Transformation of tef (*Eragrostis tef*) by *Agrobacterium* through immature embryo regeneration system for inducing semi-dwarfism

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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 (IEs) of *E. tef* cultivar DZ-01-196 were used for embryogenic callus formation and the callus was transformed with GA inactivating gene *PcGA2ox* under the control of a triple CaMV 35S promoter using *Agrobacterium* transformation procedure. Embryogenic callus was induced from immature embryos in a medium containing KBP minerals in the presence of 2,4- dichlorophenoxiyacetic acid. The embryogenic calli were further inoculated with *Agrobacterium* and the calli were grown in co-cultivation medium (CCM) followed by selection in KBP and regeneration (K4NB) media. Putatively transformed *E. tef* embryogenic calli were tolerant to treatment with the selectable marker kanamycin, while 75mg l^{-1} geneticin inhibited growth of non-transformed shoots derived from matured embryos completely after 12 days. A total of 55 plants were regenerated from all the embryogenic calli 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 amplified PCR products to have 97.2 and 99.8% sequence identity to *PcGA2ox* and *nptII*, respectively. However, detection of the transgene, *PcGA2ox* or *nptII,* in T_1 plants was inconsistent although phenotypic analysis of T_1 plants showed changes in pheno-morphic and agronomic characters such as plant height, number of internodes, tillering, panicle length, biomass, yield as well as GA content. 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. GA analysis showed plants with semi-dwarf phenotype to be associated with a low level of bioactive GA_1 and its immediate precursors. Up to 3.7 fold increase in grain yield per plant was found in some semi-dwarfed plants.

1.   Introduction

Inducing dwarfism in the small grain cereal tef, whose productivity is severly affected due to sensitiveity to lodging owing to a tall slender stem and plant height, has been a prime target for its genetic imrpovement. Conventional tef breeding has so far failed to improve lodging resistance (Teklu and Teferea, 2005; Ketema, 1983)   while in other cereals induction of dwarfism through interfering with gibberellins (GA) metabolic reactions has shown a great success (Sakamoto et al. 2003; Hedden and Phillips, 2000).

With the view to improve the low grain productivity in one of the least studied cereal crops, *E. Tef*, that occurs due to sever lodging problem, a previous study using GA inhibitors (Gebre et al.

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2012) have shown the highly responsiveness of the crop to GA inhibition. This implied that GA biosynthesis or signalling could be a prime target to modify plant height using a transgenic approach. However, the effect of a constitutive gene expression for GA inhibition on morphological and yield parameters using genetic transformed *E. tef* plants modified in their GA biosynthesis/signalling pathway is unknown. On the other hand transfomration itself in *E. tef* has been at optimization stage so far where instances of attempting transient expression of the reporter genes GUS by Ketema (1997) and Mengiste (1991), and Green florescent protein (Gfp) expression and stable transformed plant regeneration by Gugsa (2005) have been indicated.

It is also known that *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, and 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. An existing preliminary transformation protocol was used with some modification to transform *E. tef* over expressing the GA inactivation gene (*GA2 ox*) in the biosynthesis pathway. *In vitro* regeneration protocols for *E. tef* have been

reported by several research groups to regenerate plants from various explants sources such as roots, leaf bases, seeds and recently using immature embryos (Bekele et al. 1995; Mekbib et al. 1997; Assefa et al. 1998; Gugsa and Kumlehn, 2011). Gugsa et al. (2006) reported also regeneration of haploid plants using gynogenic tissue of *E. tef*. Regeneration of transformed plants, on the other hand, has so far only been obtained by Gugsa (2005) expressing the Green florescent protein (Gfp). However, Mengiste (1991) and Mekbib et al. (1997) also attempted to develop a transformation protocols for tef using reporter gene.

The objectives of this study were therefore further optimization of the transformation and regeneration procedures to transform the gene of interest based on protocols developed for tef and other closely related cereals such as barley and rice. Our gene of interest was the transgene, *PcGA2ox,* which is a GA inactivating gene involved in plant height control for the purpose of developing semi-dwarf phenotype for the purpose of lodging resistance improvement. The method of transformation of IEs and production of transformed embryogeneic callus was applied to regenerate transformed shoots that develop into fertile plants. Regenerated putatively transformed *E. tef* plants were further characterized for yield component traits as plant height, tillering capacity, stem diameter, panicle length, physiological (biomass and yield), stem diameter and biochemical characteristics (GA content).

