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ORIGINAL PAPER

Enhancing *Agrobacterium tumefaciens*-mediated transformation efficiency of perennial ryegrass and rice using heat and high maltose treatments during bacterial infection

Minesh Patel · Ralph E. Dewey · Rongda Qu

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Abstract Perennial ryegrass is one of the most widely cultivated grasses in temperate regions. However, it is recalcitrant for in vitro manipulation. In this study, various parameters affecting *Agrobacterium tumefaciens*-mediated infection were tested to optimize transformation efficiency in perennial ryegrass. The effects of heat shock and maltose concentration during *Agrobacterium* infection were evaluated along with variations in callus induction medium, bacterial infection media and callus age. Our results suggest that *Agrobacterium* infection at 42 °C for 3 min and co-cultivation of *Agrobacterium*-infected callus on a high maltose medium (6 %) significantly enhances the transformation efficiency in perennial ryegrass. The most optimal conditions proved to be use of four-month-old embryogenic callus induced on a modified N6 medium, infected with *Agrobacterium* grown on a modified Murashige and Skoog (MSM) medium, and a 42 °C heat shock treatment followed by the co-cultivation of the *Agrobacterium* and the callus on medium containing 6 % maltose (instead of 3 %). Using this optimized protocol, we were able to increase the transformation efficiencies for regenerated plants from approximately 1 % to over 20 %. Significant improvement in rice stable transformation efficiency was also observed when the optimized conditions were applied to this important cereal, indicating the method described here may apply to other monocots as well.

Keywords Heat shock · Maltose · Rice · Perennial ryegrass · Transformation

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Introduction

Perennial ryegrass is one of the most important diploid, cool-season grasses grown in the U. S., Europe and other areas of the world (Watschke and Schmidt 1992). Perennial ryegrasses have better disease resistance and wear tolerance than annual ryegrass; however, they show low tolerance to prolonged drought (McCarty 2000). In some regions, perennial ryegrass is used primarily as forage. This versatile species is also commonly used as a turfgrass for winter overseeding on golf courses and athletic fields (Qu et al. 2008). Although genetic gains have been made through conventional plant breeding, further enhancement of agronomic performance would be expected if plant transformation and molecular biological approaches were also integrated into perennial ryegrass improvement programs (Qu et al. 2008).

Over the past three decades, plant transformation technologies have been applied successfully toward introducing agronomically useful traits in numerous crop species (Vain 2007). *Agrobacterium tumefaciens* -mediated transformation is the preferred technique for genetic modification in most plant species largely because of its simplicity of use and its propensity to introduce transgenes in low copy number (Komari and Kubo 1999). For any given plant species, the efficiency of transformation can often be enhanced by manipulating the various parameters of the process, such as the culture media and its components, explant type, selection agents, and temperature treatments (Opabode 2006). Transformation frequency is species and genotype dependent and thus expansion of the *Agrobacterium* host range and enhancement of its transformation efficiencies are among the top targets in the studies of transformation of higher plants as it helps to reduce the costs and required resources (van Wordragen and Dons 1992; Cheng et al. 2004).

Table 1 Composition of medium used in the study

	Induction medium		Infection medium	Co-culture medium			
Medium components	MSM	N6M	MSMG	MSM3	MSM6	N6M3	N6M6
MS basal salt	+	−	+	+	+	−	−
N6 basal salt	−	+	−	−	−	+	+
Thiamine-HCl (mgL^{-1})	1	1	1	1	1	1	1
Myo-inositol (mgL^{-1})	250	250	250	250	250	250	250
Casein hydrolysate (gL^{-1})	1	1	1	1	1	1	1
Proline (mgL^{-1})	690	690	690	690	690	690	690
Maltose (gL^{-1})	30	30	–	30	60	30	60
Phytagel (gL^{-1})	3	3	–	3	3	3	3
2,4-D (mgL^{-1})	5	5	1	5	5	5	5
BA (mgL^{-1})	0.1	0.1	–	0.1	0.1	0.1	0.1
Glucose (gL^{-1})	–	–	30	–	–	–	–
Acetosyringone (μM)	–	–	200	200	200	200	200
pH	5.9	5.9	5.4	5.9	5.9	5.9	5.9

Perennial ryegrass is generally regarded as a species recalcitrant to genetic transformation. A range of gene transfer protocols have been described for the production of transgenic perennial ryegrass plants, but efficiencies are generally low and success has therefore been limited (Spangenberg et al. 1995; Altpeter 2006; Cao et al. 2006; Wu et al. 2007). In several plant species, improvements in *Agrobacterium*-mediated transformation efficiencies have been observed by applying a heat pretreatment to the explant prior to infection (Khanna et al. 2004; Hiei et al. 2006; Gurel et al. 2009). In many particle bombardment-mediated transformation protocols, exposure of the target cells to an osmotic pretreatment has been shown to increase the frequency of transformation (Schmidt et al. 2008; Jagga-Chugh et al. 2012). To our knowledge; however, the effect of osmotic treatments in an *Agrobacterium*-based protocol has not been reported. Here, we describe the development of an optimized perennial ryegrass protocol. Key to the enhancement of *Agrobacterium*-mediated transformation efficiency in perennial ryegrass was the combination of both a heat and an elevated maltose treatment during the infection and co-cultivation, respectively. Application of these parameters to rice substantially improved its transformation efficiency as well, indicating their potential use in other monocot species.

