

**Manipulation of gibberellin biosynthesis for the control of plant  
height in *Eragrostis tef* for lodging resistance**

By

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Thesis submitted in partial fulfilment of the requirements for the degree

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May 2012

## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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## ABSTRACT

### **Manipulation of gibberellin biosynthesis for the control of plant height in *Eragrostis tef* for lodging resistance**

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Lodging is a key agronomic problem in *E. tef*. due to morpho-physiological features, such tall and slender phenotype of the plant. Gibberellins metabolic genes are key targets in the control of plant height. Plant growth regulators (PGRs) that inhibit GA biosynthesis are used to shorten stem length thereby increasing lodging resistance. *E. tef* responded to treatment with PGRs such as GA, chlormequat chloride (CCC) and paclobutrazol (PBZ). Both PGRs reduced *E. tef* plant height but CCC treatment did not affect grain yield. Stem diameter was not affected by PGR treatment and also not the poor tapering (acropitally increasing diameter).

Putatively transformed *E. tef* plants carrying a bean GA 2-oxidase (*PcGA2ox*) coding sequence were further produced via embryogenic callus after *Agrobacterium*-mediated transformation and plants were successfully grown into mature fertile plants. Eight putative transformed plants were finally generated carrying the insert (*PcGA20 ox* or *nptIII* gene

sequence) at the T<sub>0</sub> generation. Constitutive expression of the GA 2-oxidase (*PcGA2ox*) coding sequence in *E. tef* resulted in phenotypic changes such as reduction in culm height, change in biomass, reduction in amount of GA in putative transformant semi-dwarf plants. The challenges found in the transgene detection in the T1 generation has been highlighted. Pheno-morphic changes occurred with little or no effect on yield.

Genes involved in height control (orthologs to the rice *sd-1* gene) and signaling (*Rht*) in *E. tef* were also identified and characterized. Activity of the protein for the putative rice *sd-1* orthologs was further confirmed by heterologous expression. The three putative sequences in *E. tef* were named *EtGA20ox1a*, *EtGA20ox1b* and *EtGA20ox2*. Expression analysis showed that *EtGA20ox2* were much less transcribed compared to the others and *EtGA20ox1b* could be the functional equivalent to the rice *sd-1* (*OsGA20ox2*) gene in *E. tef*.

Further, *E. tef* mutants with a semi-dwarf phenotype could be developed through mutagenesis and TILLING. However, regardless of height, grain yield was severely reduced in all mutants except in the semi-dwarf mutant GA-10. This line also had significantly higher diameter in most internodes which might contribute to the stiffness of stem. G-10 is therefore a promising line for further investigations.

## Thesis composition

**Chapter 1** of this thesis provides a summary of the lodging problem in cereals and alternative methods (chemical and genetic approaches) used to control lodging as well as the traits involved. An up-to-date review of the lodging problem in *E. tef* including phenomorphic features relating to lodging and experiences in other crops as well as in the “green revolution” are outlined. Approaches solving the lodging problem and genes that play a key role in plant architecture modification in cereal crops for improving lodging resistance are discussed. The rationale, aim, and objectives for carrying out this study are further outlined at the end of the introduction. In **Chapter 2**, results obtained from treatment of *E. tef* plants with GA biosynthesis inhibitors in controlling plant height are presented. This includes treatment with GA<sub>3</sub>, CCC and Paclobutrazol and changes in plant height and other phenomorphic and agronomical features due to PGR treatment are reported. **Chapter 3** reports about transforming *E. tef* plants using immature somatic embryos via embryogenic callus for *Agrobacterium*-mediated transformation. Successful regeneration of putative transformed plants after a transformation procedure using combinations of different media is outlined. Moreover, characterization of plants over-expressing GA2 oxidase from *Phaseolus coccineus* (*PcGA2ox1*) for inducing dwarfism is presented and results of characterizing putatively transformed T0 generation plants regarding their morpho-physiological features and expression of a semi-dwarf phenotype with reduced height are reported. The inconsistent PCR results at T1 and the possibility that any found differences could also be due to somaclonal variations owing to the relatively higher rate of auxin applied is indicated. **Chapter 4** outlines the identification, and characterization of height-controlling genes. This includes the rice homologous *SD-1* in *E. tef*, the wheat *Rht* orthologue and two Cytochrome P450 monooxygenase genes (*Eui* and Brassinosteroid deactivation genes). Also an activity

