

A Preliminary Experiment on *Agrobacterium tumefaciens*-Mediated Transformation of the *P5CS₁* GENE in Tall Fescue

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Abiotic stress conditions can be imposed on plants through either poor water quality or adverse climate condition. These types of stress affect plant growth and development throughout their life cycle. Plants that are tolerant to drought are therefore valuable, and turf grasses have the potential to grow tolerant. Turf grasses provide ground cover and offer multiple benefits, from their aesthetic value which affects our daily lives to their preservation of land against erosion. The objectives of the present work were to find the ideal media for callus induction and regeneration followed by overexpressing the *P5CS₁* gene encoding proline for improving drought stress resistance in *Festuca arundinacea* Schreb. The husks of tall fescue seeds were removed and the seeds were then sliced longitudinally. The result of this treatment showed a greater callus induction efficiency in comparison with intact seeds in the culture media. In addition, an increase in regeneration efficiency was observed in media supplemented with 2, 4-D along with BAP. The average of G418-resistant calli obtained in the experiment was around 10%. The heterologous transformation of *P5CS₁* in *F. arundinacea* background was confirmed by PCR and the transient Gus assay. More than 90% of calli expressed the *Uida* gene which can most probably convey resistance to drought stress.

Abstract

Keywords: Drought stress, *F. arundinacea*, Gus assay, *Uida* gene.

INTRODUCTION

Tall fescue is a hexaploid outcrossing species with a high degree of self-incompatibility, which makes its conventional breeding relatively difficult (Barnes, 1990). An increasing interest in tall fescue in Western Europe and elsewhere is mainly because of its better drought resistance and yield potential compared to perennial ryegrass (*Lolium perenne* L.) (Cougnon *et al.*, 2013). In comparison to other cool-season perennial grasses, tall fescue exhibits a high degree of stability when confronting drought stress. This relative drought tolerance makes it an ideal option for cultivation in urban landscapes throughout transitional climates.

Plants experience drought stress either when the water supply to roots becomes difficult or when the transpiration rate is high. These two conditions often coincide under arid and semi-arid climates. Drought stress is known to inhibit photosynthetic activity of plants likely due to an imbalance between light capture and its utilization (Foyer *et al.*, 1994). In response to water stress, plants exert adaptive modifications in their morphological, physiological and biochemical properties. Extreme morphological changes such as the reduction of vegetative growth and leaf wilting are often the first recognizable indications of water deficit. In addition, biochemical changes such as osmolyte accumulation and pigmentation changes have also been reported (Sarmast *et al.*, 2015). Numerous reports have shown the proline accumulation during the course of drought (Choudhary *et al.*, 2005), high salinity (Yoshida *et al.*, 1995), high light and UV irradiation (Saradhi *et al.*, 1995), heavy metal (Schat, 1997), oxidative stress (Yang *et al.*, 2014) and biotic stress condition (Haudecoeur *et al.*, 2009). Proline metabolism has mostly been studied in response to osmotic stress (Verbruggen and Hermans, 2008). Proline is synthesized in cytosol mainly from glutamate which can then be converted to the proline in two steps. First, glutamate is reduced to glutamate-semialdehyde (GSA) and then spontaneously converted to pyrroline-5-carboxylate (P₅C) by the pyrroline-5-carboxylate synthetase (P₅CS) and P₅C reductase (P₅CR), respectively (Hu *et al.*, 1992; Savoure *et al.*, 1995; Szabados and Savoure, 2009).

The *indica* rice over-expressing a P₅CS gene exhibited better growth performance, biomass production, higher proline accumulation and lower rate of lipid peroxidation in contrast to that of non-transgenic plants subjected to 150 mM NaCl treatment (Kumar *et al.*, 2010). Furthermore, the ameliorating effects of proline on heavy metal stress have been reported in *Chlamydomonas reinhardtii* (Siripornadulsil *et al.*, 2002). In 1992, particle bombardment-genetic transformation of *F. rubra* L. species reported for the first time by Ha *et al.* (1992). Twelve years later the first successful *F. arundinacea* *Agrobacterium*-mediated transformation was published (Bettany *et al.*, 2003). Later on *F. arundinacea* *Agrobacterium*-mediated transformation was more improved by other researchers (Gao *et al.*, 2008). Zhao *et al.* (2007) examined whether salt tolerance can be improved stably by overexpressing vacuolar Na⁺/H⁺ (AtNHX1) antiporters in tall fescue. Dong and Qu (2005) reported that *Agrobacterium*-mediated transformation of tall fescue yields 34% hyg B-resistant calli and had 8% overall transformation efficiency. Hu *et al.* (1992) had improved the cold resistance of tall fescue through *Agrobacterium* transformation of *ipt* gene. But to the best of our knowledge, the *Agrobacterium*-mediated transformation of *F. arundinacea* by P₅CS₁ gene has not yet been reported. The most important goal of this work was to pave the road for stable transformation of P₅CS₁ gene in *F. arundinacea* callus through *A. tumefactions*.

