CHAPTER 3

TRANSFORMATION OF TEF (*Eragrostis tef*)

3.1 Abstract

Successful application of genetic transformation for integration of a transgene is much dependent upon availability of an efficient *in vitro* plant regeneration procedure and detection of transgene insertion and expression. Isolated immature embryos of *E. tef* cultivar DZ-01-196 were used for embryogenic callus formation and callus was transformed with the GA inactivating coding sequence (*PcGA2ox*) under the control of a triple CaMV 35S promoter using the Agrobacterium transformation procedure. Media K99 was applied as basal medium and both the MS-based co-cultivation medium (CCM) and regeneration medium (K4NM) were used for embryogenic callus induction from immature embryos, *Agrobacterium* transformation and regeneration of embryogenic calli. Transformed *E. tef* callus was tolerant to treatment with the selectable marker kanamycin which inhibited growth of non-transformed shoots derived from matured embryos. A total of 55 plants were regenerated from callus to fully viable plants setting seeds at maturity. Eight putatively transformed T_0 plants were produced carrying the transgene in their genome which was detected by PCR. Sequence analysis confirmed that the amplified PCR product had 97.2 and 99.8% sequence similarity to *PcGA2ox* and *nptII,* respectively, but detection of the *PcGA2ox* or *nptII* transgene in the T_1 generation was inconsistent although phenotypic characterization of semi-dwarf T_1 generation plants showed changes in agronomic characters such as plant height, number of internodes, tillering, panicle length, biomass and yield as well as changed GA content. Results showed a GA-deficient growth characteristic (semi-dwarf phenotype) in putatively transformed plants associated with a low level of bioactive GA_1 and immediate precursors. Culm reduction was due to absence of elongation of the upper-most

internodes. Panicle length in semi-dwarfed plants showed no relation with Culm length. Up to 3.7 fold increase in grain yield per plant was found in some semi-dwarfed plants. Lack of detection of transgene insertion in T_1 generation is still a major concern and further studies are necessary to rule out that somaclonal variation has not been the source of variation in plant height and other plant characteristics.

3.2 Introduction

Agrobacterium-based transformation still imposes a considerable challenge in cereal transformation. Some of the salient features that determine success of the method have been previously extensively investigated (Shrawat and Lörz, 2006). Further, variations in transgene expression are influenced by several factors including somaclonal variation induced by tissue culture process, the copy number of the transgene incorporated into the host genome, truncation of the transgene, and epigenetic gene silencing (Shrawat, 2007). Silencing of the transgene is often associated with a high transgene copy number or transgene promoter activity and occurs either at the transcriptional or post-transcriptional level.

The successful application of *Agrobacterium*-based genetic transformation systems and progress in precision of integration of the transgene is dependent upon availability of an optimized efficient *in vitro* plant regeneration procedure. This has been one of the objectives for this study. *In vitro* regeneration protocols for *E. tef* have been reported by several research groups to regenerate plants from various explants such as roots, leaf bases and seeds (Bekele *et al*. 1995; Mekbib *et al*. 1997; Assefa *et al*. 1998). However, obtained regeneration was poor to consider further application (Gugssa, 2006). Moreover, immature reproductive organs, such as embryos, had not been used until recently (Gugssa *et al*., 2006) where regeneration of haploid plants has been achieved using gynogenic tissue of *E. tef* and immature zygotic embryos (Gugssa, 2008). However, regeneration of transformed plants has so far only been obtained by Gugssa (2008) regenerating a single plant expressing the Green florescent protein (Gfp).

The objectives of this study were therefore establishing a transformation and regeneration procedure using protocols developed for various cereals and detecting integration and expression of the GA inactivating gene (*PcGA2ox*) in *E. tef*. The method of transformation of immature embryos and production of transformed embryogeneic callus was applied to regenerate transformed shoots that develop into fertile plants. Putatively transformed *E. tef* plants with changed plant stature (dwarf/semi-dwarf) were also characterized for such traits as plant height, tillering, stem diameter, panicle length, physiological (biomass and yield) and biochemical characteristics (GA content).

3.3 Materials and Methods

3.3.1 Preparation of plant material and culture

Seeds of improved variety DZ-01-196 were obtained from the Ethiopian Institute of Agricultural Research (EIAR). The seeds were germinated on germination mix soil and the seedlings were grown in pots under a 26±2 / 18°C day/night temperature and a 14 hr day length. Plants were further supplemented with a full-strength Hoagland nutrient solution until immature zygotic embryos, referred to as immature embryos (IEs) in this study, could be harvested from the developing panicle 2-3 weeks after panicle emergence.

3.3.2 IE isolation, callus induction and culture growth

Immature embryos (IEs) from *E. tef* plants of cultivar DZ-01-196 were isolated and callus was induced according to the protocol reported by Gugssa (2008). Callus inoculation and co-cultivation with Agrobacterium during *E. tef* transformation and further regeneration was carried out following various protocols (Gugssa, 2008; Hensel and Kumlen, 2004; Rao *et al*., 2007; Toki, 1997; O'Kennedy *et al*., 2004). Immature embryos were collected from flower spikes 7 to14 days post anthesis and IEs were isolated using a binocular microscope (Gugssa *et al*., 2008). The middle segment was selected for isolation of embryos. Freshly detached spikes were used for immediate isolation and culture after sterilization, or spikelets were pre-treated at 4^{o}C for a day before isolation and disinfection of the immature embryo. For sterilization, the intact spikelet was cut short to 3 - 5 cm segments before isolation, this allowed better handling and culture of IEs. Intact spikelets were surface-sterilized with 70% ethanol for 1 min followed by washing in 2.0% chlorox containing 0.1% Tween 20, 2 - 3 drops of savlon for 12 min under shaking (modified from Gugssa, 2006 and O'Kennedy *et al*., 2004), which was followed by a 4 - 5-times rinse in ddH_2O (sterile) by working in a laminar air-flow cabinet (LAFC). The IEs were isolated aseptically with forceps under sterile conditions and were placed, scutellum side-up, on petri-dishes containing K99EM embryogenic callus induction medium (Table 3.1; Gugsa *et al*., 2008). Embryonic calli initiated from IEs were used in this study as explants source for Agrobacterium-mediated transformation. Viable looking proliferating embryogenic calli were transferred to fresh CI medium (Table 3.1) every second week. Infection with Agrobacterium for transformation was done at this stage using 2-3 weeks old young calli.

