli of rice and ryegrass showed 4 fold increase in GFP expression as compared to control whereas 1 min of heating had no impact on GFP expression and prolonged 5 min of heating showed detrimental effect on callus growth and regeneration (Patel et al. 2013). Improved transformation efficiency between various heat treatment durations (30–45 min at 43 °C) was observed for rice immature embryos but prolonged incubation period and increased temperature were shown to reduce the viability and callus induction capability of the explants (Hiei and Komari 2006). On the other hand, heat-treated maize immature embryos were found to be genotype dependent as

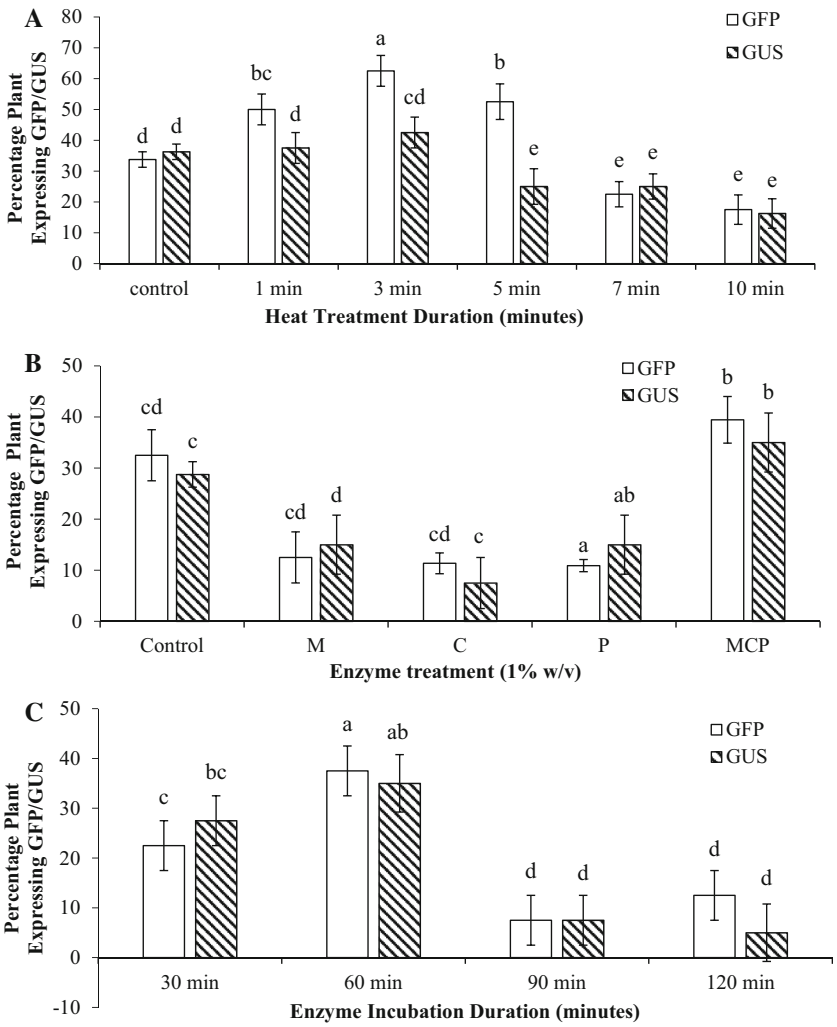

Fig. 1 Effects of **a** Heat treatment duration (minutes), **b** various enzyme treatments (*M* macerase; *C* cellulase; *P* pectinase) and **c** enzyme incubation duration on the percentage of plants expressing GFP and GUS after 3 days of co-cultivation. Data represents mean ± standard deviation (n = 15). Different letters indicate the values are significantly different ($p \leq 0.05$)

certain cultivars were sensitive to heat thereby affecting the transformation efficiency (Ishida et al. 2007).