MATERIALS AND METHODS

Surface sterilization and callus induction

Tall fescues (*Festuca arundinacea* Schreb.) seeds were submerged into 25% sulfuric acid for 35 min then washed in tap water overnight. These seeds were moved into air flow cabinet hood and treated with 25%-50% clorox (containing 5.25 % sodium hypochlorite) solution contained 0.02 % household detergent for 30 min for surface sterilization then rinsed six times with sterilized distilled water. Finally removed-husk seeds were cut longitudinally and placed onto MS (Murashige and Skoog, 1962) basal medium supplemented with 0-16 mg/L 2, 4-D in which explants gained

the ability of callus production at dark in two weeks. These calli were subcultured consequently for more than a year every 8 weeks on MS media contained 5-8 mg/L 2,4-D. We tried to keep the embryogenic callus in subcultures which was identifiable easily by yellowish color and firm texture as compare to non-embryogenic calli. In all these time, calli were kept at dark. BAP (0.1 and 0.5 mg/L) and kinetin (Kin) (0.1 and 0.2 mg/L) without 2,4-D, have been considered for plant regeneration of tall fescue calli. The pH of the all media was adjusted to 5.8 by 0.1 N HCl before autoclaving for 15 min at 121°C. Those cultures that did not need darkness were kept at 25°C under cool-white fluorescent light ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$) with 16/8 h day/night photoperiod.

Sensitivity of *Festuca arundinacea* Schreb. calli to G418

Tall fescue calli have been examined in MS media which has had 5, 25, 50, 75, 100, 125, 150 and 200 mg/L of G418 for just about 2 weeks. We preferred to pick 100 and 150 mg/L G418 as an ideal concentration for our goal soon after co-cultivation in selective media so as to identify discrepancy between transformed and non-transformed calli.

Strains, binary vectors and transformation

pGV3101 strain of *Agrobacterium tumefaciens* harboring pBI121 plasmid as a binary vector, chosen for this experiment. The reporter and selectable marker genes of aforementioned plasmid were *Gus* and *nptII*, respectively. Bacteria were grown and selected in rotator in LB liquid media (tryptone 10 g/L NaCl 5 g/L, yeast extract 5 g/L) supplemented with 50 $\mu\text{g/ml}$ kanamycin for 24 h at 28°C. The cells were harvested by centrifugation (8000 rpm for 10 min at 4°C) and further re-suspended in 10-15 ml MS medium. Acetocyringon (AS) was added to the medium, up to 100 μM . For transformation, 4 × 4 mm of calli -grown at dark considered for inoculation with *A. tumefaciens* ($\text{OD}_{600}=0.5-1$) under 400 mgHg pressure for 10-15 min, then the callus pieces and *Agrobacterium* were incubated for 20 min with gentle shaking. Excess bacteria were removed after the incubation, the infected callus pieces were transferred onto filter papers for a few minutes then placed onto co-cultivation MS medium supplemented with 100 μM AS and 5 mg/L 2,4-D for almost 3 days (in dark) at 25°C. An empty Petri dish contained a Whatman filter paper–moisturized with sterile water–applied for co-cultivation in some experiments. After cocultivation, explants were rinsed with sterilized distilled water once and for the second time explant incubated with either 500 $\mu\text{g/ml}$ cefotaxime or 400 mg/L timentin for about 30 min along with gentle shaking to prevent *Agrobacterium* overgrowth (in regeneration MS media). Explants then blotted onto sterilized filter papers and placed onto MS medium supplemented with 5-8 mg/L 2,4-D, 150-200 $\mu\text{g/ml}$ G418 for selection of transformed explants and 200 mg/L timentine to prevent *Agrobacterium* overgrowth for 2 weeks. For plant regeneration, explants were transferred to regeneration media supplemented either with BA (6-benzyl-amino-purine) or Kin in light. Cultures were kept at 25 ±3 °C under cool white fluorescent light ($30 \mu\text{mol m}^{-2} \text{s}^{-1}$), for 16 h each day.