assay through heterologous expression of *EtGA20ox1* in *E. coli* and specific tissue expression of the three *EtGA20ox* homologs as well as copy number of these genes in the *E. tef* genome are presented. In **Chapter 5**, data on phenotype (plant stature) characterization is outlined for selected mutant *E. tef* lines developed through mutagenesis and TILLING to generate sufficient variability for semi-dwarfism in *E. tef* for lodging resistance. Morphological and physiological attributes and agronomic relevance of these mutant lines are described in terms of plant height reduction, tillering, biomass and yield. **Chapter 6** finally summarizes the findings and relevant information developed in this PhD study. It also outlines the salient features that need to be considered further in a lodging-resistant *E. tef* ideotype. This is followed by the list of citations (References) used in this dissertation. The **Appendix** provides further sequence results (nucleotides and translated amino acid) from the gene cloning and characterization study, alignment and phylogenetic analysis of *E. tef* sequences with different species.

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## DEDICATION

I dedicate this thesis to my wife Meseret Worku, whose understanding, love, kindness and help enabled me to pursue the work to the end; and to my parents, brothers and sisters who occupy such a special place in my heart for their love and inspiration uplifting my spirit through their prayers.

## ABBREVIATIONS AND SYMBOLS

%	Percentage
µg	Microgram
µL	Microlitre
2-ODD	2-Oxoglutarate dependent dioxygenase
bp	Base pair
BSA	Bovine serum albumin
CCC	Chlormequate chloride
CCM	Co-cultivation medium
CaMV	Cauliflower mosaic virus
cDNA	complementary deoxyribonucleic acid
cDNA	Complimentary DNA
CPS	<i>ent</i> -copalyl diphosphate synthase
Ct	Cycle threshold
CTAB	hexadecyl-trimethyl-azanium bromide
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxy nucleotide triphosphate
DZ	Debre Zeit
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra acetic acid
EMS	ethylmethanesulphonate
ER	Endoplasmic reticulum
EUI	elongated uppermost internode
g	Grams
GA <sub>n</sub>	Gibberellin A <sub>n</sub>
GA-ox	Gibberellin -oxidase
GA 2-ox	Gibberellin 2-oxidase
GAI	Gibberellin insensitive
gDNA	genomic deoxyribonucleic acid
GGPP	geranyl-geranyl diphosphate
GID	Gibberellin insensitive dwarf
GUS	β-Glucuronidase
h	hours
H <sub>2</sub> O	Water
IE	Immature embryo
IPTG	Isopropyl-β -d-thiogalactopyranoside
KAO	<i>ent</i> -kaurenoic acid oxidase
KO	<i>ent</i> -kaurene oxidase
KS	<i>ent</i> -kaurene synthase
L	Litre
LB	Luria broth
LCM	Laser capture microdissection

M	Molar
min	minute
mL	Millilitres
mM	Millimolar
NaAC	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NTC	No-target control
°C	Degree Celcius
ORF	Open reading frame
PBZ	Paclbutrazol
PcGA2ox	<i>Phaseolous coccinuous</i> GA 2-oxidase
PCR	Polymerase chain reaction
PPFR	Photosynthetic photonflux rate
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RACE	Random Amplified cDNA Ends
RGA	repressor of <i>gal-3</i>
RHT	Reduced height
RNA	Ribonucleic acid
RNAase	ribonuclease
rpm	Revolutions per minutes
s	Second
sd H <sub>2</sub> O	Sterile distilled water
SD1	semi-dwarf 1
SDS	Sodium dodecyl sulphate
SLN	Slender
SNP	Single Nucleotide Polymorphism
TILLING	Targeting induced local lesions in genomes
UV	ultra violet
v/v	volume per unit volume
w/v	weight per unit volume
wk	week (s)

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# **CHAPTER 1**

## **INTRODUCTION**

## 1.1 The problem of lodging

Lodging, as a mechanical plant stress, is a complex interaction of the plant with the environment including factors such as light, wind, temperature, rain, topography, soil type, nutrition, plant density and diseases (Berry *et al.*, 2004) and is frequently associated with plant pheno-morphic characteristics (Pinthus, 1973). It is a process by which the shoot of a small grained cereal is displaced from their vertical stance. The standing strength of the stem, or the root, to hold up the shoot leverage is threatened by adverse weather conditions. Lodging is also variety (genotype)-dependent and a tall, weak-stemmed variety is more prone to lodge than a semi-dwarf variety with stiffer stem. It occurs in the form of stem lodging or failure of the anchorage system (root lodging) (Pinthus, 1973; Thomas, 1982; Crook and Ennos, 1993; Graham, 1983 and Delden *et al.*, 2010).