The role of heat in the *Agrobacterium* T-DNA transfer mechanism was correlated to various identified proteins that interacted with the bacterial vir proteins. The expression of the *Agrobacterium* ATP-dependent *Lon* (*La*) protease was induced during elevated temperature and the mutant *Agrobacterium* that lacked this gene has proposed several possible roles of *Lon* in T-DNA strand processing, transfer and integration into the plant genome (Su et al. 2006). A total of 4 genes encoding for the small heat shock proteins (*sHsps*) were also identified in *Agrobacterium* during T-DNA transfer. The alpha-crystallin-type small heat-shock protein (α-*Hsp*) *HspL* was found to be highly expressed at elevated temperatures (Balsiger et al. 2004). The increased *HspL* transcripts, induced by both acetosyringone and heat, were found to correlate with the accumulation of *HspL-virB* protein complexes, escalated *virB/D4*-mediated DNA transfer activity via the type IV secretion system (T4SS), and increased chaperone activity of *HspL* which prevented the irreversible protein aggregation of *virB* during heat stress (Tsai et al. 2009, 2010, 2012). Overall, applying heat treatment at optimal conditions could have contributed to the improvement of *Agrobacterium* T-DNA transfer through the upregulated expressions of either *HspL* or *Lon* proteins.

Cell wall degrading enzymes or hydrolytic enzymes such as cellulases, macerases and pectinases were regularly utilized in protoplast isolation protocols. The application of these enzymes in pre-treating shoot apices were reported to be less destructive as compared to mechanical wounding which could greatly reduce the recovery and regeneration of transformed cells (Weber et al. 2003). According to Fig. 1B, the screening of cellulase (11.5 % GFP and 7.5 % GUS), macerase (12.5 % GFP and 15.0 % GUS) and pectinase (11.0 and 15.0 %) when used individually at concentrations of 1 % w/v showed very weak expression of reporter genes after an hour of incubation. When these enzymes were added in a concentration ratio of 1:1:1, the GFP expression increased by 4 fold (40.0 %) and almost 2

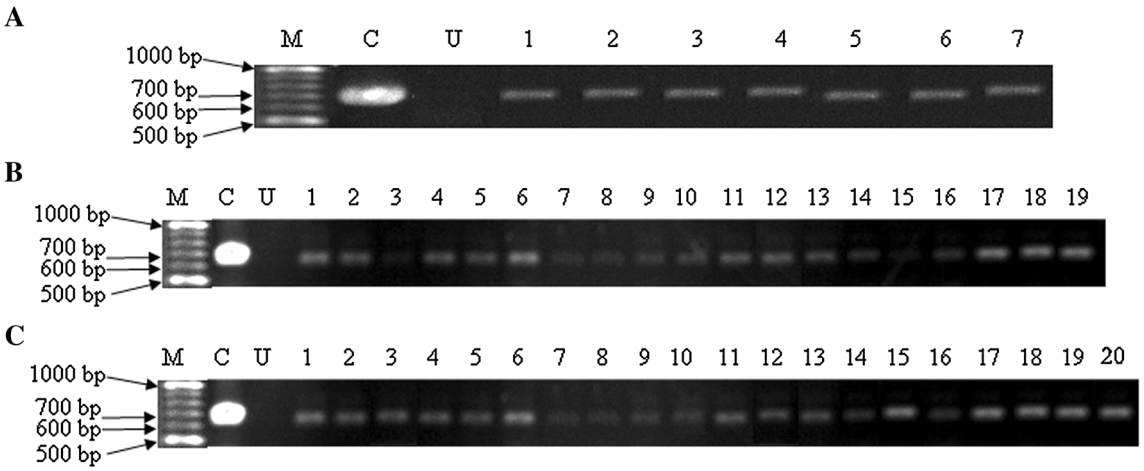

Fig. 2 The PCR product of GFP (~678 bp) using *mGFP* specific primers. The PCR product was electrophoresed on 0.8 % (w/v) agarose gel. Lane M: 100 bp PLUS DNA ladder (Fermentas, USA); C: plasmid DNA and U: genomic DNA from untransformed plants. The numbered lanes represents the putative transformed lines generated from different transformation protocols—**a** normal (control), **b** 3 min heat treatment, and **c** hydrolytic enzyme pre-treatment