PCR confirmation and *Gus* histochemical staining

The presence of *uida* gene in embryogenic calli was confirmed by PCR amplification of tall fescue-calli genomic DNA with gene specific primers: *Gus-F* (GCTGTGCCAGGCAGTTT-TAAC) and *Gus-R* (ATATCGTCCACCCAGGTGTTC). The predicted size of the amplified DNA fragment of *uida* was 425 bp. DNA amplifications were performed in a total volume of 20 μl containing 1 μl of 10 μM forward primer, 1 μl of 10 μM reverses primer, 2 μl of 10X Ex Tag Buffer, 0.5 μl of dNTP mixture (2.5 mM each), 0.1 μl of TakaRa Ex Taq™ enzyme (5 unit/ μl) and 1 μl of gDNA (50 ng) (Takara, Shuzo, Kyoto, Japan), by thermocycler (Bio-Rad). The PCR was carried out for screening of regenerated transformed plantlets with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s. Final extension carried out in 75 °C for 10 min. The PCR products were separated in 1% agaros gel containing 0.5 $\mu\text{g/ml}^1$

ethidium bromide. The size of the amplification products was estimated using a 100 bp DNA ladder (Gene ruler DNA Ladder mix # SM 0331, Fermentas). Gus histochemical staining was performed based on Jefferson (1987).

RESULTS AND DISCUSSION

Osmotic stress tolerance in plants can be enhanced assuringly by proline overproduction. This subsequently spurs the overexpression of the *P5CS* gene encoding proline which can improve drought stress resistance in *F. arundinacea*. The preliminary work on callus induction resulted in a high amount of callus during a year. Whereas the callus regeneration rate was astonishing but plant regeneration was far behind callus production. An increase in callus regeneration efficiency was observed in media supplemented with 8 mg/l 2,4-D which was almost six times greater than 2 mg/l 2,4-D treatment by dehusked-longitudinally sliced seeds. The research results obviously revealed that removing husks of festuca's seeds would progress callus induction. Using 2, 4, and 16 mg/l 2,4-D did not affect callus induction and production significantly, hence we decide to apply 8 mg/L 2,4-D for all our experiments in which the goal was callus induction and production (Table 1). Apart from 2,4-D concentration, dehusked tall fescue mature seeds showed a further callus induction efficiency as opposed to intact seeds. The callus regeneration frequency preceded in dark condition compared to light situation (data not shown). Acetosyringone in co-cultivation media greatly improved *Agrobacterium* growth while acetosyringone free MS media delayed *Agrobacterium* growth and subsequently could decrease *Agrobacterium*- mediated transformation efficiencies.

Table 1. Callus induction rate of tall fescue seeds after 36 days.

2,4-D (mg/L)	Intact seed	Dehusked seed	Dehusked sliced seed-longitudinally	Dehusked sliced seed -vertically
control	-†	-	-	-
2	-	+	4.7 bc	7 bc
4	5 ab	+	11 b	9 b
8	9 a	+	25 a	19 a
16	5 ab	-	4b c	2c

†In each column, means with the same letters are not significantly different at $P < 0.05$.

Festuca mature seeds showed great amount of fungi contamination. Due to this problem, a small piece of *Festuca* callus was induced to produce a high amount of callus. This strategy has two advantages. First, genetic variability is slacked off during subcultures. Secondly, the chances of infection undermining seed sterilization can be vividly diminished. *Festuca* callus were able to growth onto 2,4-D media for 2 months even without subcultures. High level of plant regeneration was impossible unless we used either Kin or BA in MS media (Table 2). Regarding to the plant regeneration, Kin to some extent was superior compared to BAP. Some embryogenic explants were able to produce leaves even in media supplemented with 2,4-D alone, in dark condition with an albino phenotype.

Table 2. Effects of different PGRs on overall plant regeneration of tall fescue calli.