Materials and methods

Plant material and tissue culture

Perennial ryegrass (*L. perenne* L.) cultivar 'Citation fore' and rice (*Oryza sativa* L.) cultivar 'Taipei 309′ seeds were used for callus induction. Seeds of perennial ryegrass were soaked in 50 % H_2SO_4 for 30 min to remove the husks. The de-husked seeds were surface sterilized with 3 % (w/v) sodium hypochloride for 15 min, then rinsed 3–4 times with sterile distilled water. The surface sterilized seeds were sliced longitudinally cutting the embryos into two halves before culturing on a modified Murashige and Skoog (MSM) medium (Murashige and Skoog 1962) or a modified N6 medium (N6M) (Chu et al. 1975). The compositions of the various media used in this study are shown in Table 1. Powder of basal MS and N6 media were purchased from Caisson Laboratories (North Logan, UT, USA). All other chemicals used in the culture media were purchased from Sigma Aldrich unless otherwise specified. For rice, seeds were de-husked using sand paper then surface sterilized and cultured on MSM or N6M media. Every 3 weeks, friable type II embryogenic callus (Armstrong and Green 1985) were selected from both species and were sub-cultured onto fresh callus induction medium.

Agrobacterium transformation

For perennial ryegrass transformation, *Agrobacterium* strain EHA105 harboring binary vector pTJN33-GFP or pJLU13 (Lu et al. 2008) was used. Plasmid pTJN33-GFP contains a *bar* selectable marker gene driven by the CaMV 35S promoter and GFP driven by the rice *rubi3* promoter (Sivamani and Qu 2006) whereas pJLU13 contains a hygromycin B (hyg B) resistance gene, *hph*, driven by the CaMV 35S promoter with the GFP driven by the *rubi3* promoter. The *rubi3* promoter was isolated from a rice polyubiquitin gene and provides strong constitutive expression in monocots (Sivamani and Qu 2006). For rice transformation, only plasmid pJLU13 was used. *Agrobacterium* was grown overnight in 5 ml YEP (10 g Yeast Extract, 10 g Bacto

peptone, 5 g NaCl per liter) medium in a shaking incubator at 28 °C. Forty-five ml of either fresh YEP or MSMG medium and 200 μM acetosyringone (final concentration) were added to the overnight cultures and growth was continued until the OD_{600} reached ~0.6 (about 3 h). The *Agrobacterium* suspensions were used directly for infection without further resuspension. For *Agrobacterium* infection, 2–6 month-old callus from perennial ryegrass or 2–3 month-old rice callus were collected in 50 ml centrifuge tubes (100–120 callus per tube). Callus were incubated with 30 ml of *Agrobacterium* suspension at room temperature for 10–15 min, and then blotted onto three layers of sterile filter paper to remove the excess *Agrobacterium* suspension. For experiments using a heat treatment, callus incubated with *Agrobacterium* suspensions were immediately placed in a 42 °C water bath for 3 min then transferred to room temperature for another 10 min. For vacuum treatment, callus incubated with *Agrobacterium* suspensions were immediately placed in a vacuum chamber for 3 min then transferred to room temperature for 10 min. When the heat and vacuum treatments were combined, callus were incubated with *Agrobacterium* suspensions and immediately placed in a 42 °C water bath for 3 min, then subjected to a vacuum for 3 min before transferring them to room temperature for 7 min. Agro-infected callus were subsequently cultured on one of the four co-cultivation media (MSM3, MSM6, N6M3 and N6M6; Table 1) and incubated at 25 °C in a dark growth chamber. After 3 days of co-cultivation, infected callus were transferred to a resting medium [either MSM or N6M plus 200 mgL^{-1} timentin (GlaxoSmithKline, Research Triangle Park, NC, USA)] and incubated at 25 °C in the dark for 5 days. Callus were subsequently sub-cultured 3 times at 2 week intervals onto fresh selection medium (same as the resting medium plus either 5 mgL^{-1} bialaphos or 100 mgL^{-1} hyg B depending on the selectable marker gene). Callus grown on selection medium were transferred to regeneration medium [MS salts, MS vitamins and glycine, 0.5 mgL^{-1} 6-benzyladenine (BA), 30 gL^{-1} maltose, 3 gL^{-1} phytagel, 150 mgL^{-1} timentin, and either 10 mgL^{-1} phosphinothricin (PPT) or 25 mgL^{-1} hyg B, pH 5.9]. After 3–4 weeks, well-developed plantlets were transferred to rooting medium (same as regeneration medium without BA but containing either 15 mgL^{-1} PPT or 100 mgL^{-1} hyg B). Plants with developed shoots and roots were transferred to soil. Bialaphos and PPT were purchased from Gold Biotechnology (St. Louis, MO, USA) and hyg B was purchased from A.G. Scientific (San Diego, CA, USA).

Analysis of induced callus and regenerated plants

A fluorescence microscope (SMZ1000, Nikon, Melville, NY, USA) mounted with a long pass GFP filter (Excitation: 480/40) was used to identify the callus expressing GFP. Callus were typically examined ~7 weeks after infection (after about 6 weeks under selection, just prior to transfer onto regeneration medium). Regenerated plants were also checked for GFP expression using fluorescence microscopy. Genomic DNA was isolated from transgenic and non-transgenic plants using the CTAB method (Murray and Thompson 1980), and the integration of *bar* and GFP were further confirmed by Southern blot analysis. Fifteen μg of genomic DNA of each putative transgenic line was digested with *Hind*III, separated by electrophoresis in a 1.0 % agarose gel then transferred to Hybond-N nylon membranes (Amersham). Blots were hybridized with [α-^{32}P]-dCTP-labeled *bar* gene probe. Hybridizations were conducted as described by Sambrook et al. (1989).