Lodging, causes a direct yield loss due to falling-over of the plant and also an indirect loss due to limiting optimum use of nitrogen fertilizer. Problem of reduced lignifications of the stem also reduces stem strength following high nitrogen fertilizer use, which is counter-productive, and any increase in grain biomass is offset by increased lodging (Crook & Ennos 1994). Lodging also restricts mechanical seed harvesting and reduces seed quality. Furthermore lodging can also limit efficient light interception; reduce translocation and assimilation, increase respiration and chlorosis thus affecting growth and development processes in the plant system (Berry *et al.*, 2004). Because of the significance in the global agricultural economy, wheat and rice were the two main field crops in which it was desirable to substantially reduce significant yield losses due to lodging of tall, wild-type genotypes. Applying nitrogen fertilizer was not successful, since it aggravated the lodging problem. Shorter, sturdier, semi-dwarf varieties were developed that have been far more resistant to

lodging and also exhibited an unexpected benefit of improved assimilation into reproductive organs due to pleiotropic effects (Hanson *et al.*, 1982). Thus an essential trait of the higher-yielding varieties was reduced (dwarf) stature that enabled large increases in yield to be obtained.

Therefore, in many cereal crops, plant height has been the main target for improvement of lodging resistance incorporating the trait in modern cultivars. For instance the semi-dwarf wheat variety from Japan (Norin 10) contains the most important dwarfing genes that confer reduced height trait used today in wheat. This variety originated from a cross between the native Japanese dwarf variety Daruma with two American wheat varieties, Fultz and Turkey Red. The genes conferring semi-dwarfing traits are for culm shortening with a semi-dominant gain-of-function mutation in the *REDUCED HEIGHT (RHT)* homologous genes in wheat (Gale and Marhsall, 1976). These genes were introduced into many other varieties grown in many parts of the world (Silverstone *et al.*, 2001) and 70% of modern wheat varieties carry at least one of the dwarfing genes (Hedden, 2003). In rice, the recessive semi-dwarf (*sd-1*) gene has been used to reduce plant height and considerably improved lodging resistance. The mutated *sd-1* in rice existed in semi-dwarf native varieties. In the 1960s the dwarfing trait was incorporated into improved rice lines to develop a semi-dwarf phenotype in rice (Ashikari *et al.*, 2002). In barley, the value of short variety was understood earlier and shorter barley varieties such as ‘Valticky’ that originated from local landraces was widely grown replacing taller varieties in Moravia at the beginning of the twentieth century (Bouma and Ohnoutka 1991).

Generally, taking in to account wheat and rice, mutations in the *Rht* and *sd-1* genes have been among the main factors responsible for higher yields obtained in the “green revolution”

(Yamaguchi, 2008; Kashiwagi and Ishimaru, 2004; Hedden, 2003). As a result the average yield in wheat has increased from 2.2 t ha<sup>-1</sup> to 6.0 t ha<sup>-1</sup>, whereas in rice yield has increased due to introduction of semi-dwarf varieties (Berry *et al.*, 2004) from 1.5 t ha<sup>-1</sup> to 4.2 t ha<sup>-1</sup>. Thus the identification and subsequent introgression of stem height controlling genes was considered the principal factors for reduced lodging allowing higher amount of fertilizer use in wheat and rice during the 70s of the last century (Tong, 2007; Kashiwagi and Ishimaru, 2004; Berry *et al.*, 2004; Hedden, 2003).

## 1.2 Plant architecture and lodging

Plants architectures refers to the degree of branching, internodal elongation, and shoot determinacy (Wang and Li, 2008). The architecture of plants is linked to plant functions including efficient water transport, light interception, soil resource acquisition, and the maintenance of a mechanically stable structure. To optimize their functions plants keep building their architecture gradually in response to changes in environmental conditions (Sterck, 2005). When plants lose its optimum architecture orientation or vertical stance required for optimal function in a given set of environment, due to mechanical failure, conditions that promote plant growth is interrupted. A favourable environment is created for diseases increasing harvesting cost and yield loss. The culm structure, such as length, diameter, shape, composition, degree of branching, leaf arrangement and orientation, internodal elongation, and shoot (determinate or not), define the type of architecture of a crop. These factors determine the specific pheno-morphic and physio-morphic features and define the specific interaction the plant may have with environmental elements such as wind, rain, soil, nutrient and light (Berry *et al.*, 2004).