3.3.3 *GA2ox* and *nptII* marker gene plasmids

The hybrid binary plasmid *pGPTV-kan* containing the coding sequence for neomycin phosphotransferase (*nptII*), which confers resistance to kanamycin and its analogue geneticin (G418), under the control of the *nos* promoter and terminator sequences was used in *E. tef* transformation. The plasmid T-DNA region also contained the coding region of GA2 oxidase (about 1 kb) isolated from runner bean (*Phaseolus coccineus)* (*PcGA2ox1*) obtained through functional screening (Thomas *et al*., 1999). The transgene is under the control of a triple CaMV 35S promoter sequence located next to the right border of the T-DNA (Fig. 3.1). The full construct containing the transgene, promoter and Kan resistance was a gift of Dr. Hedden, Rothamsted Research, UK. The *E. coli* strain JM109 (Invitrogen, USA) was used to maintain the plasmid before transforming cells of *Agrobacterium tumefaciens* strain LBA4404. The presence of the insert in the plasmid was confirmed using agarose gel electrophoresis for plasmid DNA digested with restriction enzymes.

Competent Agrobacterium cells were used for transformation with the plasmid. Agrobacterium cells were transformed with the plasmid DNA of *pGPTV-kan* by mixing 60 µl competent cells with 10 µl plasmid DNA harbouring the transgene and incubating on ice for 5 min before transferring the mixture to liquid nitrogen for 5 min. The mixture was then incubated at 37^{o}C in a water bath for 5 min. LB medium (1 ml) was added to the tube containing the Agrobacterium-plasmid mixture, the tube was sealed and was shaken on a rocking table for 2 - 4 h at room temperature. After briefly spinning the tube in Eppendorf microcentrifuge to collect the cells, 150 µl of the mixture was poured onto

solid LB medium containing the two antibiotics kanamycin (50 mg l^{-1}) and rifampicin (25 mg l^{-1}) and plates were incubated for 2 days at 28°C for selection of transformed cells. Single colonies were randomly selected and cultured on a new antibiotic containing LB plate for two more days. A liquid culture of the re-streaked colony was established to verify after plasmid isolation by PCR the presence of the transgene in the plasmid.

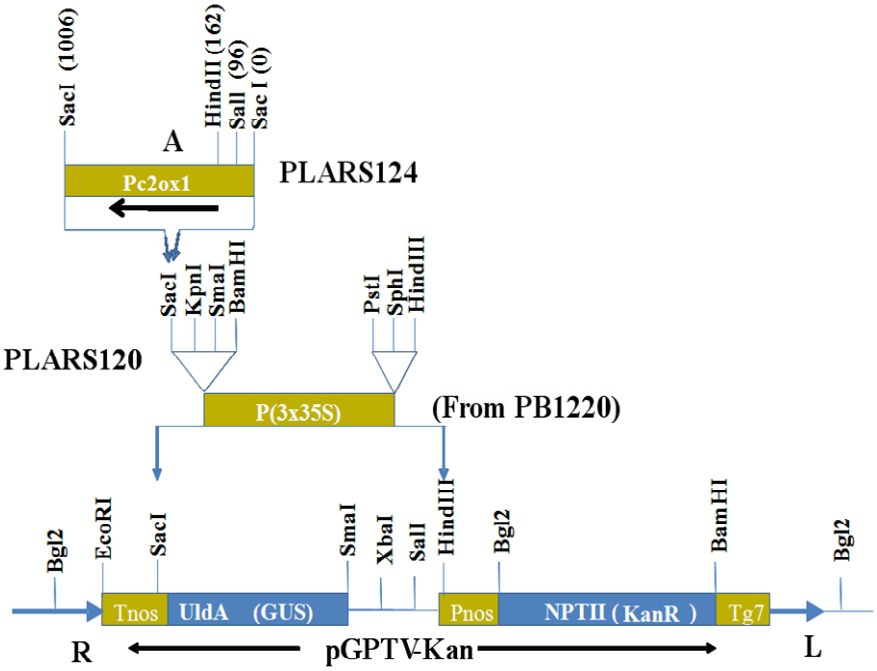


Figure 3.1 Construction of plasmid pGPTV-Kan harbouring *Phaseolus coccineus GA2ox1* (*PcGA2ox1*), the triple 35S CaMV promoter sequence, the *nos* terminator (Tnos) sequence and also the *nptII* selectable marker gene.

3.3.4 Agrobacterium culture, inoculation and co-cultivation

For *E. tef* transformation, transformed Agrobacterium (strain LBA4404) cells (500 µl) were transferred into a 500 ml Erlenmeyer flask containing 250 ml LB/YEP medium and kanamycin (50 mg l^{-1}). The culture was shaken at 200 rpm at 28°C until the OD_{660} was about 1.0. About 30 ml of Agrobacterium cells were centrifuged (3,500 rpm, 10 min) in a bench top centrifuge and the cell pellet was re-suspended in 30 ml liquid co-cultivation medium (CCM; Table 3.2) (Hensel and Kumlehn, 2004). The virulence activator acetosyringone (Table 3.1) was added immediately before inoculation. The cultures were stirred at 50 rpm for about 1 h before infection. After infection for 6 – 12 h, calli were blotted onto sterile tissue paper and briefly rinsed with liquid CCM. Washed calli were then co-cultivated by growing on K99EM callus induction medium (K99EM-CIM) for 2-3 days with 2.0 mg l^{-1} of the auxin 2, 4-dichlorophenoxyacetic acid added (2, 4-D) but without addition of any antibiotic (Table 3.1). The surviving calli were then transferred to a selection medium. For the control, embryogenic calli were kept uninfected with Agrobacterium but were subject to all post infection treatments excluding antibiotic treatment.

After co-cultivation, and further growth for 2-3 days in antibiotic free medium, calli were then transferred to CI - SL media (Table 3.1) supplemented with 2.17 mg l^{-1} 2,4-D, 250 mg l^{-1} cefotaxime (or 250 mg l^{-1} timetin) and 100 mg l^{-1} kanamycin and then cultured for 2 to 3 weeks. After this time, calli were transferred to a CI - SL medium supplemented with 2.17 mg l^{-1} 2,4-D, 200 mg l^{-1} cefotaxime (or 200 mg l^{-1} timetin) and 100 mg l^{-1} kanamycin before plant regeneration. All culturing until the regeneration stage was done

using culture plates (50 mm x 10 mm) which were kept in dark at 24±2^{0}C. Developing embryos (sometimes turning green) transferred to K4NM regeneration medium were grown under a 16 h photoperiod maintaining a temperature of 24±2^{0}C.

3.3.5 Plant regeneration

After selection, calli that were still creamy-white were transferred to K4NM pre-regeneration medium (Table 3.1). The medium had no 2, 4-D addition and a reduced concentration of kanamycin (50mg l^{-1}) and cefotaxime (125 mg l^{-1}). After two rounds of selection on this medium, calli were transferred to a regeneration medium (Table 3.2) for 6 to 8 weeks which was refreshed after 3 weeks (with no antibiotics added). Developed regenerated shoots (2 to 4 cm long) were transferred for 1 week to partly ventilated baby jars containing the regeneration medium. Plantlets were then transferred to an environmentally controlled phytotron for hardening-off.