2. Materials and Methods

2.1 Preparation of plant material and culture

Seeds of tef (cv. DZ-01-196) were obtained from the Ethiopian Institute of Agricultural Research (EIAR). The seeds were germinated on commercially available germination soil mix suitable for fine seed germination. Then the seedlings were transplanted in pots containing the same soil mix as above and grown 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 development of immature zygotic embryos (IEs). Immature embryos (IEs) were harvested from 17±2 days old developing panicle after its emergence.

2.2 Immature embryo isolation, callus induction and shoot growth

Immature seeds were collected from middle spikelets 7 to14 days post anthesis and IEs (Fig. 1a) were isolated and visually assorted into small, medium and large size groups using a binocular microscope and embryonic calli were induced according to the protocol reported by Gugsa and Kumlehn (2011). Freshly detached spikelets were used for immediate isolation and disinfection of the immature embryo. For sterilization, the intact spikelet was cut short to 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 drops of savlon for 12 min shaking (modified from Gugsa and Kumlehn (2011) and O'Kennedy et al. 2004), which was followed by a 4 – 5 -times rinse in ddH$_2$O. The IEs were

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isolated aseptically with forceps under sterile conditions and were placed, scutellum side-up, on Petri-dishes containing KBP EM embryogenic callus induction (CI) medium (Kumlehen et al. 2006). Embryonic calli induced from the cultured IEs were used for *Agrobacterium* -mediated transformation. Proliferating embryogenic calli (Fig. 1b and 1c) were transferred to fresh CI medium every second week. Callus inoculation and co-cultivation with *Agrobacterium* was carried out following various protocols (Gugsa, 2005; Hensel and Kumlen, 2004; Rao et al. 2007; O'Kennedy et al. 2004). Infection with *Agrobacterium* for transformation was done using 3 weeks old young calli (Fig1 b and c).

2.3 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. 2). The full construct containing the transgene, promoter and Kanamycin resistance was provided by 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. 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 $28^{o}C$ 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.

2.4 Agrobacterium culture, inoculation and co-cultivation

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 $28^{o}C$ 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 2) (Hensel and Kumlehn, 2004). The virulence activator acetosyringone was added immediately before inoculation. The cultures were stirred at 50 rpm for an hour before infection. After infection for 6 h, calli were blotted onto sterile tissue paper and briefly rinsed with liquid CCM. Washed calli were then co-cultivated by growing on KBP embryogenic (KBPEM) callus induction medium (CIM) for 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. The surviving calli were then transferred to a selection medium (Table 1). For the control, embryogenic calli were kept uninfected with *Agrobacterium* but were subject to all post infection treatments excluding antibiotic treatment.

After co-cultivation, infected calli were collected by placing them into a sterile petri dish and they were washed with liquid CCM containing cefotaxime (250 μg ml^{-1}) for 20 min to suppress further *Agrobacterium* growth.Washed and blotted calli were then transferred to CI - SL media (Table 1) supplemented with 2.17 mg l^{-1} 2,4-D, 250 mg l^{-1} cefotaxime (or 250 mg l^{-1} timetin) and 200 mg l^{-1} kanamycin and then cultured for 3 weeks. After this time, calli were transferred to a CI - SL medium supplemented with 2.17 mg l^{-1} 2,4-D, 250 mg l^{-1} cefotaxime (or 200 mg l^{-1} timetin) and 200 mg l^{-1} kanamycin before plant regeneration. All culturing procedure until the regeneration stage was done using culture plates (50 mm x 10 mm) which were kept in dark at 24 ± 2^0C. Developing prolific embryonic calli that were transferred to regeneration medium (K4NB) were grown under a 16 h photoperiod maintaining a temperature of 24 ± 2^0C.