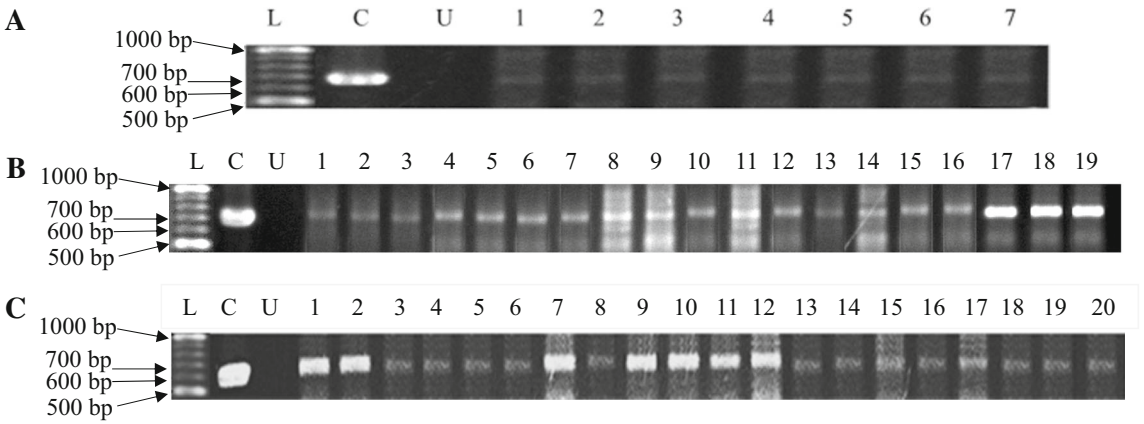

Fig. 3 The PCR product of GUS (~640 bp) using GUSA specific primers. The PCR product was electrophoresed on 0.8 % (w/v) agarose gel. Lane M: 100 bp PLUS DNA ladder (Fermentas, USA); C: plasmid DNA and U: genomic DNA from untransformed plants. The numbered lanes represents the putative transformed lines generated from different transformation protocols—**a** normal (control), **b** 3 min heat treatment, and **c** hydrolytic enzyme pre-treatment

fold (35.0 %) for GUS expression. A typical plant cell wall composed of cellulose, hemicellulose, lignin and pectic polysaccharides (Keegstra 2010) which could be easily digested by cell wall degrading enzymes. Adding a consortium of enzymes could have increased cell wall porosity and therefore, allowing the *Agrobacterium* to penetrate easily into the plant cells (Cantu et al. 2008). As shown in Fig. 5a–j, the transient expressions of both GFP and GUS genes were improved when a consortium of enzymes was used to treat the shoot apices as compared to individual enzyme and non-enzyme treated explants.

Following that, the enzyme incubation period as shown in Fig. 1c indicated that 60 min remained as the suitable period for producing relatively high transient expression levels of GFP (37.5 %) and GUS (35.0 %) when compared to the control (GFP 32.5 % and GUS 28.5 %) in which no enzyme pre-treatment was carried out. A 30 min of incubation showed a relatively high GFP (22.5 %) and GUS (27.5 %) expressions per plant whereas longer incubation period of 90 min (7.5 and 7.5 %) and 120 min (12.5 % and 5.0 %) reduced the percentage of GFP and GUS expression respectively (Fig. 1c). Prolonged enzyme treatments has led to necrotic and dead explants during the co-cultivation stage.