Rice transformation

Rice callus was generated by culturing rice seeds on N6M media for 2–3 months. Rice callus were transformed according to the conditions shown to be favorable for perennial ryegrass transformation. *Agrobacterium* harboring the pJLU13 binary vector was grown in MSMG (Table 1) infection media. During Agro-infection, callus incubated with *Agrobacterium* suspensions were placed in a 42 °C water bath for 3 min and co-cultivated in the elevated maltose medium (N6M6). Control callus did not receive the heat treatment and were co-cultivated in medium with standard maltose content (N6M3). After co-cultivation, all transformed callus were cultured on N6M resting medium with 200 mgL^{-1} timentin for 5 days followed by N6M selection media containing 100 mgL^{-1} hyg B. GFP fluorescence was examined in both callus (7 weeks after Agro-infection) and green plants.

Experimental design and statistical analysis

A randomized block design was used for all experiments with each treatment replicated three times. For transient expression assays, GFP expressing callus were recorded 10 days after Agro-infection. For stable transformation, the numbers of callus expressing GFP were counted just before transfer to regeneration media (about 7 weeks after Agro-infection). The number of regenerated green plants expressing GFP was also recorded for each treatment. Treatment means, the standard error of the means and LSDs (Least Significant Differences) were calculated using SAS (Statistical Analysis System, Cary, NC, USA) software.

Results

For perennial ryegrass transformation, callus were induced on callus induction medium from mature seeds. Every

3 weeks, the embryogenic Type-II callus were visually selected from induced callus and transferred to fresh medium. A series of experiments were performed to develop an efficient transformation system for perennial ryegrass by evaluating the effects of several factors, including variations in infection medium, infection conditions, callus induction medium, callus age, and the use of elevated maltose during co-cultivation.

MS-based infection medium performs better than YEP medium

To study the effects of infection media, *Agrobacterium* carrying binary vector pTJN33-GFP were grown in YEP or MSMG media for callus infection. MSMG infection medium is the same as MSM, without BA and phytagel, but with reduced 2,4-D (1 mgL^{-1}), and glucose (30 gL^{-1}) instead of maltose, and the pH lowered to 5.4 (Table 1). In each of the four different experiments, 60–80 pieces of callus from each treatment were analyzed for GFP expression. As shown in Fig. 1, a nearly two fold increase in GFP-expressing callus was observed using MSMG as the infection medium compared with YEP medium, both containing 200 μM acetosyringone. No GFP expression was observed in the absence of acetosyringone (data not shown).

Heat shock improves *Agrobacterium* infection

In several studies, the application of a heat treatment has been shown to improve *Agrobacterium* transformation efficiencies (Khanna et al. 2004; Hiei et al. 2006; Gurel et al. 2009). Vacuum treatment during the initial exposure of the explant to the *Agrobacterium* is also used in many protocols. To establish whether either of these treatments could influence transformation frequency in perennial ryegrass, each was tested, both alone and in combination. As shown in Fig. 2, a four-fold increase in the number of callus expressing GFP was observed when they were exposed to 42 °C for 3 min during infection, followed by 10 min at room temperature. Although a modest increase in efficiency was observed with a 3 min vacuum application alone, combining the two treatments showed no improvement in callus transformation efficiency (Fig. 2). When the transformed callus were placed on regeneration media, an overall plant transformation efficiency of about 3 % was observed for the materials receiving a heat shock during Agro-infection, compared to ~1 % without this treatment (data not shown). In addition to applying the 42 °C heat shock for 3 min, heat treatments of 1 and 5 min were also tested. A 1 min heat shock had no impact on transformation efficiency, whereas the 5 min heat shock was very detrimental, with no GFP expression being detectable in callus under this regime (data not shown).

N6-based medium and 6 % maltose enhances transformation frequencies

Next, we tested whether the transformation efficiency could be influenced either by the media used for callus induction, or the maltose content of the co-cultivation

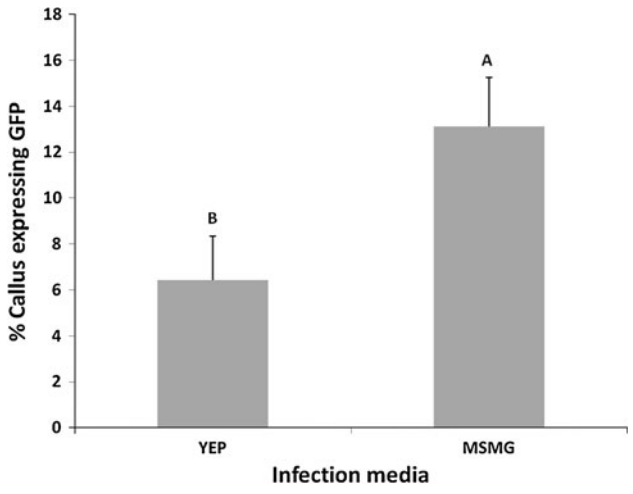

Fig. 1 Transient expression assays in perennial ryegrass using YEP or MSMG as the infection media. GFP foci were counted for 60–80 callus per treatment for each of four independent experiments. The letters *a* and *b* indicate that the mean values for the two treatments are significantly different ($p < 0.05$)

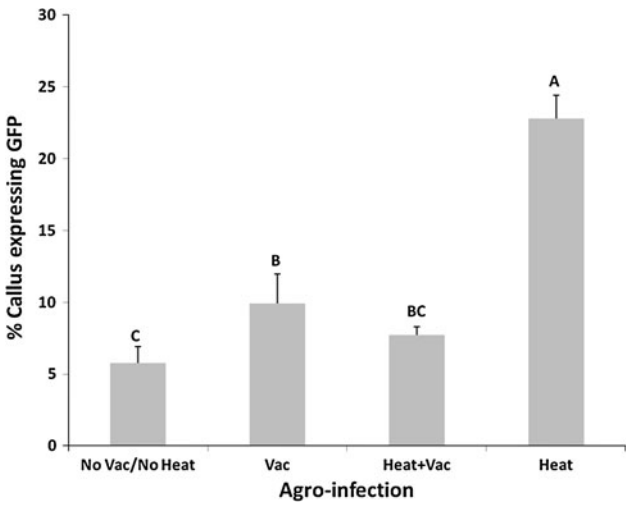

Fig. 2 Effect of heat shock and vacuum treatments on callus transformation. During Agro-infection, callus were exposed to a 3 min vacuum treatment, a 3 min treatment at 42 °C, or both, and compared to controls (No Vac/No Heat). Data shown represent the analysis of 60–100 callus per treatment for each of three independent experiments. Mean values with different letters are significantly different from each other ($p < 0.05$)