3.3.6 Preparation of plant material and culture

Regenerated T_0 plants were acclimatized and grown in an environmentally controlled greenhouse with a 16-h photoperiod provided by natural light supplemented with light from sodium lamps to maintain a minimum PAR of 350 µmol$m^{-2}s^{-1}$. The temperature was maintained at 23-27°C (day) and 15-18°C (night). Seeds from selected T_0 plants that showed positive PCR amplification of *GA2ox1* insert were further grown in pots [15 cm diameter (top) x 12.5 cm (height) and 10 cm (bottom)]. A soil mixture consisting of peat

(75%), sterilized loam (12%), vermiculite (3%) and grit (10%) was used supplemented with a slow release fertilizer.

3.3.7 DNA isolation and PCR screening of *E. tef* regenerants

All regenerated plants grown in the phytotron were screened for the presence of the transgene by PCR using gene specific primers for *PcGA2ox* and *nptII*. Leaf tissue from putatively transformed plantlets was used to extract genomic DNA using a modified CTAB method (Harini *et al*., 2008). Same procedure was applied to extract DNA from untransformed plants (control) that however were regenerated through the whole process except the Agro-infection. DNA amplification was carried out in a 25 µl reaction mixture with template DNA (ranging between 100-150 ng), 0.5 µl dNTPs (10 mM stock), 1.2µl $MgCl_2$ (25 mM stock), 0.5 µl primer (10mM), 5 µl of a 5X reaction buffer, and 0.15 µl Taq polymerase (Fermentas, Canada). Amplifications were carried for 35 cycles (DNA denaturation: 94^0C, 30 sec.; primer annealing: 60^0C, 30 sec.; DNA extension: 72^0C, 30 sec.). Sequences of the *PcGA2ox* and *nptII* gene primers used for PCR amplification were: one sense primer (*PcGA2ox*): 5'- TCA TAG TGA ACG CCT GTA GG- 3' and two anti-sense primers: 5'-TGT TCT TCA CTG CTG TAA TG - 3' and 5'- ACC TGC TTA ACG TAT TCC TCT G – 3'obtained from NCBI database mRNA nucleotide sequence (Acc. No. AJ132438 for *PcGA2ox*). Expected fragment size after amplification of *GA2ox* gene were 321 and 391 bp, respectively. PCR amplification of the *nptII* gene was performed under identical conditions as used for *PcGA2 ox*. Sequences of the *nptII* primers used for PCR amplifications were: primer 1: 5'-AGA CAA TCG GCT GCT CTG AT-3' and primer 2: 5'- ATA CTT TCT CGG CAG GAG CA-3'. PCR products

with expected size of 365bp were analyzed by gel electrophoresis on a 1.0% agarose gel (Sigma, St. Louis, MO) to confirm that a correct size product was amplified. The sizes of the amplified fragments were determined using a molecular weight marker after ethidium bromide staining to view fragments on the gel (GIBCO BRL, Gaithersburg, MD).

3.3.8 Phenotypic measurements and characterization of T_1 generation

T_1 generation transformed plants were grown from seeds after selfing putative transformed plants (T_0 generation) that have shown *GA2ox1*amplification by PCR from isolated genomic DNA. T_1 generation plants that showed a dwarfed phenotype at seedling stage were further phenotypically characterized for growth and yield. Control plants that were subjected to transformation and had a wild-type phenotype were used for comparison. Measurements were taken at plant maturity to determine plant height, length of culm, length and diameter of individual internodes, above ground biomass, tillering, yield and yield components. Dry weight for above ground biomass was determined by drying fresh material at 80^{o}C for 2 days in an oven. Grain yield was determined by measuring the weight of seeds from main and secondary tillers. All data were collected at plant maturity and analyzed using GenStat statistical package.

3.3.9 Analysis of endogenous GA content

From selected dwarfed T_1 plants, sample of near equal weight were harvested during the stem elongation stage before panicle initiation from the secondary tillers. The upper-most

two internodes including its nodes were cut and weighed and stored at -80^{0}C until analysis. The same procedure was followed as described in Chapter 2 Section 2.3.5.

Table 3.1 Media used for induction of embryogenic callus, co-cultivation, selection and regeneration of transformed *E. tef* shoots.

	K99EM* based callus induction medium
K99EM (CIM)	Modified MS salts and Organic I* and Organic II* containing in 1 L medium: 1.023 mg (7 mM) glutamine; 250 mM casein hydrolysate; 213.2 mg (1 M) MES; 90 g (250 mM) maltose H_2O; 2.17 mg (10 µM) 2,4-D; pH 5.8
	MS based co-cultivation medium
CCM	Medium (1 L) contains: MS salts and vitamins (4.4 g); 30 g maltose; 800 mg L-cysteine; 500 mg L-proline; 300 mg casein hydrolysate; 350 mg myo-inositol; 98 mg acetosyringone; 2.5 mg DICAMBA, 2.0 mg 2, 4-D; pH 5.8
	K99EM based selection medium
CI- SL	Modified MS salts and Organic I and Organic II containing in 1 L: 1.023 mg (7 mM) glutamine; 250 mM casein hydrolysate; 213.2 mg (1 M) MES; 90 g (250 mM) maltose H_2O; 2.17 mg (10µM) 2,4-D; pH 5.8, and 250 mg cefotaxime; 100 mg kanamycin
	K4NB based regeneration medium**
PRE-RE	Medium (1 L) contains: 0.25 M glutamine, 10 mM $CuSO_4$, 36 g (100 mM) maltose H_2O; 1 mM BAP; 50 mg kanamycin; 125 mg cefotaxime; pH 5.8
RE	Medium (1 L) contains: 0.25 M glutamine; 10mM $CuSO_4$, 100 mM maltose H_2O; 1 mM BAP; pH 5.8

* (See Table 3.2); ** (See Table 3.3); CIM = callus induction medium is equivalent medium to K99EM (Gugssa, 2008); CCM = co-cultivation medium + antibiotics (Hensel and Kulmen, 2004); CI-SL = callus induction and selection medium (K99EM + antibiotics); PRE-RE = pre-regeneration medium; RE= regeneration medium.

Table 3.2 Composition of the K99EM medium (Gugssa, 2008) used for embryogenic callus induction from *E. tef* immature embryos.