2.5 Plant regeneration

After selection, calli that were still creamy-white were transferred to pre-regeneration K4NB medium (Table 1) without 2, 4-D and addition of a reduced concentration of cefotaxime (125 mg l^{-1}). After two rounds of selection on this medium, calli were transferred to a regeneration medium (K4NB) for 6 to 8 weeks depending on growth of the regenerated shoot, which was refreshed after every 3 weeks (with no antibiotics added). By this time regenerated shoots have

grown roots without any addition of rooting hormone in the regeneration medium. 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 and subsequent growth to maturity.

2.6 Handling transformed planlets regeneration of T1 plants

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 $\mu molm^{-2}s^{-1}$. 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)] at the same controlled environment. A soil mixture consisting of peat (75%), sterilized loam (12%), vermiculite (3%) and grit (10%) was used supplemented with a slow release fertilizer (3.5 kg Osmocote m^{-3} with 15-11-13 NPK granular fertilizer plus micronutrients).

2.7 DNA isolation and PCR screening of the 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 non-transformed control plants that passed through the whole process except exposure to 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).

2.8 Phenotypic characterization of T1 generation

T_1 generation transformed plants were grown from seeds after selfing putative transformed plants (T_0 generation) that have shown *GA 2ox1* 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 such as panicle length and weight. Dry weight for above ground biomass was determined by drying fresh material at 80^oC 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 (VSN International Ltd. Release 4.23DE, UK).

2.9 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. Additional sample also was taken from a known short stature (dwarf) landrace variety, Gea Lammie, that was not transformed but only for the purpose of comparison. The upper-most two internodes including its nodes were cut and weighed and stored at $^-80^0C$ until analysis. The upper most two internodes including their nodes at shoot elongation stages and just before heading were harvested and stored at $^-80^0C$ until analysis. Endogenous GA levels were monitored using stored internodal tissue after samples were freeze-dried and grinded using a ball mill for extraction, purification, and analysis of GAs. Powdered replicate samples of about 0.5g were re-suspended in 80% aqueous MeOH with addition of mixture of 2H- and 3H-labeled GA internal standards. The aqueous extract was then subjected to a rotation vacuum evaporator at $40\text{-}45^0C$ to remove methanol. The pH of the aqueous extract was adjusted to 3.0 using 1 mol/l HCl before further partitioning three-times with water-saturated ethyl acetate. The combined organic phases were reduced to dryness under vacuum at 42^0C to remove ethyl acetate. After column purification and

full methylation with ethereal diazomethane, samples dissolved in methanol were injected onto an analytical C18 reversed phase high performance liquid chromatography (HPLC) column for fractionation with online radioactivity monitoring. Recovery of fractions was monitored using tritiated (3H) internal standards and GAs were quantified using gas chromatography- mass spectrometry (GC-MS) system (Hewlett Packard 5890 Gas Chromatograph with a Hewlett Packard 5970 Series Mass Selective Detector (MSD) (now Agilent, S. Queensferry, West Lothian, UK) using selective ion monitoring.

3. Results

3.1 Plant transformation

Agrobacterium (strain LBA4404)-mediated transformation was carried out using the embryogenic callus from the scutellum region of *E. tef* IEs. The immature embryo produced embryogenic callus (Fig 1b and c) from the scutellum side within 2 weeks of culturing on embryogenic callus induction medium and further proliferation of the callus was promising on the same medium (Figs. 1a). In some cases, when embryos were small, embryonic calli already appeared within a week while in intermediate ones only after 2 weeks. This result is in agreement with Gugsa and Kumlehn (2011). Calli that were formed late were not used for *Agrobacterium* transformation.

In the mean time, the antibiotic-containing selection medium (CL-SL) (Table 1) was optimized for selecting kanamycin-resistant using germinating mature embryos (Table 2). At 25 mg l^{-1}

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G418, up to 75% of shoots derived from non-transformed embryos wilted after 12 days exposure to the antibiotic without complete collapse. The shoots also turned yellow at the 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 1).

Immature embryos developed in to embryogenic callus (Figs. 1a and b) and further proliferated into shoots after 2 months of culturing on K4NB regeneration medium (Fig. 1c). Regenerated green plantlets were made to root in the same regeneration medium without adding hormone for root formation in agreement with Gugsa and Kumlehn (2011). Plantlets were hardened-off and grown to maturity in an environmentally controlled phytotron. A total of 55 plants were regenerated from all IE cultures to fully viable plants setting seeds at maturity (Fig 1g). 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 phytotoron 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 producing seeds.