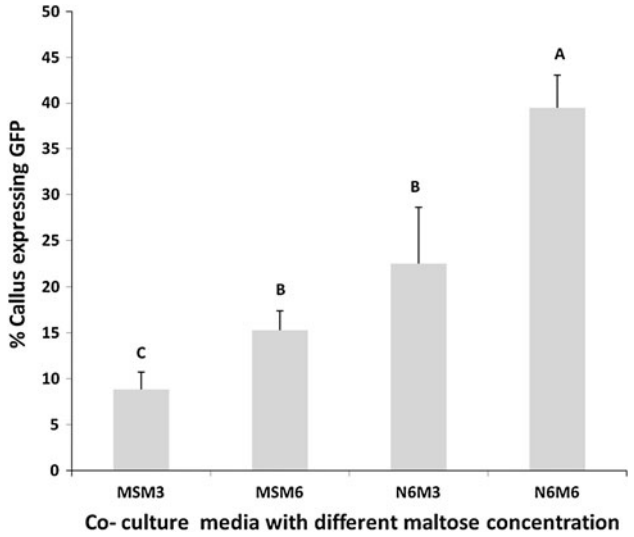

Fig. 3 Transient expression assays measuring the effect of callus induction media and the maltose concentration in the co-culture media. Perennial ryegrass callus induced and subcultured on MSM (columns 1 and 2) or N6M media (columns 3 and 4) were divided into two groups and co-cultured on same media containing 3 or 6 % maltose after *Agrobacterium* infection. Each experiment included 60–100 callus per treatment and the means represent the averages of three independent replications. GFP foci were counted 10 days post-infection. Mean values with different letters are significantly different from each other ($p < 0.05$)

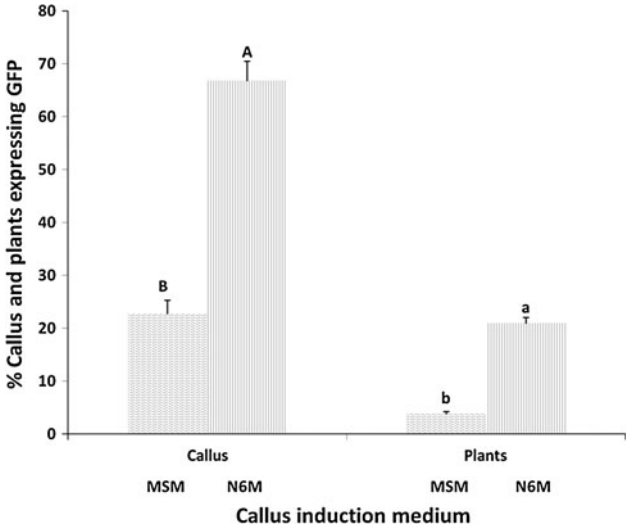

Fig. 4 Effect of callus induction medium on transformation efficiency in perennial ryegrass. Callus were initiated using two different induction media (MSM and N6M) and infected with *Agrobacterium* grown on MSMG infection medium. After *Agrobacterium* inoculation, callus initiated on MSM medium were co-cultured on MSM6 and callus initiated on N6M medium were co-cultured on N6M6 co-culture media. For each treatment, 75–125 callus were used and the means shown represent the averages of three replications. Statistical analysis for callus and plant transformation was performed separately. Mean values with different letters are significantly different from each other ($p < 0.05$)

media. In this study, callus were induced on either a modified MS (MSM) or modified N6 (N6M) media. N6M medium is similar to MSM medium except that N6 basal (Chu et al. 1975) salts are used instead of MS salts. After Agro-infection (using MSMG infection medium) callus were divided into two groups to culture on co-cultivation media with either normal (30 gL^{-1}) or elevated (60 gL^{-1}) concentrations of maltose in MS- or N6-based media.

A transient expression study was performed to evaluate the effect of the elevated maltose in the co-cultivation media, in combination with using MSM versus N6M media for callus induction. The number of GFP-expressing callus nearly doubled when they were co-cultivated on 6 % maltose media (Fig. 3). The results also demonstrated that callus cultured on N6M media were more competent for transformation than those cultured on MSM media (Fig. 3). This conclusion was further reinforced by a stable expression study performed to compare the two callus induction media (MSM and N6M) in combination with Agro-infection at 42 °C (heat shock) and co-cultivation in media with higher maltose (6 %). As shown in Fig. 4, three times the number of transformed GFP-expressing callus was obtained when using callus originating from N6M media as opposed to MSM media. In addition, the number of callus that ultimately regenerated into GFP-expressing plants was six times higher.