Media Components					
Inorganic salts	**Conc. (mg l^{-1})**	**Organics I**	**Conc. (mg l^{-1})**	**Organics II**	**Conc. (mg l^{-1})**
$(NH4)_2SO_4$	80 (1 mM)	Retinol	1.01 (0.04 µM)	Malic acid	40 (0.3 µM)
KNO_3	2,022 (20 mM)	Thiamine HCl	1.0 µM	Citric acid	40 (0.1 µM)
KH_2PO_4	340 (2.5 mM)	Riboflavin	0.2 (0.5 µM)	Fumaric acid	40 (0.3 µM)
$CaCl_2.2H_2O$	441 (3 mM)	Ca-panthenate	1.0 (4.2 µM)	Na-pyrovate	20 (0.2 µM)
$MgSO_4.7\ H_2O$	246 (1 mM)	Folic acid	0.4 (0.9 µM)	Glutamine	1.023 (7 mM)
NaFeEDTA	27.5 (75 µM)	Pyridoxine HCl	1.0 (4.9 µM)	Casein-hydrolysate	250 µM
$MnSO_4.4\ H_2O$	11.2 (50 µM)	Cobalamine	0.02	MES	213.2 (0.1 M)
H_3BO_3	3.1 (50 µM)	Ascorbic acid	2.0 (11.4 µM)	Maltose H_2O	90,000 (250 mM)
$ZnSO_4.7\ H_2O$	7.2 (25 µM)	Calciferol	0.01 (0.03 µM	2,4-D	2.17 (10 µM)
$Na_2MoO_4.2\ H_2O$	0.125 (5 µM)	Biotin	0.01 (0.04 µM)		
$CuSo_4.5\ H_2O$	0.025 (0.2 µM)	Cholin chloride	1.0 (7.1 µM)	Phytagel	0.3%
$CoCl_2.6\ H_2O$	0.025 (0.2 µM)	p-aminobenzoic acid	0.02 (0.1 µM))	pH	5.8
KI	0.17 (1µM)	Myo-inositol	100 (0.6 µM)		
		Nicotinic acid	1.0 (8.1 µM)		

Table 3.3 Ionic composition of the K4NB regeneration media used for *E. tef* immature embryo cultures.

Components of K4NB regeneration medium			
Inorganic salts	**Concentration (mg l^{-1})**	**Organics**	**Concentration (mg l-1)**
Macro-nutrients		**Organics I**	**T-vitamins (1000x)**
(NH4)2SO4	320 (4 mM)	Thiamine HCl	10.0
KNO3	3640 (36 mM)		
KH2PO4	340 (2.5 mM)	Pyradoxine HCl	1.0
CaCl2.2H2O	441 (3 mM)		
		Organics II	
MgSO4.7 H2O	246 (1 mM)		
Na-FeEDTA	27.5 (75 µM)	Glutamine	0.25 mM
		$CuSO_4$	10.0 mM
Micro			
		MaltoseH_2O	100 mM
$MnSO_4.4\ H_2O$	11.2 (50 µM)		
H_3BO_3	3.1 (50 µM)	2,4-D	1µM
$ZnSO_4.7\ H_2O$	7.2 (25 µM)	Phytagel (Sigma-PB169)	0.3%
$Na_2MoO_4.2\ H_2O$	0.12 (0.5 µM)	pH	5.8
$CuSo_4.5\ H_2O$	1.25 (5 µM)		
$CoCl_2.6\ H_2O$	0.024 (0.1 µM)		
KI	0.17 (1 µM)		

3.4 Results

3.4.1 Plant transformation

Agrobacterium (strain LBA4404)-mediated transformation was carried out using the embryogenic callus from the scutellum region of immature *E. tef* embryos. The immature embryo produced embryogenic callus from the scutellum side within 2 weeks of culturing the embryos on embryogenic callus induction medium (Figs. 3.1 A and B). In some cases, callus already appeared within a week, this callus was also used for transformation. Further, not all embryos formed callus and some embryos only formed callus after 2 weeks. This callus was not further used.

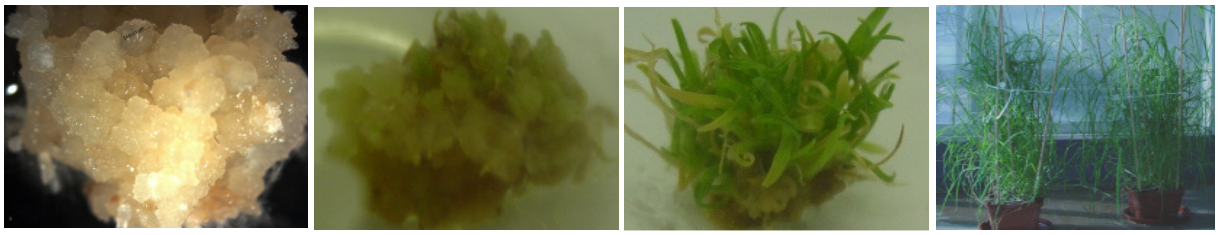
The antibiotic-containing selection medium was optimized for selecting kanamycin-resistant germinating mature embryos (Table 3.1). At 25 mg l^{-1} G418, up to 75% of shoots derived from non-transformed embryos wilted after 12 days exposure to the antibiotic without completely collapsing. The shoots also showed a yellowing of leaf tips. At 40 mg l^{-1} G418, up to 90% of non-transformed shoots collapsed and all shoots turned brown. Germinating shoots did not survive treatment with 75 mg l^{-1} G418 after 12 days of treatment with G418 (Table 3.1).

Immature embryos developed in to embryogenic callus (Figs. 3.2A and B) proliferated into shoots after 2 months of culturing the embryogenic callus on induction medium followed by 2 months of culturing on K4NM regeneration medium (Fig. 3.2C). Several regenerated shoots turned white or failed to survive while growing *in vitro* (data not

shown). Regenerated green plantlets were obtained which were hardened-off and grown to maturity in an environmentally controlled phytotron (Fig. 3.2E). A total of 55 plants were regenerated to fully viable plants setting seeds at maturity (Fig 3.1D). The regenerated putative transformed plants had generally a slower growth when compared to non-transformed plants. Putative transformed plants were kept under high humidity in the phytotron with a perforated polyethylene bag covering the pots and growing plants for about 1 to 2 weeks. *E. tef* being strictly selfing, no bagging was required to avoid crossings. At maturity, all transplanted and successfully grown plants produced fertile panicles setting seeds.

3.4.2 Transgene detection

In 8 of the 55 putative transformed plants (T_0 generation), which were regenerated and grown in the phytotron, the genome-inserted *PcGA2ox* or *nptII* sequences were detected by PCR in isolated genomic DNAs (Fig. 3.3). Sequence analysis of the amplified PCR product confirmed that amplified products using two sets of *PcGA2ox* primers with the sizes of 321 bp and 391 bp (*PcGA2ox*), and 365 bp (*nptII*) had a 97.2 to 99.8% sequence similarity to *PcGA2ox* and *nptII*, respectively. However, detection of *PcGA2ox* or *nptII* sequences was inconsistent in the T_1 generation where from several plants with a semi-dwarfed phenotype the two sequences could not be consistently amplified by PCR from isolated genomic DNA in repeated amplifications.