3.2 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). Sequence analysis of the amplified PCR product confirmed that

amplified products using two sets of *PcGA2ox* primers with sizes 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.

3.3 Phenotypic characterization

3.3.1   Culm, internode and panicle length

Despite inconsistent PCR results, significant (P<0.001) variation in mean culm height was found amongT_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 (culm + panicle) of 157 cm (Fig. 4, 5 and 6). *In vitro* regenerated plants of line number 18 had the shortest culm length (65 cm) followed by plants of line 23 (88 cm). Line 18 (1) showed enternode elongation at a later stage than shown in Fig. 4, since heading was much later than the control. In plants with a dwarf or semi-dwarf phenotype, major reduction in plant height was 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. In some dwarf plants elongation of the 7th and/or 8th 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 semi-dwarf plants and wild-type control plants. Generally, internode diameter increased up to the 3rd internode when overall mean values were compared (data not shown). However, control plants had a small and steady increase in diameter up to the 6th 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 plant (67 cm). Panicle elongation was not associated with any change in plant height but somewhat negatively correlated with tiller number (data not shown). Panicle emergence (heading) was delayed by a few days to 2 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 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.

Up to four-fold increase in grain yield per plant was also found in some semi-dwarf plants (Figure 6). Higher biomass was found in the majority of semi-dwarf plants when compared to control plants (Table 5). Further, most of above-ground shoot weight increase was due to more tillering (Table 5).

3.4 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 7). The content of the most abundant bioactive GA form, GA_1, in semi-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} and GA_{20}, in particular GA_{19}, were also reduced in these plants (Figure 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) except in some cases like GA_{29} and GA_{51} in putative transformed plants was much lower than in Gea Lammie.

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 the induction (KBP) and regeneration (K4NB) media have been successfully applied for embryogenic callus induction, *Agrobacterium* inoculation, co-cultivation and plant regeneration. These media have been previously used for plant regeneration from immature embryos in barley, rice as well as in *E. tef*

using the same cultivar (Hensel and Kumlehn, 2004; Rao and Rao, 2007; Gugsa and Kumlehn 2011). However, in contrast to previous reports about *E. tef* transformation either for expressing the Gfp protein, which resulted in a single transformed *E. tef* plant (Gugsa, 2005), or integrating the *gus* gene into callus tissue (Mengiste, 1991), in this study 8 putative transformed plants carrying the insert of *PcGA20 ox* or *nptII* gene sequence at the T_0 generation were regenerated.

Success in embryogenic callus induction using zygotic immature embryos as explants and regeneration into shoots was dependant on the age (size) of the embryos confirming to previous report (Gugsa and Kumlehn 2011). Older immature embryos developed callus later with limited differentiation, whereas 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. The reason for failure to induce callus in some IEs 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 DZ-01-196 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. This antibiotic completely controlled shoot regeneration from mature *E. tef* embryos at 75 mg l^{-1}. Gugsa (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 screening system for transformed *E. tef*. However, Gugsa 2005 reported hygromicine at low concentration was more effective than biolaphos in the Agrobacterium transformation protocol development for *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} of cefotaxime. 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 in control plants. Reduction was across internodes except for the most dwarfed culms. 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 et al. 1999). In this study, reduction in plant height in the semi-dwarf regenerated plants was also associated with reduced amounts of bioactive GA_1, a metabolite of 13β-hydroxylation and a dominant GA biosynthesis product in 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 mostly due to increase in number of 2^0 tillers per plant. However, 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), it shows yield increase was not always associated with increase in number of 2^0 tillers. Yield increase was also highly and positively related to shoot and panicle dry weight. Semi-dwarf plants had generally delayed heading up to 2 weeks, shoot growth, including panicle growth in turn delayed maturity but without fertility of panicle and seed set being 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.

5. Conclusion

Lodging is a primary bottle neck problem of tef productivity and attempts to produce lodging resistant varieties so far through conventional breeding including mutation breeding has failed.