Lodging occurs when the stem strength, which depends on stem diameter and the composition and width of the stem wall, is insufficient to hold the shoot up against leverage. It also occurs due to failure of the anchorage system, which depends on the spread and depth of the root plate and the strength of surrounding soil, causing the root to have insufficient strength to hold the shoot up. Stem lodging takes place in the form of “buckling” of the lower internodes or the middle internodes called “brackling” (Thomas, 1982; Neenan and Spencer-Smith, 1975), or the breaking of the peduncle known as “necking” found in wheat, barley, oats (White, 1991). It can also occur in the form of root lodging (failure of anchorage system) which results in a permanent displacement of the stem found in wheat, barley, and oats and also in *E. tef* (Pinthus, 1973; Crook and Ennos, 1993; Graham, 1983 and Delden *et al.*, 2010). Berry *et al.* (2004) have further shown that root lodging could be predominant. Reports, however, have often been in favour of stem (straw) lodging indicating that misconception can occur about the reason of failure in lodged crops. In *E. tef* grown in sandy soils, root anchorage failure and insufficient stem strength have been found (Delden *et al.*, 2010). However, earlier studies with *E. tef* have shown that stem length thickness or diameter of basal internodes, panicle length and weight, and earliness to be important traits for lodging resistance (Berhe, 1981; Ketema, 1983; Mengesha *et al.* 1965; Hundera *et al.*, 1999; Asefa *et al.*, 2000 and Yu *et al.*, 2007).

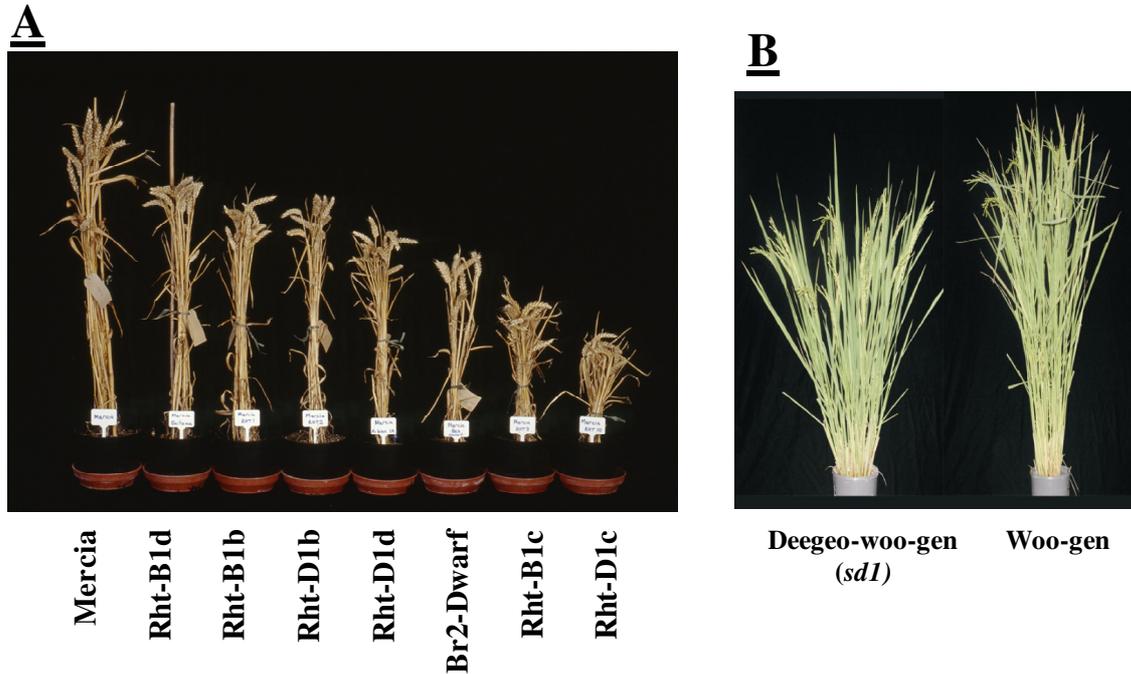
Further, optimum fertilizer application for increasing grain yield promotes lodging due to increasing plant height. However, under conditions of high fertilizer (N) and moisture, varieties having a semi-dwarf stature are less prone to lodging. If semi-dwarf varieties are further have thick-straw, resistance to lodging is greatly improved.