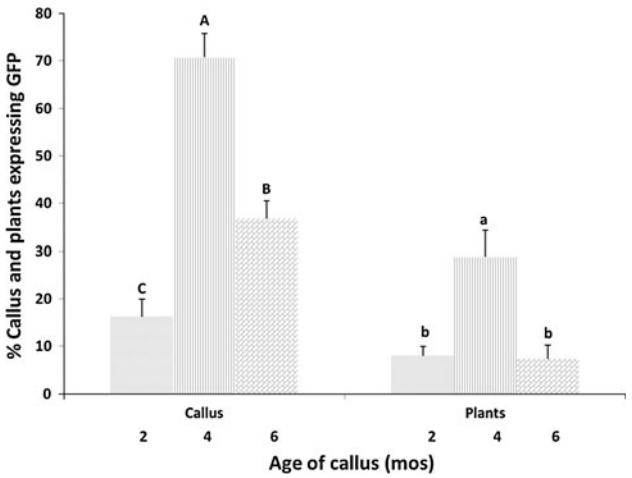

Fig. 5 Effect of callus age on transformation efficiency in perennial ryegrass. Callus initiated on N6M induction medium were grown for two, four and 6 months (2, 4, 6 mos) prior to Agro-infection, heat treatment, and co-culture on N6M6 media. These experiments were conducted using the pJLU13 vector that uses hygromycin selection. Callus were counted for GFP expression 7 week post-infection. For each experiment, 50–100 callus were used per age group and the data shown represents the averages of three independent replications. Statistical analysis for callus and plant transformation was performed separately. Mean values with different letters are significantly different from each other ($p < 0.05$)

Effect of callus age on transformation efficiency

To test the effect of callus age, perennial ryegrass callus were induced on N6M media and sub-cultured every 3 weeks for 2, 4 and 6 months prior to infection with *Agrobacterium* carrying the binary vector pJLU13 (grown in MSMG infection media). During infection the callus were exposed to the 42 °C heat treatment and subsequently plated onto N6M6 (6 % maltose) co-culture media (followed by N6M-based resting and selection media). The results shown in Fig. 5 illustrate that the transformation efficiency was greatly influenced by callus age, with 4-month-old callus yielding the highest transformation frequencies both in callus as well as in regenerated plants. When 2-month-old callus tissue was used for transformation, nearly 50 % of the GFP expressing callus regenerated into plants, whereas less than 20 % of the GFP positive, 6-month old callus regenerated into plants (Fig. 5). These results demonstrate that younger callus have a higher regeneration capability compared to older callus and there is a narrow window in which callus are most competent for transformation without losing their regeneration ability.

Optimized transformation protocol for perennial ryegrass

The results shown in Fig. 5 suggested that high efficiency perennial ryegrass transformation could be achieved by combining the following factors: (1) using 4-month-old select callus induced on N6M media; (2) growing the *Agrobacterium* suspension cultures in MSMG infection medium; (3) applying a heat shock (42 °C, 3 min) during infection; and (4) co-cultivating on N6M6 medium after infection. To partition the effects of the heat shock from the maltose treatment, a final set of experiments was designed. Four-month-old Type II embryogenic callus induced on N6M medium were incubated with *Agrobacterium* (harboring the pJLU13 vector) grown on MSMG infection medium. For the control treatment, no heat was applied and the callus were co-cultured on N6M3 media (normal maltose). In the second treatment, the 3 min 42 °C heat shock was applied during the Agro-infection, followed by co-cultivation on N6M3 media. The third treatment combined the 3 min 42 °C heat shock with co-cultivation on the high maltose containing N6M6 media. As shown in Fig. 6, transformation efficiencies were the highest when the heat and high maltose treatments were combined. For the combined treatment, callus transformation efficiency averaged 76 % and plant transformation efficiency reached 21 %, which is three- and five-fold higher, respectively, than the control callus that did not receive these treatments.

A pictorial representation of perennial ryegrass transformation and GFP expression in callus and plants is shown in Fig. 7. Southern blot analysis suggests that the majority of transformed plants have a low copy number gene insertion.

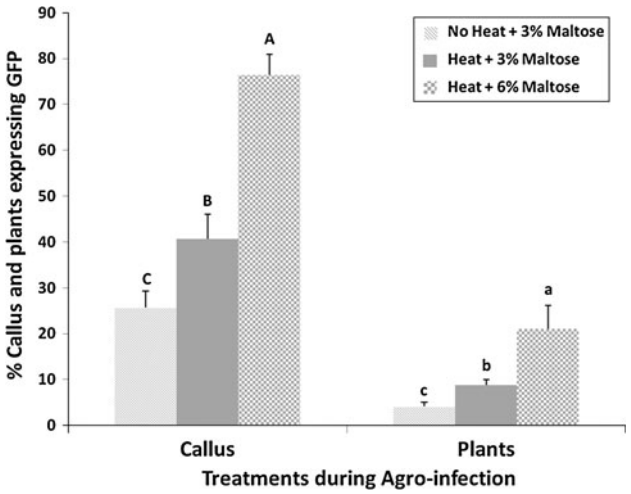

Fig. 6 Combining heat shock and maltose treatments during Agro-infection enhances transformation efficiency in perennial ryegrass. Four-month-old callus initiated on N6M induction medium were infected with *Agrobacterium* grown on MSMG infection medium. Control callus received no heat shock and were co-cultured on medium with a standard maltose concentration (3 %) during co-cultivation. Other callus received either the heat shock treatment alone (42 °C for 3 min), or both the heat shock and high maltose (6 %) treatments. For each experiment, 100–200 callus were used per treatment, and the means represent the averages of three replications. Statistical analysis for callus and plant transformation was performed separately. Mean values with different letters are significantly different from each other ($p < 0.05$)

Heat shock and elevated maltose treatments also enhance rice transformation efficiency

To investigate whether the treatments found to enhance perennial ryegrass transformation may also apply to other monocot species, we used the optimized perennial ryegrass transformation protocol to transform rice (cv. Taipei 309). In a preliminary set of experiments, rice transformation was performed using callus induced from MSM medium, with YEP as the infection medium, no heat shock during infection and no elevated maltose in the co-culture medium. GFP expression was observed in 40–45 % of the infected callus under these conditions (data not shown). In addition, we also observed that the response of 2–3 month-old callus was better for transformation and plant regeneration than 4–6 month-old callus (data not shown). To evaluate the effects of heat shock during Agro-infection and co-cultivation with elevated maltose on rice transformation, 2–3 month-old callus induced using N6M medium were incubated with *Agrobacterium* (containing the pJLU13 binary vector) grown in MSMG infection medium. In the absence of a heat shock and co-cultivation in the