Therefore genetic transformation using semi-dwarf genes from the cultivated species is the modern approach. A workable transformation procedure for tef genetic improvement is still lucking. Our study shows success in inducing embryogenic callus using IEs in *E. tef* and regeneration potential into shoots after transformation. This regeneration potential of IEs through *Agrobacterium* based transformation protocol applied here appears to be a promising procedure for genetic improvement in tef for modification of plant stature or any other agronomically useful trait. Here the use of *A. tumefaciens* strain LBA4404 to transfer GA inactivating transgene (*PcGA2ox*) in to tef is described and show molecular evidence at primary transgenic stage but with inconsistent results at T_1 progeny. The results also showed produced semi-dwarf plants with reduced level of endogenous bioactive GA, increased tillering and delayed heading indicating the promise of the technique to bring significant impact on lodging resistance improvement in tef.

6. Acknowledgement

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Table 1. Media used for induction of embryogenic callus, co-cultivation, selection and regeneration of transformed *E. tef* shoots.

KBPEM* based embryogenic callus induction medium	
KBPEM	Modified MS salts and Organics* 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
KBPEM 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; 250 mg cefotaxime and 200 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; 50mg G418; 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

*, **(Kumlehn et al., 2006); CIM = callus induction medium is equivalent medium to KBP (Kumlehen et al., 2006); CCM = co-cultivation medium + antibiotics (Hensel and Kumlehn, 2004); CI-SL = callus induction and selection medium (KBP + antibiotics); PRE-RE = pre-regeneration medium; RE= regeneration medium.

Table 2. 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	
Mean	46.11	
SE	17.07	
p	0.027	

Table 3. Ground biomass (gm) of putatively transformed dwarf DZ-01-196 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). (*** =$P<0.001$).

Figure 1. Plant regeneration of tef using immature embryo (a) of cv. DZ-01-196 as explants. Embryogenic callus deriving from immature zygotic embryos two weeks after culture (b); proliferation of embryogenic callus (c), shoots proliferating on embryogenic callus after 2 months of culture on embryogenic callus induction medium (d) followed by two months of culture on K4NB regeneration medium (e); rooting of shoots on same medium (f) and fertile regenerated T_o plants (g).

Figure 2. 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.

Figure 3. PCR amplification of *PcGA2 ox* (A and C) and *PcGA2ox* and *nptII* (B); DNA amplifications from putative transformed plants of T_0 (A & B) and T_1 (C) generations. (M) 1kb and 100 bp ladder molecular size markers for (A & B) and (C), respectively; (NC) negative control without template DNA added in a reaction mix; (PC) a positive control with plasmid *pGPTV-kan.* ([a]) Events 1-9 in A represent PCR result of putative transformants (T_0) for the insert *PcGA2ox* with positive PCR results. ([b,c]) Same events in B as in A (eight samples excluding one event) show amplification with a different set of primer for *PcGA2ox* and *nptII* genes. In C, Lines with asterisk (*) are those selected among semi-dwarf phenotypes at T_1 and used for further phenotypic analysis (Figures 4-6).

Figure 4. Morphological attributes such as culm and panicle length (cm) (Y1) and number of tillers per plant (Y2) of putatively transformed *E. tef* plants showing a semi-dwarf phenotype, which include selected lines (including lines indicated with asterisk in Fig. 3C) Lines 19(2), 19(1), 4, 19(3), 10, 21, 25, 28, 23, 18(1) at T1. 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 5. Selected *E. tef* representative lines 18(1), (19(3), 19(2), 28 and 10 plants at T_1 generation showing dwarf or semi-dwarf phenotypes. Heading is advanced in the control plant compared with the rest semi-dwarf lines which are at panicle emergence (Lines 19(3) and 19(2)) or about to begin (Line10) or late to head (Line 18(1)). Control is a plant subjected to the transformation process without addition of *Agrobacterium* and antibiotic selection.