Treatments	Callus regeneration frequency (%)	Overall plant regeneration frequency (%)
8 mg/L 2,4-D + 0.1mg/L BAP	65	25
8mg/L 2,4-D + 0.5mg/L BAP	38	20
0.2 mg/L kinetin	35	18
0.1 mg/L kinetin	38	28

Embryogenic compact and yellowish calli were ideal for transformation because they were embryogenic callus and were able to regenerate plants compared to other types of callus (Fig. 1, B-C). Using vacuum device in inoculation stage of agro-transformation for just 10-15 min had pivotal influence on expression of *Gus* gene. Robust *uidA* expression was observed in transformed calli by means of vacuum device as opposed to those that just gently shaken for quite a while. *Gus* histochemical assay was carried out in 3-4 days after co-cultivation when we realized that timentin stopped the bacteria overgrowth in selective media at dark. Fig. 1 (D and E) are representative images for transient *Gus* assay, 4 days after cocultivation on MS media.

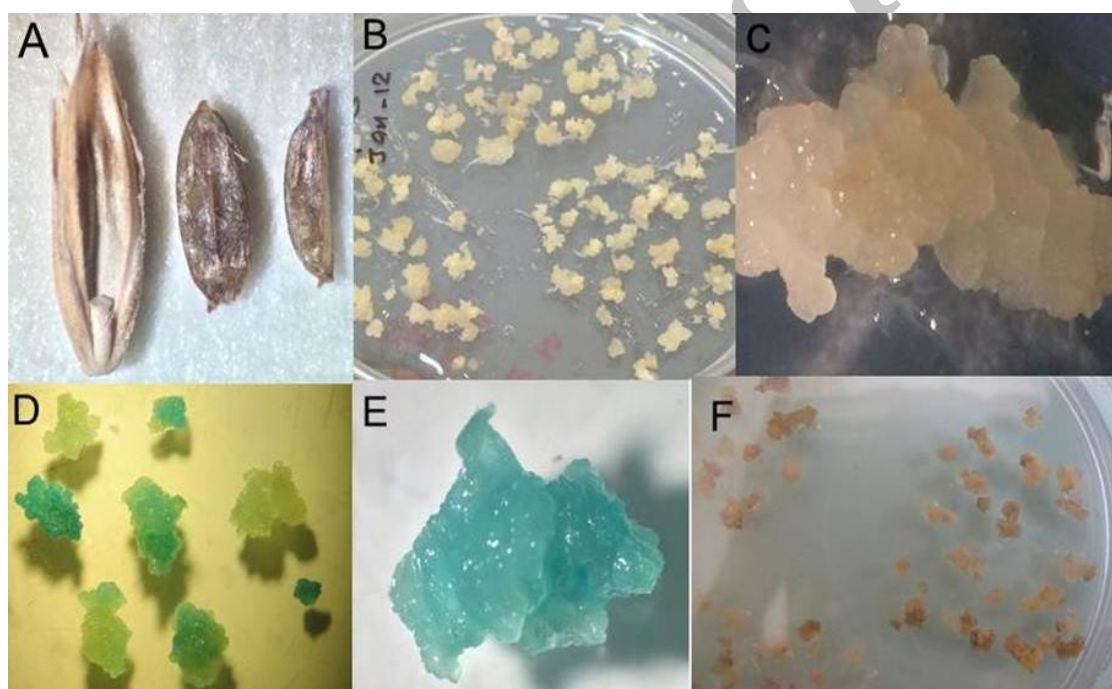


Fig. 1. (A) Tall fescue mature seeds and sliced longitudinal explant for callus induction. B-C. Embryogenic callus induced from seeds/caryopses. (D) Transient *Gus* expression three days after co-cultivation of calluses with *Agrobacterium*. (E) Detailed view of *Gus* expression three days after cocultivation. (F) G418 resistant calli obtained 3 weeks after *Agrobacterium*-mediated transformation and selection of infected callus pieces on medium containing 200 mg/L timentin.

Selective MS media were supplemented with 100-150 mg/L of G418 to show discrepancy between the transgenic cells which have received the tDNA and non-transformed cells which slowly turned brown and stands out from transformed cells in two or three weeks. The total genomic DNA of resistant calli subsequently tests for presence of *Gus* gene via *uidA* gene specific primers (GSPs) in PCR reaction (Fig. 2). The predicted size of *uidA* gene after PCR amplification with GSPs was 425 bp.