Several studies have reported improved transformation efficiency by pre-treating target tissues using hydrolytic enzymes prior to bacterial immersion. For instance, barley mature embryos pre-treated with a combination of 3.0 % macerase and 0.5 % cellulase for 30 min generated 29.6 % of GUS positive mature embryos as compared to the non-

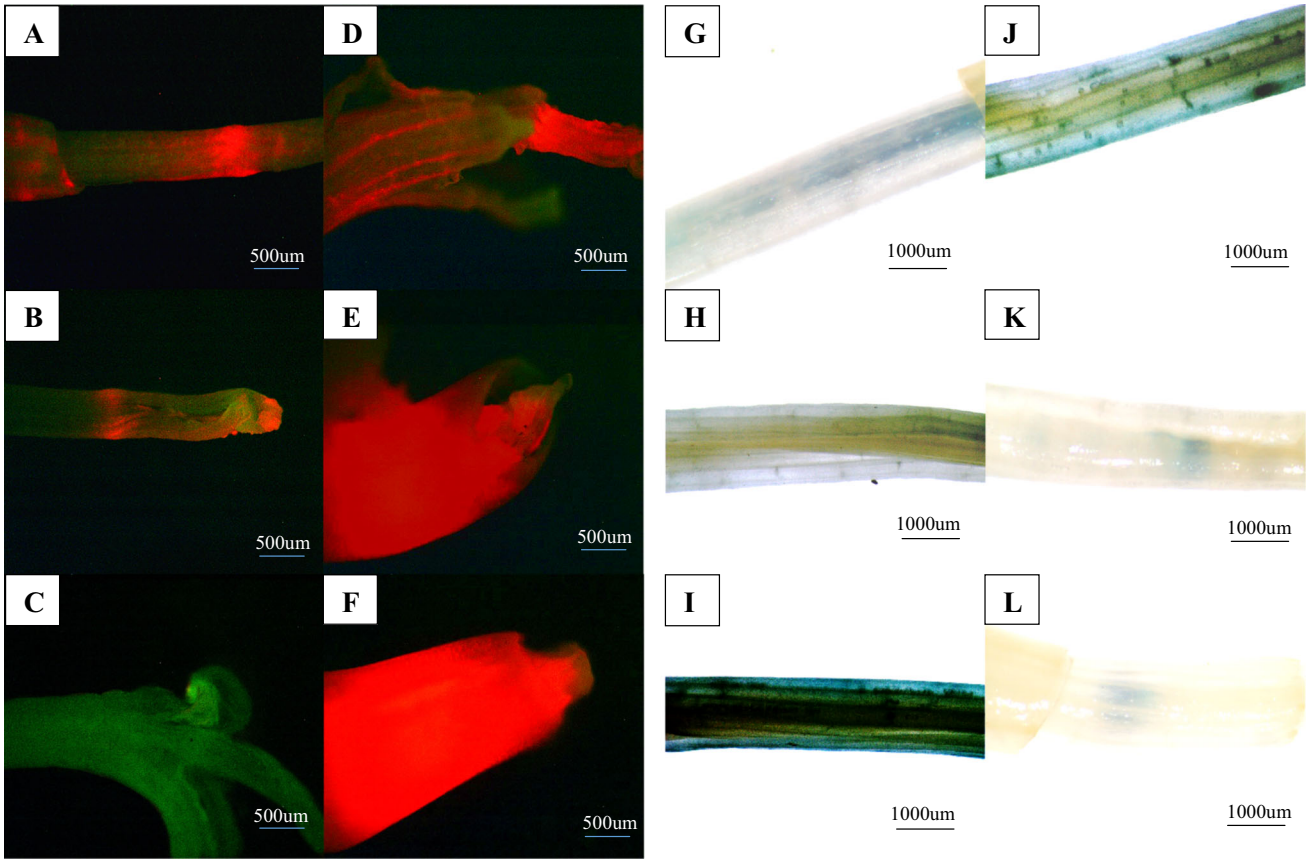

Fig. 4 Effect of different heat treatment duration—(**a**, **g**) control (without heat treatment), (**b**, **h**) 1 min, (**c**, **i**) 3 min, (**d**, **j**) 5 min, (**e**, **k**) 7 min, and (**f**, **l**) 10 min on the transient GFP (*left panel*) and GUS (*right panel*) expression in shoots of regenerated MR219 rice shoot apices. GFP images were taken at ×50 magnification under fluorescence microscope (Leica MZFL III) equipped with GFP 2 filter (Excitation filter: 480/40 nm) while GUS images were taken at ×32 magnification under light stereomicroscope (Zeiss, Axio)