A B C D

Figure 3.2 *E. tef* shoot regeneration using immature embryo from young emerging panicle as explants. (A) Embryogenic callus deriving from immature zygotic embryos; (B) embryogenic callus proliferating into shoots; (C) embryogenic callus tissue proliferating into shoots after 2 months of culture on embryogenic callus induction medium followed by two months of culture on K4NM regeneration medium; (D) fertile regenerating plants.

Table 3.4 Survival of non-transformed *E. tef* seedlings derived from 100 mature embryos on antibiotic (G418)-containing selection medium.

G418 (mg l^{-1})	Survival (%)	Remarks
0	100	
20	100	<50% yellowing of leaf
25	100	<75% wilting and yellowing
30	100	100% yellowing of leaf with 1/3rd leaf top area burning
40	10	90% collapsed and brown
50	5	95% collapsed and brown
75	0	
100	0	
125	0	

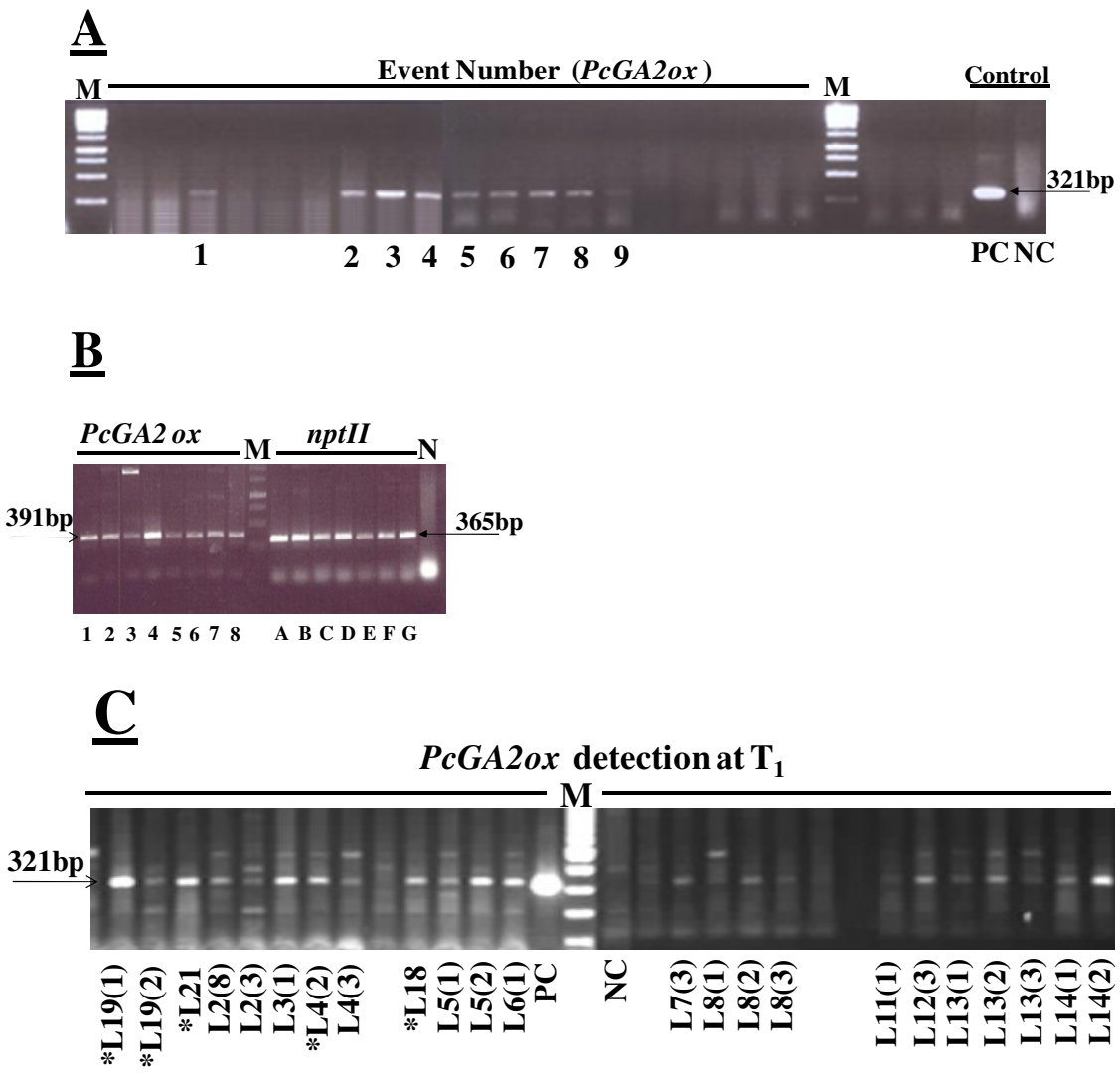


Figure 3.3 PCR amplification of *PcGA2 ox* (A and C) and *PcGA2ox* and *nptII* (B); sequences from putative transformed plants of T_0 (A & B) and T_1 (C) generation. (M) 1kb (A & B) and 100 bp (C) ladder molecular size markers; (NC) negative control without template DNA added in a reaction mix; (PC) a positive control with plasmid *pGPTV-kan*. Lines with asterisk (*) are among semi-dwarf phenotypes used for further phenotypic analysis.

3.4.3 Phenotypic characterization

3.4.3.1 Culm, internode and panicle length

Despite inconsistent PCR results, significant (*P<0.001*) variation in mean culm height was found between T_1 plants, with a dwarf/ semi-dwarf phenotype, and wild-type non-transformed plants. Plants with a dwarf/semi-dwarf phenotype had a Culm height ranging from 65 cm to 117 cm whereas the wild type control plants had a height of 157 cm (Figures 3.4 and 3.5). *E. tef* plants of line number 18 had the shortest Culm length (65 cm) followed by plants of line 23 (98 cm). In plants with a dwarf or semi-dwarf phenotype, major reduction in plant height originated from reduction of the Culm and not from reduction of the panicle. Culm reduction was mostly due to absence of elongation of the upper-most internodes (data not shown). In some dwarf plants elongation of the 7^{th} and/or 8^{th} internodes did not occur when compared to control plants. In most dwarfed plants reduction in length was found in all internodes (data not shown).

There was no significant variation in internode diameter between dwarf plants and wild-type (control) plants. Generally, internode diameter increased up to the 3^{rd} internode when overall mean values were compared (data not shown). However, control plants had a small and steady increase in diameter up to the 6^{th} internode. Plants showed acropetal increase in diameter upwards in both semi-dwarf and control plants demonstrating a weak tapering in these plants.