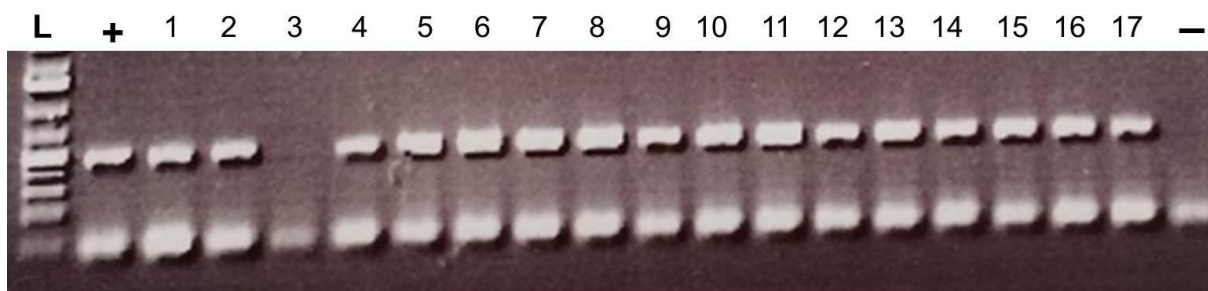


Fig. 2. PCR screening of DNA samples of tall fescue embryogenic callus by *uida* gene specific primes after *Agrobacterium*-mediated transformation with *P5CS1*. Predicted band is 425 bp. L is gene ruler DNA Ladder, + is positive control (pBi121 plasmid), – is negative control and numbers represent transgenic events.

Ever since the successful report on rice transformation in the early 1990s, researchers have been contemplating the possibility of making turf transgenic plants. It has been a long time that many researchers have put effort towards producing drought-resistant turf cultivars through genetic transformation but due to low regeneration rate and low transformation efficiency in *Festuca* spp. its transformation efficiency still need to be improved. Proline is one of the major abundant osmolytes which so far has been found in plants especially under salinity and water deficit conditions (Delauney and Verma, 1993; Sarmast *et al.*, 2015). Proline predominantly acts as osmoprotectant. It can also function as a protein stabilizer and functions as a hydroxyl radical scavenger. It can also stabilizes cell membranes by interacting with phospholipids, and serves as a source of carbon and nitrogen inside the cells (Szabados and Savoure, 2009).

The average transformation efficiency in 4 lines of *F. arundinacea* Schreb calli was reported to be 10.5% (Gao *et al.*, 2008). In this report, *Agrobacterium*-mediated transformation appears to be the preferred method for producing transgenic tall fescue plants than that of particle bombardment. The overexpressing vacuolar Na^+/H^+ (AtNHX1) antiporter was investigated in the tall fescue (Zhao *et al.*, 2007). Previous research led to the identification of a single copy inheritance of AtNHX1 which performs better in the presence of 200 mM NaCl than in the control plants regarding most of the T1 and T2 lines of tall fescue after *Agrobacterium*-mediated transformation occurred (Zhao *et al.*, 2007). Regarding to antibiotic-resistant calli, we have examined callus resistance to G418 and determined that, in contrast to Dong an Qu (2005) experiment that yields to 34% *hyg B*-resistant calli, we could only get around 10% G418-resistant calli (200 calli were used in this experiment). This indicates that, apart from experimental design, *nptII* not the best option for tall fescue transformation. However, more than 90% of G418-resistant calli were positive when we used them for PCR amplification of *uida* gene. Dehusked tall fescue seeds that have sliced longitudinally, influenced callus induction and regeneration to great extent compared to intact seeds. This most likely is due to a higher contact surface of seeds to the MS media. Recently Zhuang *et al.* (2017) reported that during drought stress in tall fescue, genes involved in strigolactone biosynthesis and axillary bud dormancy were up-regulated. In one report by Fariaszewska *et al.* (2016), drought stress significantly increased the content of proline in tall fescue which is in line with Sarmast *et al.* (2015) results on the same species. In response to drought stress aquaporin genes in tall fescue leaves down regulated (Pawlowicz *et al.*, 2017). These experiments collectively suggest that proline is an important compatible osmolyte which serves as a protectant for enzymes and cellular structures of tall fescue under severe drought stress. The heterologous transformation of *P5CS1* in *F. arundinacea* was confirmed by PCR and transient Gus assay which most probably can improve this cultivar's resistance to drought stress. Although due to high callus regeneration cycles and 2, 4-D concentration, it was not possible to produce transgenic plants, mostly due to DNA hypermethylation-derived somaclonal variation (Sarmast, 2016). However, by means of as-

sessing the callus induction strategy and *Agrobacterium*-mediated transformation protocols in this species, this preliminary study represents a first step towards developing transgenic tall fescue's callus regeneration guidelines and strategies which can be of more general interest.

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