treated explants (20.3 %) (Karakaş et al. 2011). A concentration of 0.1 % cellulase coupled with a 15 min of vacuum infiltration were found to produce 7 % of sunflower meristem-derived shoots showing uniform GUS expression whereas macerozyme and pectinase produced chimaeric shoots and inhibited regeneration capacity (Weber et al. 2003). The flax hypocotyl tissues immersed for 10 min in *Agrobacterium* suspension with the addition of 0.02 % cellulase also produced explants with the highest GUS expression as compared to macerase and pectinase treatments (Vrbová et al. 2008). On the contrary, the application of enzymes drastically reduced the TE of *Agrobacterium rhizogenes* on tobacco (Kumar et al. 2006). Cotyledon derived sunflower shoots that were pre-treated with enzymes suffered from severe bacterial contamination and no GUS expression was observed (Sujatha et al., 2012). From these studies, it was suggested the types of plant, cell wall component of the target tissues, types of hydrolytic enzymes, concentration of enzymes, and duration of incubation might be the important parameters that could either improve or decrease transformation efficiency.

The monosaccharides such as glucose, galactose, arabinose, galacturonic acid, glucuronic acid, mannose, fucose, cellobiose and xylose derived from cell wall degrading enzyme activity were some of the characterized monomers of plant cell wall polysaccharides that were discovered as *vir* inducers in *Agrobacterium* T-DNA transfer mechanism (Brencic and Winans 2005). A composition study of the rice cell wall, specifically hemicellulose, revealed the presence of more than 95 % of arabinose and xylose (Wu et al. 2013) which might be the potent *vir* inducers. Similar to the mode of action of phenolic compounds, the presence of monosaccharides induced the expression of the sugar binding protein *ChvE* of *Agrobacterium* in addition to playing a role in the bacterial chemotaxis response towards the *vir* signaling source. The activated *ChvE* then interacted with *virA* which triggered an expression cascade of other *vir* genes (Liu et al. 2001). The concerted effect of three macerating enzymes used in this study could have produced monosaccharides, digested from rice cell walls, which served as *vir* inducers. Such hypothesis should be investigated to further clarify