Panicle length of the semi-dwarf plants ranged from 60 - 80 cm when compared to panicle length of the control (67 cm) (Figure 3.4). Panicle elongation was not associated with any change in plant height but positively correlated with tiller number. Panicle emergence was delayed by a few days to a few (2 - 3) weeks in more dwarfed plants. In semi-dwarf plants the number of tillers per plant varied between 18 - 67 and the number was significantly higher (*P<0.001*) (1.3 - 4.8 fold) than in control plants (Figure 3.4). Grain weight per main tiller was not significantly different (P > 0.05) between semi-dwarf plants and control plants and differences originated from secondary tillers.

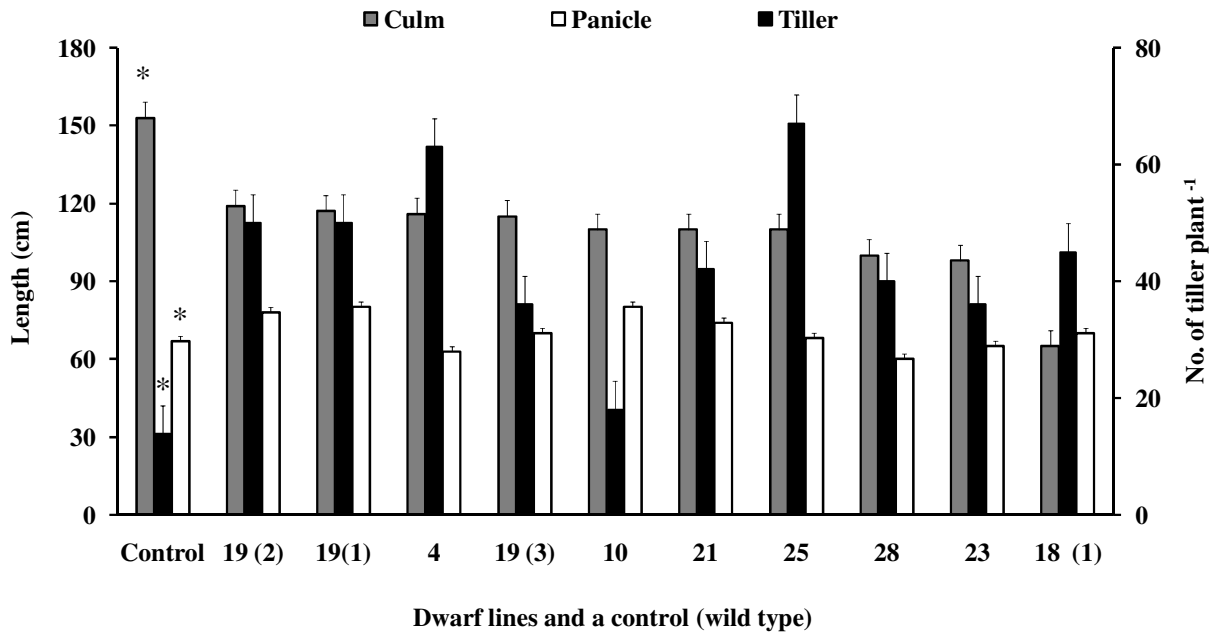


Figure 3.4 Culm and panicle height (cm) and number of tillers per plant of putatively transformed dwarf *E.tef* plants. Standard error (SE) values and significance level was determined by Student's *t*-test using GenStat Discovery Edition (VSN International Ltd) ($P \leq 0.001$).

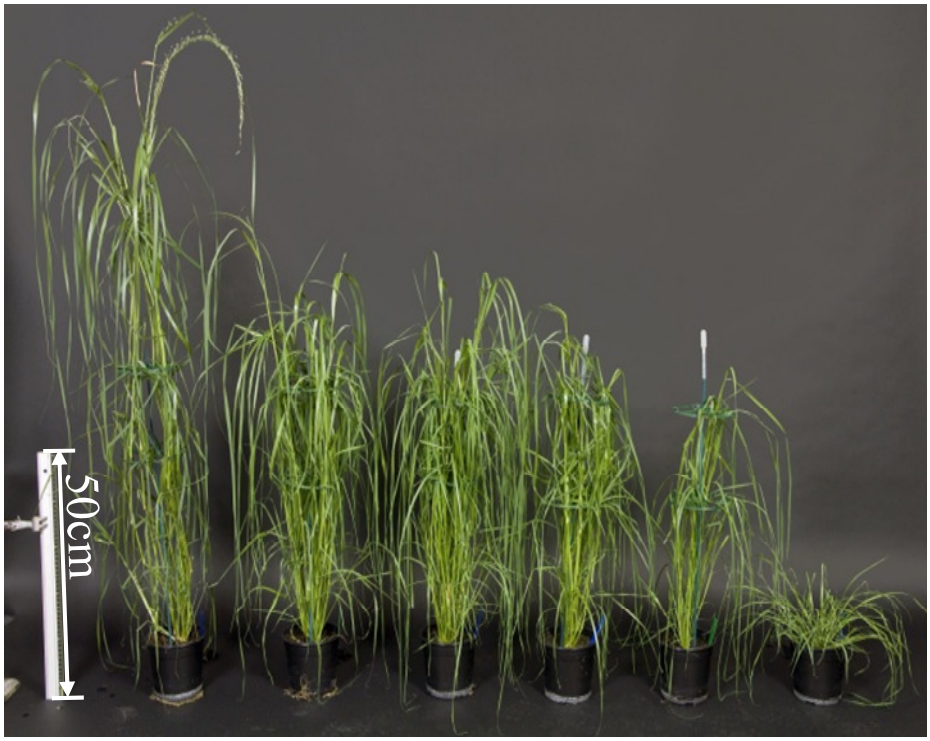


Figure 3.5 Selected *E. tef* dwarf (18) and semi-dwarf (19(3), 19(2), 28 and 10 T_1 generation plants. Control is a plant subjected to the transformation process without addition of Agrobacterium and antibiotic selection.

Up to four-fold increase in grain yield per plant was also found in some semi-dwarf plants (Figure 3.6). Higher biomass was found in the majority of semi-dwarf plants when compared to control plants (Table 3.5). Further, most of above-ground shoot weight increase was due to more tillering (Table 3.5).