Figure 6. Seed weight of primary and secondary panicles of putatively transformed semi-dwarf *E. tef* lines (Lines 4, 10, 18(1), 18(2), 19(1), 19(2), 19(3), 21, 23, 25 and 28) and a wild type control (DZ-01-196). 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 7. Comparison of endogenous GA levels in the GA biosynthetic pathway between semi-dwarf plants (Lines 18(2), 19(2), 9(1), 21, 18 and 10) and wild-type controls (D1a = DZ-01-196, a tall phenotype and G1a = Gean Lammie, a short phenotype). Semi-dwarf lines represent semi-dwarf phenotypes. Standard error (SE) values (bar) were determined by Student's *t*-test using GenStat Discovery Edition (VSN International Ltd).
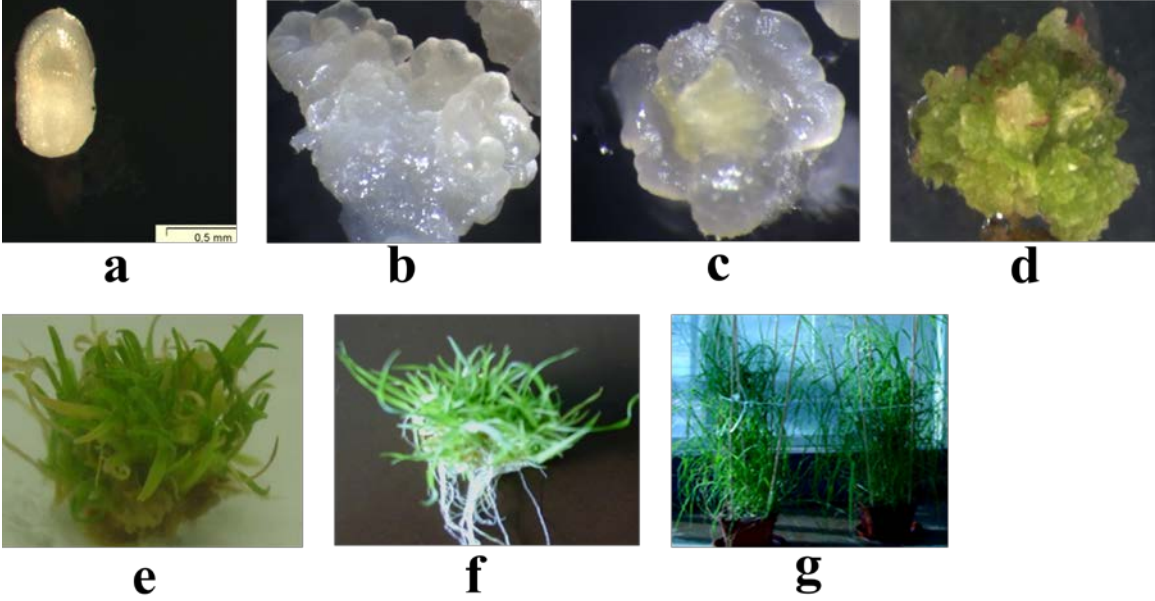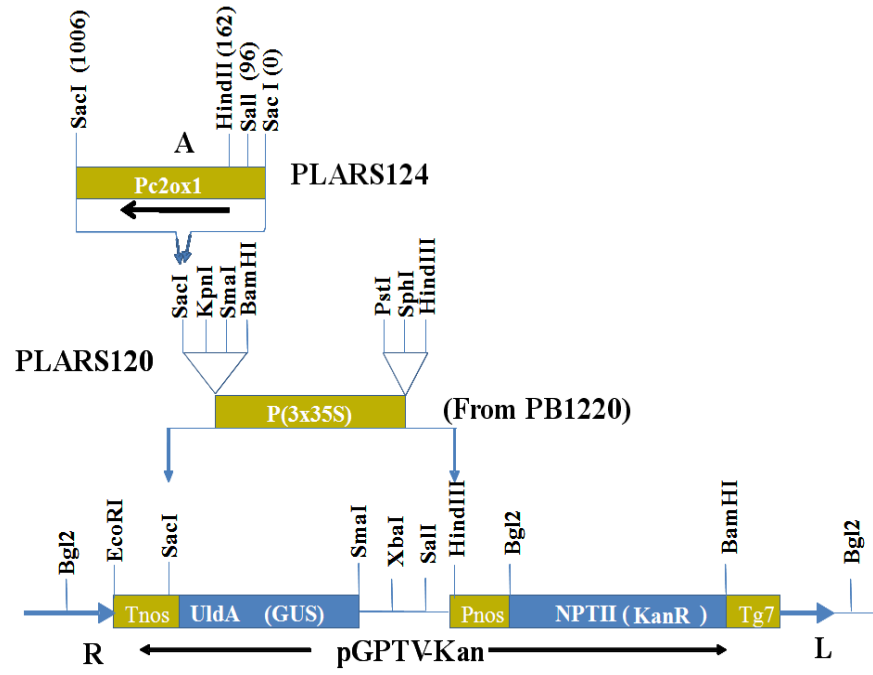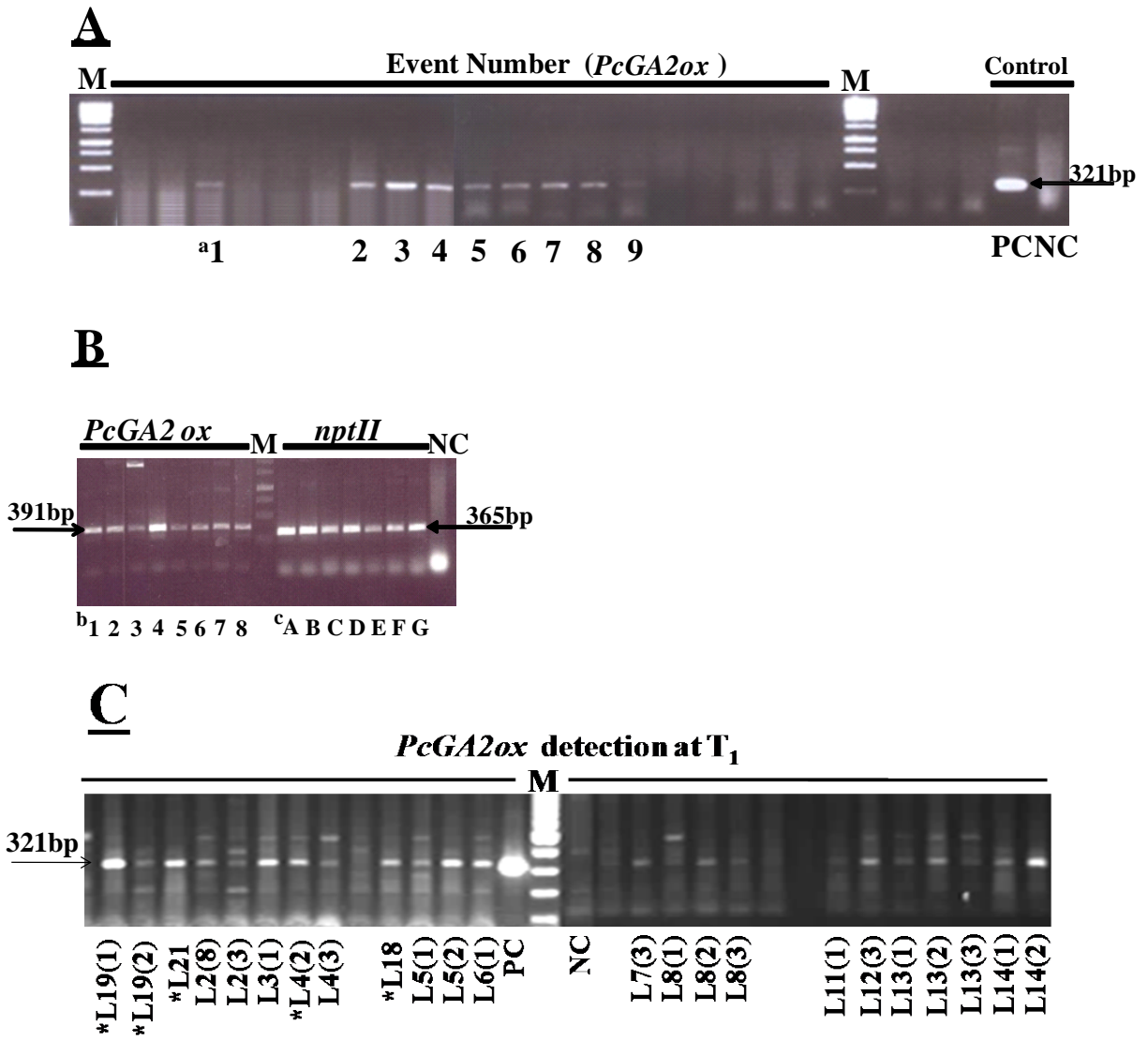
Figure 1