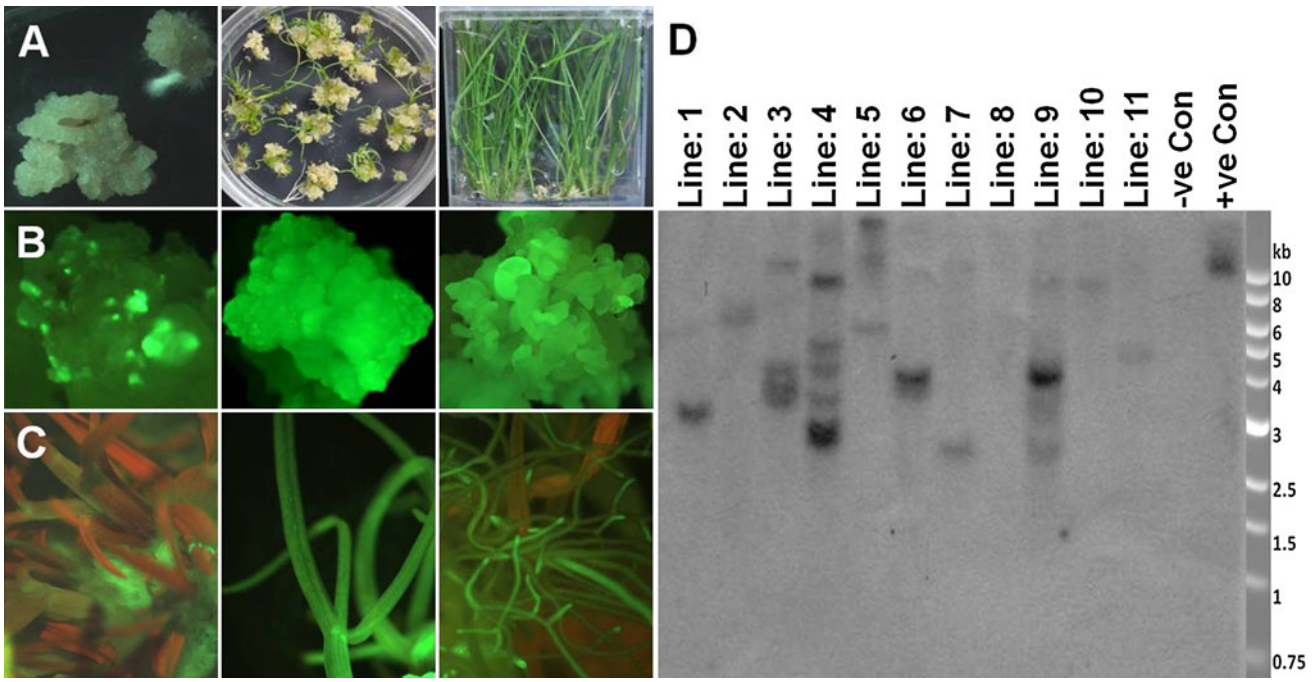

Fig. 7 Perennial ryegrass tissue culture and transformation. Row *a*, perennial ryegrass plant regeneration from callus derived from mature seeds. Row *b*, GFP expression in transgenic callus. Row *c*, GFP expression in transgenic shoots and roots of perennial ryegrass. **d** Southern blot hybridization of transgenic perennial ryegrass using *bar* gene coding sequence as a hybridization probe. (Lines 1–11 are putative transgenic lines, −ve Con is a non transgenic plant control and +ve Con is a *bar*-containing plasmid for positive control)

standard N6M3 media, 69 % of the callus scored positive for GFP expression (Fig. 8). Thus it would appear that using N6M callus induction media and MSMG media for *Agrobacterium* growth may enhance rice transformation efficiency, though this conclusion must be considered tentative since this experiment was not conducted simultaneous with the preliminary experiment that utilized MSM and YEP media, respectively. Addition of the 42 °C heat shock during infection plus 6 % maltose during co-cultivation on N6M6 medium further increased the percentage of GFP positive callus from 69 % to 93 % (Fig. 8). When GFP expressing callus were transferred to regeneration medium, 30–35 % of those callus regenerated into fully developed plants (data not shown). These results further support the conclusion that combining a heat shock during Agro-infection and an elevated maltose treatment during co-cultivation enhances *Agrobacterium*-mediated transformation of monocot species. Representative stages of the rice transformation procedure are shown in Fig. 9.

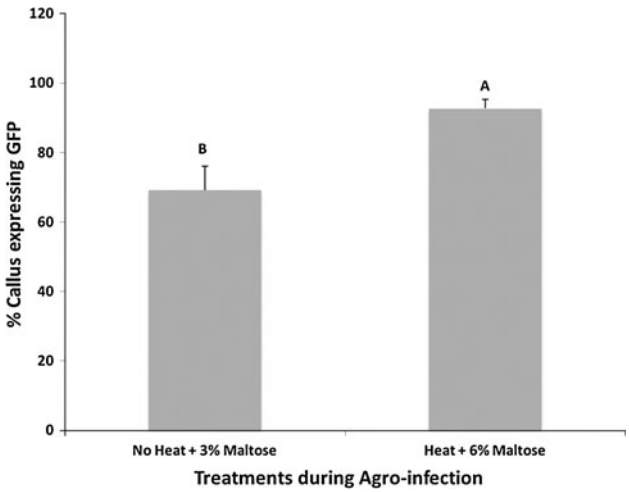

Fig. 8 Rice transformation efficiency is increased by including a heat shock and high maltose treatment. Rice callus initiated on N6M induction medium were infected with *Agrobacterium* grown on MSMG infection medium. GFP expression was assessed 7 weeks post infection in callus exposed to a 42 °C heat shock and elevated maltose co-cultivation treatment (*right*) or control callus lacking these treatments. For each experiment, 100–140 callus were used per treatment and the means represent the averages of three replications. Mean values with different letters are significantly different from each other ($p < 0.05$)