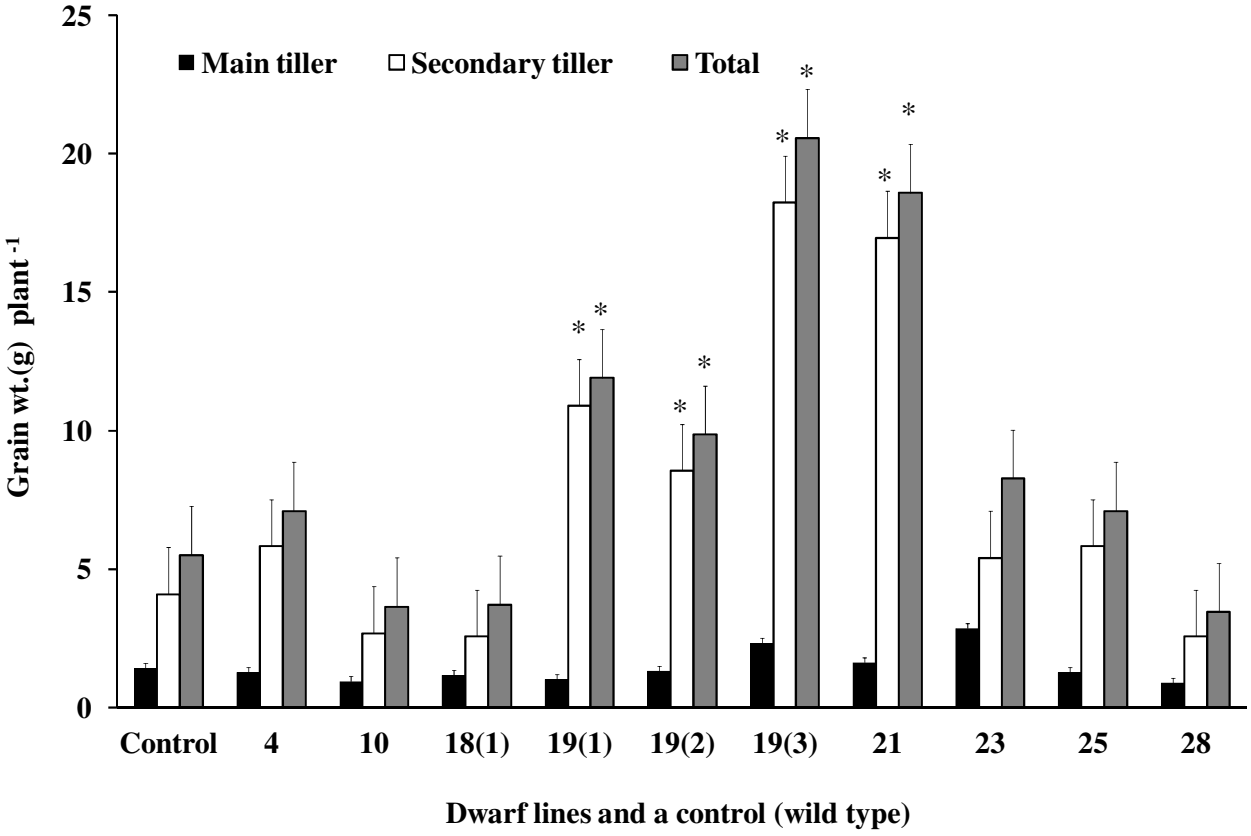


Figure 3.6 Seed weight of primary and secondary panicles of putatively transformed dwarf *E. tef* lines and a control. Standard error (SE) values and significance level was determined by Student's *t*-test using GenStat Discovery Edition (VSN International Ltd) ($P \leq 0.001$).

Table 3.5 Above ground biomass (gm) of putatively transformed dwarf tDZ-01-196 *E. tef* plants

Lines	Shoot FW	Culm + Leaf DW	Panicle DW	Tillers Culm + Leaf DW	Tillers panicle DW	Total Shoot DW
Control	213.7	5.9	2.7	54.9	10.8	74.2
4	429.9	4.7	2.3	120.8	28.9	177.7
10	375.3	3.8	2.2	103.2	12.8	174.0
18 (1)	155.9	4.2	2.6	42.0	10.1	144.2
19(1)	493.3	4.5	2.3	134.4	36.5	156.7
19 (2)	504.1	4.4	2.8	140.4	32.8	122.1
19 (3)	418.8	5.6	4.3	116.4	50.0	180.3
21	465.7	4.1	3.0	125.3	41.6	176.4
23	171.2	4.3	4.5	43.1	14.7	113.9
25	424.4	3.6	2.3	113.3	25.0	66.6
28	346.0	3.2	1.9	89.5	19.3	58.8
Mean	363.5	4.4	2.8	98.5	25.7	131.4
SE	38.35	0.24	0.25	10.90	4.07	14.24
Significance	***	***	***	***	***	***

FW = Fresh weight (gm); DW = Dry weight (gm)

Standard error (SE) values and significance level was determined by student's *t*-test using GenStat Discovery Edition (VSN International Ltd). (*** =*P<0.001*).

3.4.4 Analysis of endogenous GA content

Semi-dwarf plants had lower amounts of bioactive GA as well as lower amounts of precursors than the control plants when the endogenous GA content of plant tissues taken from the upper-most two internodes at shoot elongation stages were analyzed (Figure 3.7). The content of the most abundant bioactive GA form, GA_1, in dwarf plants such as L18(2), L19(2), L9(1), L21, L18 and L10, was considerably less when compared to the control. Amounts of immediate GA_1 precursors, such as GA_{44}, GA_{19}, GA_{20} and in particular GA_{19}, were also reduced in the dwarf plants (Figure 3.7). An expected increase in GA_{29} due to GA2ox over-expression was, however, not found. GA amount was also compared with the GA amount in the *E. tef* dwarf landrace variety, Gea Lammie, grown under similar conditions. Bioactive GA amount (including GA precursors in the 13β-hydroxylation pathway (See Table 2.1) except in some cases like GA_{29} and GA_{51}) in putative transformed plants was much lower than in Gea Lammie.