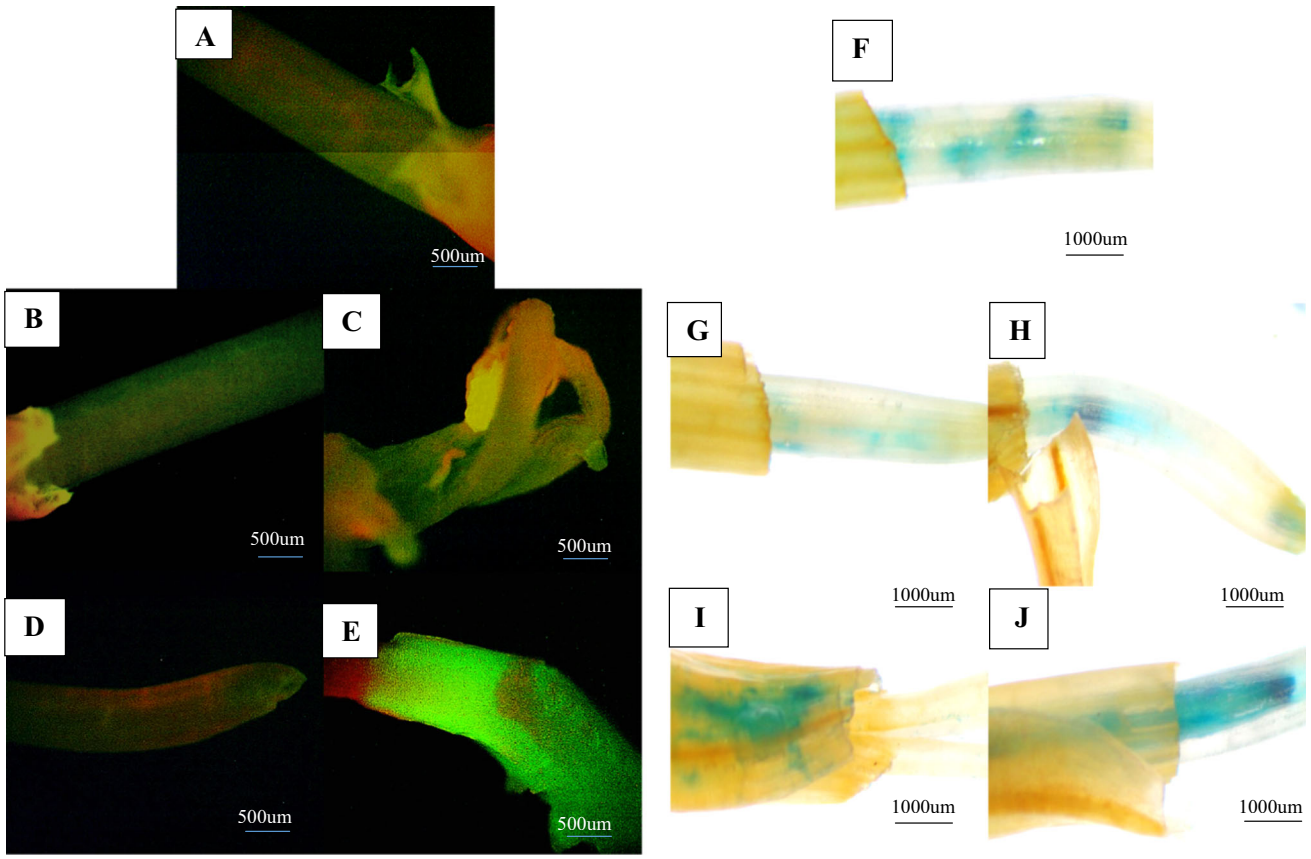

Fig. 5 Effect of different enzyme treatment—(**a**, **f**) control (without enzyme treatment), (**b**, **g**) macerase, (**c**, **h**) cellulase, (**d**, **i**) pectinase and (**e**, **j**) a combination of macerase, cellulase and pectinase (1:1:1 w/v) on the transient GFP (*left panel*) and GUS (*right panel*) expression in shoots of regenerated MR219 rice shoot apices. GFP images were taken at ×50 magnification under fluorescence microscope (Leica MZFL III) equipped with GFP 2 filter (Excitation filter: 480/40 nm) while GUS images were taken at ×32 magnification under light stereomicroscope (Zeiss, Axio)

the role, presence and quantity of these sugars from enzymatically treated rice shoot apices.

The presence of GFP and GUS reporter genes in the putative transformants were verified with gene specific primers. From Fig. 2a–c and Fig. 3a–c, PCR product for GFP (~678 bp) and GUS (~640 bp) was successfully amplified. The pCambia 1304 plasmid (with amplifications of both reporter genes) and genomic DNA from untransformed rice plant (with no amplification of PCR bands) both served as the positive and negative control respectively. In addition, stable expression of GFP and GUS were evident in multiple shoots derived from regenerated transformants after 4 weeks of culturing in MS media supplemented with 4 mg/l of kinetin. The transformation efficiencies for heat treated explants (15.83 %) and hydrolytic enzyme pre-treated explants (16.67 %) were improved as compared to the reported standard transformation protocol (5.83 %) (Fook et al. 2015). Previous transformation studies of shoot apices using electroporation (Padua et al. 2001) and *Agrobacterium*-mediated (Arockiasamy and Ignacimuthu 2007) methods have reported varied transformation efficiencies that ranged from 5.6 to 8 % and 0.4 to 13.8 % respectively. Based on this study, it is evident that the addition of heat treatment and enzyme treatments have improved the transformation efficiency of shoot apices in recalcitrant indica rice cultivar. With the enhanced transformation efficiency, the generation of transgenic rice with superior traits and industrial importance could be produced more efficiently and rapidly.

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