Discussion

Perennial ryegrass is an attractive target for crop improvement by genetic engineering due to its importance as a turf species suitable for parks, cemeteries, roadsides, golf courses, etc., as well as its widespread usage as a forage crop. Generating transgenic perennial ryegrass plants has been widely considered to be challenging, as ryegrass has proven to be recalcitrant to molecular

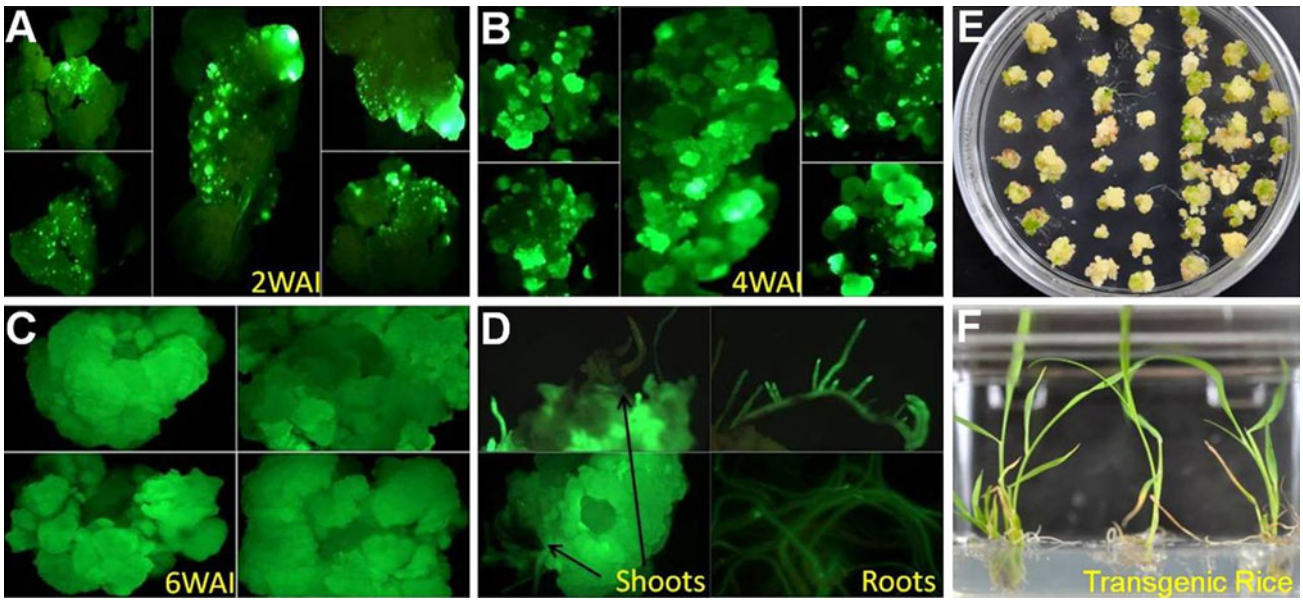

Fig. 9 Schematic presentation of rice transformation. GFP expression in callus 2 weeks after infection (**a**), 4 weeks after infection (**b**), 6 weeks after infection (**c**), and in regenerating shoots and roots (**d**). **e** transformed rice callus on regeneration medium; **f** transgenic rice plants in rooting medium

transformation. Although there are a few reports on the production of transgenic perennial ryegrass plants (Cao et al. 2006; Wu et al. 2005; Wu et al. 2007), a robust protocol that consistently yields transgenic individuals at a high frequency is needed. Here, we analyzed various factors affecting perennial ryegrass transformation and demonstrated step-by-step improvement in the transformation procedure to achieve a high overall plant transformation frequency of greater than 20 %. In addition to the culture medium and callus age, we notably found that a 42 °C heat shock treatment during Agro-infection and an increase in the maltose concentration in the co-cultivation medium, greatly enhanced efficiencies of transformed perennial ryegrass callus, as well as regenerated transgenic plants.

The most critical factors in making a transformation protocol more efficient are the induction and maintenance of high-quality callus and the transfer of T-DNA from *Agrobacterium* to the plant cell at a high frequency. In an attempt to maximize the quality of our perennial ryegrass callus, we tested the effects of various culture media, as well as the age of the callus. Our studies revealed that callus induced on N6 basal media could be transformed at a significantly higher rate than callus induced on MS basal media. Similar beneficial effects on transformation rate using N6 basal salts for *Agrobacterium*-mediated transformation have been reported for maize (Armstrong and Green 1985; Zhao et al. 2001), perennial ryegrass and tall fescue (Cao et al. 2006). N6 salts contain a lower concentration of inorganic nitrogen, but a higher ratio of nitrate to ammonium than do MS salts (Armstrong and Green 1985; Elkonin and Pakhomova 2000). Nevertheless, the specific reason for the positive effect of N6-based media over MS-based media remains unclear.