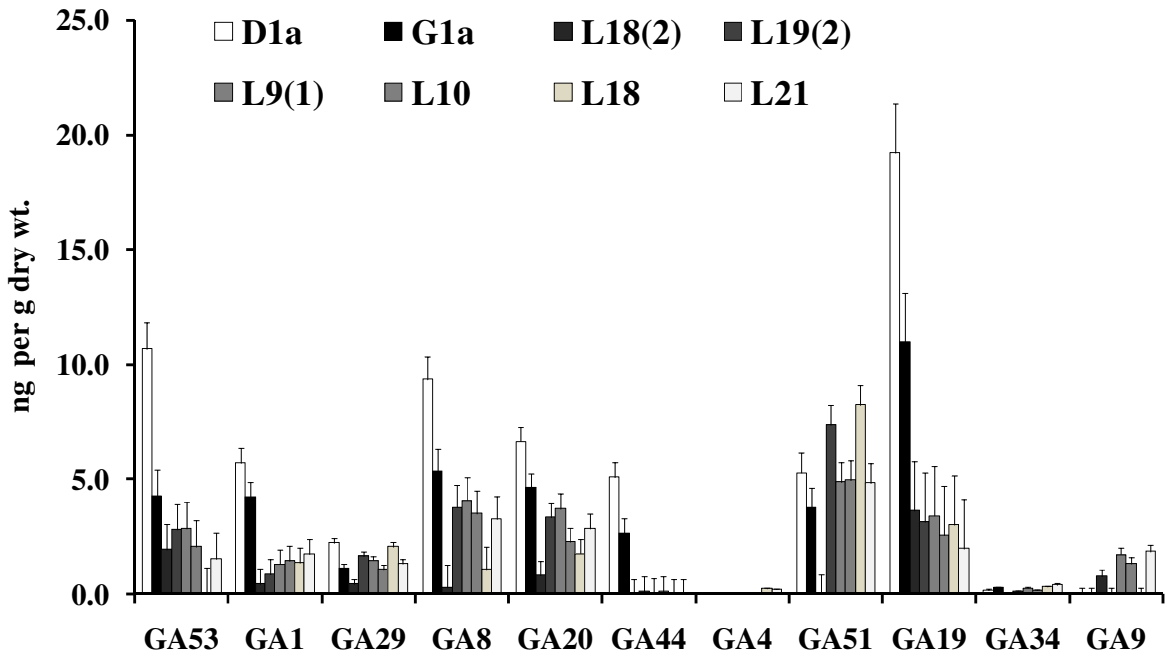


Figure 3.7 Comparison of endogenous GA levels in the GA biosynthetic pathway between dwarf plants and wild-type controls (DZ-01-196 tall phenotype and Gea Lammie short phenotype). Dwarf lines (L18(2), L19(2), L9(1), L21, L18 and L10) represent semi-dwarf phenotypes. Standard error (SE) values (bar) was determined by Student's *t*-test using GenStat Discovery Edition (VSN International Ltd).

3.4 Discussion

This is the first report about *E. tef* transformation with the aim of modifying plant stature through over-expression of a GA inactivating gene (*GA2ox*) from *Phaseolus coccineus*. *In vitro* regenerated plants were successfully grown into seed producing mature fertile plants. For Agrobacterium-mediated transformation, a combination of different media has been successfully applied for embryogenic callus induction, Agrobacterium inoculation and co-cultivation and plant regeneration. These media have been previously used for plant regeneration from immature embryos in barley and rice as well as in *E. tef* (Hensel and Kumlehn, 2004; Gugssa, 2008; Ramana Rao and Narasimha Rao, 2007). However, in contrast to previous reports about *E. tef* transformation either expressing the Gfp protein, which either resulted in a single transformed *E. tef* plant (Gugssa, 2008), or integrating the *gus* gene into callus tissue (Mengiste, 1991), 8 putative transformed plants carrying the insert (*PcGA20 ox* or *nptII* gene sequence) at the T_0 generation were regenerated in this study.

Success in embryogenic callus induction using zygotic immature embryos as explants and regeneration into shoots was dependant on the age (size) of the embryos. Older immature embryos developed callus later with limited differentiation, very small embryos died on the callus-inducing medium. Intermediate-sized immature embryos successfully developed into embryogenic callus and regenerated ultimately into a fertile seed-setting plant. Some of the *in vitro* regenerated shoots lost, however, their green pigment during growth and died during the selection/regeneration stage. The reason for this is still unclear and requires further investigation. Also, anti-necrotic compounds, such as ascorbic acid, cysteine, and silver nitrate in co-cultivation and subsequent culture media, might be applied in future work to fine-tune the transformation process.

High natural kanamycin resistance of *E. tef* callus was also found in this study. Such natural antibiotic resistance of callus has been already reported for *E. tef* (Mengiste, 1991) as well as for rice (Twyman *et al*., 2002). To overcome natural resistance against kanamycin, a more potent kanamycin derivative, geneticin (G418), was used in this study. This antibiotic completely controlled shoot regeneration from mature *E. tef* embryos at 75 mg l^{-1}. Gugssa (2008) previously found no inhibition of *E. tef* callus induction and somatic embryo formation from immature embryos with 50 mg l^{-1} G418. However, the amount required for complete killing of untransformed callus was not reported. A future study might, therefore, investigate if selection using G418 and others such as glyphosate or hygromycin resistance would be a more powerful selection system for transformed *E. tef*.

Agrobactrium growth during inoculated callus development was suppressed using 250 mg l^{-1} cefotaxime, but Agrobacterium growth was sometimes not completely blocked. A higher cefotaxime concentration (500 mg l^{-1}), in addition to better suppression of bacterial growth, has been reported inducing embryogenesis in rice and sugarcane (Mittal *et al*., 2009) whereas Ratnayake *et al*. (2010) recently found inhibition of rice embryogenesis with amounts higher than 500 mg l^{-1}. Therefore, a future study might also determine the optimum cefotaxime amount suitable for *E. tef* transformation.

Plant height significantly varied among the semi-dwarf plants and maximal reduction in Culm height was 56% when compared to the height of control plants. Reduction was across internodes except for the most dwarfed plants. In these plants, there was no elongation of the upper most three internodes. Results are in agreement with earlier observations modifying height controlling GA genes (Lo *et al*., 2008; Hedden 1999). In this study, reduction in plant

height in the semi-dwarf *E. tef* plants was also associated with reduced amounts of bioactive GA_1, a metabolite of 13β-hydroxylation and a dominant GA biosynthesis product in *E. tef*. However, accumulation of GA_8, a deactivation product of the bioactive GA_1, in the semi-dwarf plants was not proportional to the relative height differences or to deactivation of GA_1.

In rice, GA deactivation decreased height (Lo *et al*., 2008) with increasing yield (Ookawa *et al*., 2010). Results in this study also showed that yield increased in some semi-dwarf plants. This was not entirely due to an increase in the number of tillers per plant because some plants had a low total tiller number but had still a higher grain yield per plant (e.g. line 23 and 19(2) vs. 19(3)). Yield increase was also highly and positively related to shoot and panicle dry weight. Although semi-dwarf plants had generally delayed panicle initiation, shoot growth, including panicle growth, was extended due to delayed maturity with fertility of panicle and seed set not affected.

Although putatively transformed plants showed the semi-dwarfed phenotype, detection of integration of the transgene in T1 plants by PCR was inconsistent and no amplification product was found when the PCR reaction was repeated. Therefore, the final proof that plants are indeed transformed has so far failed despite several attempts repeating genomic DNA isolation and changing the PCR conditions. Currently, the possibility that any found morpho-physiological differences are due to somaclonal variations owing to the relatively higher rate of 2, 4-D applied, which is known to cause such changes, cannot not be ruled out (Banerjee *et al*., 1985). Therefore, dwarfed plants have to be further characterized to consistently show *GA2ox* transgene integration into the genome, including Southern blot analysis, and if expression of integrated *GA2ox* is always associated with reduction in plant height. However,

if somaclonal variation has caused the phenotype change, this technique might also be considered as an excellent tool to develop semi-dwarf phenotypes in *E. tef*.