a b c d

e f g

Figure 2

Figure 3

A

M — Event Number (*PcGA2ox*) — M — Control

321bp

ᵃ1   2 3 4 5 6 7 8 9   PCNC

B

PcGA2ox M *nptII* NC

391bp 365bp

ᵇ1 2 3 4 5 6 7 8 ᶜA B C D E F G

C

PcGA2ox detection at T₁

M

321bp

*L19(1) *L19(2) *L21 L2(8) L2(3) L3(1) *L4(2) L4(3) *L18 L5(1) L5(2) L6(1) PC NC L7(3) L8(1) L8(2) L8(3) L11(1) L12(3) L13(1) L13(2) L13(3) L14(1) L14(2)
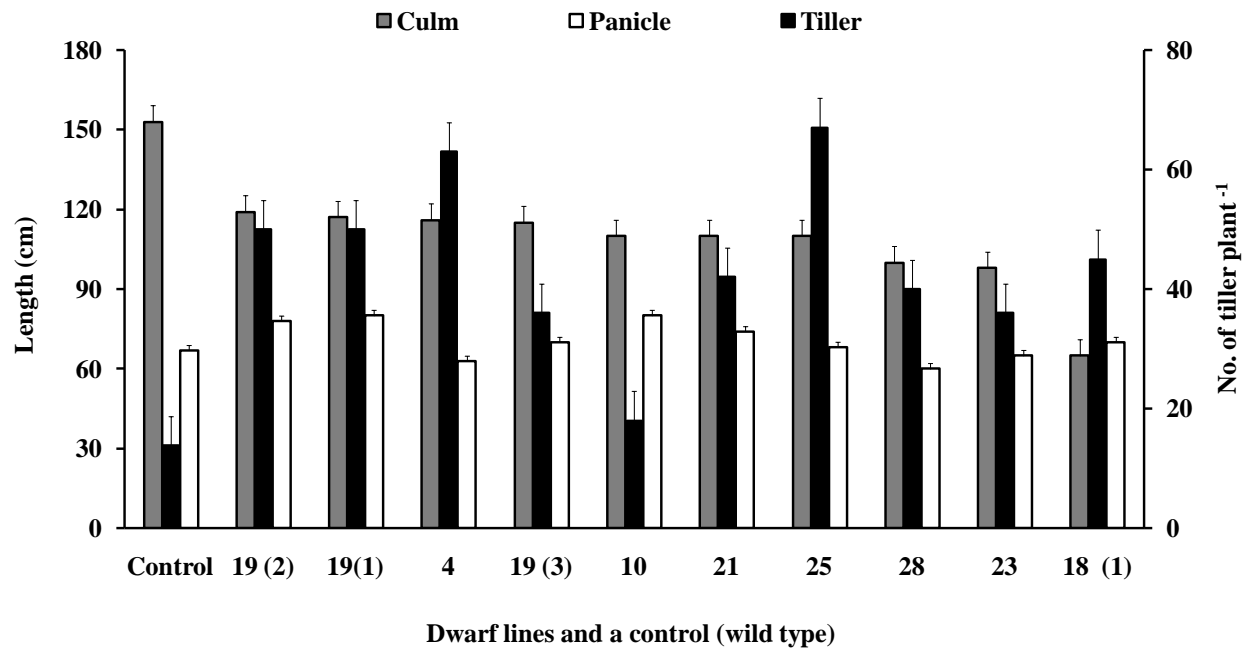
Figure 4

Figure 5

Control   19(3)   19(2)   28   10   18(1)

Figure 6

Figure 7

Analyzed intermediates and bioactive GAs