During the process of this study, we noticed that perennial ryegrass callus initially grows more slowly and takes a longer time to produce good quality embryogenic callus from mature seeds than other monocots species such as rice. Thus it became important to establish the optimal callus age for high efficiency callus transformation and subsequent green plant regeneration. In our investigations, we observed that callus age dramatically affected the transformation efficiency of the callus. "Middle-aged" (4 month-old) callus gave the highest transformation efficiency and better plant regeneration frequencies compared to the younger (2 month-old) or older (6 month-old) callus. Similar results were reported for pea by de Kathen and Jacobsen (1995) and for the wetland monocot, *Typha latifolia* by Nandakumar et al. (2004). In contrast, in rice we observed that the responses of younger callus were better for transformation and plant regeneration than middle-aged or older callus (data not shown).

The efficient delivery of T-DNA to the plant cell is a pivotal step in genetic transformation. In this study we showed that the infection media, the Agro-infection conditions and the co-culture media played significant roles in infection and the efficient delivery of T-DNA. We observed significantly higher T-DNA delivery into plant cells (as evidenced by GFP expression) when the *Agrobacterium* was grown in MSMG infection media instead of the common bacterial media YEP (both being supplemented with

acetosyringone). Han et al. (2005) also reported improved transformation efficiency in *Artemisia annua* L. when using MS as an infection medium rather than the bacterial medium LB. The positive influence of the MSMG infection media may be due to its greater acidity (pH 5.4 vs. pH 5.9) as also reported by Kumar et al. (2011), and/or the presence of glucose in the infection media, as both acidic pH and glucose can induce *vir* gene expression (Loubens et al. 1997), which in turn could enhance transfer of the T-DNA into host cells. In addition, *Agrobacterium* grows more slowly in MS media than bacterial media such as MGL, LB, AB (Li et al. 1999). This may help prevent the over-growth of the *Agrobacterium* suspensions. It is widely believed that transformation normally takes place within actively dividing cells and MS medium is better for plant cell growth compared to YEP medium. MS-based media with acetosyringone has been successfully used for Agro-infection in several reports of rice transformation (Mohanty et al. 1999; Lucca et al. 2001; Saharan et al. 2004; Meiru et al. 2011).

In many *Agrobacterium*-based transformation protocols, it is a common practice to infect the explant at 28 °C or lower. Furthermore a brief heat shock, cold shock or centrifugation just prior to infection with *Agrobacterium* has been reported in different plant species (Khanna et al. 2004; Cao et al. 2006; Hiei et al. 2006; Gurel et al. 2009; Zhang et al. 2012). Gurel et al. (2009) applied a heat treatment of 43 °C for 3 min to immature sorghum embryos before the infection, in contrast to our study where the heat shock was applied during infection after the ryegrass callus and *Agrobacterium* had been combined. To our knowledge, this is the first report to show the positive effects of using heat shock during Agro-infection in *Agrobacterium*-mediated transformation. Our results suggest that a heat shock treatment significantly increases the gene transformation efficiency in perennial ryegrass. By using a proteomics approach, Lai et al. (2006) showed that acetosyringone (AS) treatment induced HspL, an alpha-crystalline-type small heat-shock protein (α-Hsp) that exists in most organisms, including animals, plants, and bacteria. Further study of HspL regulation indicated that AS-induced HspL protein accumulation is regulated in a VirB-dependent manner and that the HspL protein is required for optimal VirB protein accumulation, which in turn may be important for efficient VirB/D4-mediated DNA transfer and virulence (Tsai et al. 2009). It is thus tempting to speculate that in our experiments the heat shock causes bacterial and/or plant cells to release heat shock proteins such as HspL in a manner similar to that induced by acetosyringone as a means to optimizing VirB protein accumulation. In addition, according to Hansen (2000), *Agrobacterium* induces apoptosis in maize cells and heat shock proteins can protect cells from cell cycle arrest and apoptosis (Iordanskjy et al. 2004); thus the production of heat shock proteins in some plant cells may function to prevent programmed cell death (Khanna et al. 2004). Despite these intriguing possibilities, the mechanism by which heat shock enhances transformation efficiency in perennial ryegrass remains unknown.

In this study, we used elevated maltose during co-cultivation with *Agrobacterium* and observed significantly higher transformation efficiencies. To our knowledge, this is also the first report to show the positive effects of use of elevated maltose during co-cultivation in *Agrobacterium*-mediated transformation. Previously, Hiei et al. (1994) used an additional 10 gL^{-1} glucose in the co-culture medium for rice transformation; however, they did not compare their results with conditions lacking the additional 10 gL^{-1} glucose in the co-culture media. Maltose is a common plant metabolite and its role in transformation enhancement remains to be elucidated. One possible explanation, however, would be that higher maltose might have acted as an osmoticum. The positive effects of osmotic treatments have been well reported by researchers using biolistic-based transformation approaches (Vain et al. 1993; Sailaja et al. 2008), but similar treatments have not been well described in *Agrobacterium*-mediated studies. Wu et al. (2005) used a mannitol pre-treatment of callus prior to *Agrobacterium*-infection and reported improvement in transformation efficiency in perennial ryegrass, indicating that an osmotic treatment may have a positive effect.

In conclusion, after dissecting various aspects of the perennial ryegrass transformation process, we combined the critical factors and observed an exceptional transformation frequency. Prior to our methodical optimization of the process, we observed a callus transformation efficiency of less than 10 % and a regenerated plant transformation efficiency of only about 1 %. By combining the optimal treatment conditions, the transformation efficiencies in callus and plants were dramatically improved to greater than 70 and 20 %, respectively. We also tested a similar approach in rice, a major food crop, to see whether these parameters could be extrapolated to other plant species. By applying our optimized perennial ryegrass protocol to rice, we were able to increase the rice callus transformation efficiency to over 90 % from an initial frequency of 40–45 %. Thus it is possible that the conditions highlighted in this study may serve to facilitate enhanced transformation efficiency in other monocots as well, particularly those that are known to be recalcitrant to *Agrobacterium*-based approaches.

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