### 1.3 Genetic control of lodging resistance

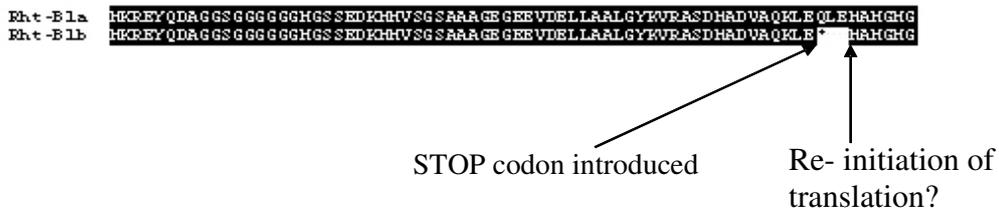
#### 1.3.1 GA genes and lodging

The first indication that GAs were endogenous growth regulators in plants was reported in the 1950s, after study showed the height of dwarf pea and maize mutants were restored to normal by applying gibberellic acid (GA<sub>3</sub>) (Hedden and Phillip, 2000). Among the many influences of GA genes on the plant growth and development, their ability to promote internodal elongation in a wide range of species that belong to the grass family has been of considerable agronomic importance (Taiz and Zeiger, 2006). Basically the importance of GA was demonstrated by the discovery of GA-deficient (reduced bioactive GA amounts) or GA-insensitive mutants in several species, including rice (Murakami, 1970), wheat (Maluszynski and Szarejko, 2005), maize (Phinney, 1956), and *Arabidopsis* (Koornneef and Van der Veen, 1980). These mutants had typically a dwarf or a semi-dwarf phenotype with a reduced bioactive GA amount in case of GA-deficient mutants or high bioactive GA concentrations in certain GA-insensitive dwarf mutants, such as *Rht3* wheat and *Dwarf-8* maize (Hedden and Kamiya, 1997) due to a negative feed-back regulation (Alvey and Harberd, 2005). The use of highly sensitive methods of physio-chemical analysis, such as gas chromatography and mass spectrometry (GC-MS), has shown that the GAs are large group of natural products, up to 126 different compounds currently known. However, based on the analysis of the GA-deficient mutants only few GAs have intrinsic biological activity and a hormonal actions (Hedden and Phillip, 2000).

Identification and isolation of the homologous *sd-1* (GA-deficient ) and *Rht* (GA-insensitive ) genes in *Arabidopsis* has further helped to understand the role of GA genes in plant height control. The *sd-1* gene encodes a GA biosynthetic enzyme, GA20 oxidase, and the rice genome carries four GA20- oxidase genes, *GA20ox1-4*. The *sd-1* corresponds to *GA20ox-2*, which is highly expressed in leaves and flowers (Yamaguchi, 2008). The enzymatic action of the two other oxidases is not well-known (Hedden *et al.*, 2002). GA 20-oxidase is a regulatory enzyme with multifunctional catalytic activity acting at several stages in the biosynthesis process and the oxidase is further a prime target in the genetic manipulation of the GA biosynthetic pathway (Hedden *et al.*, 1998). The function of *RHT* was only identified as a result of molecular genetic studies on the analogous gibberellic acid-insensitive (*ga-insensitive*) *gai* mutant of *Arabidopsis*. *GAI* encodes a GA response repressor gene in the GA response pathway which functions in the absence of GA (Peng *et al.*, 1997). The *Arabidopsis* *GAI* dwarf mutants have further been found to be orthologs of the maize dwarf (*D8*) genes (Fu *et al.*, 2001). Unlike *sd-1*, the phenotype of these dwarf mutants could not be restored to the wild-type by exogenous GA application due to a mutation in the GA response pathway (Taiz and Zeiger, 2006). Further studies revealed that *RHT* has multiple allelic variants resulting in variations of the *RHT* mutant (Figure 1.1). Three amino acid deletions and introduction of a stop codon at the N-terminus of its coding region further resulted in a semi-dwarf phenotype (Figure 1.2).



**Figure 1.1** A) Phenotypic variations for allelic diversity for semi-dwarfing traits for the wheat *Rht* gene (except Br2-dwarf which is a brassinosteroid insensitive dwarf) (Peng *et al.* 1999 and Pearce *et al.* (unpublished)) and B) for the semi-dominant (*sd-1*) semi-dwarfing gene in rice (Monna *et al.* 2002, Sasaki *et al.* 2002 and Spielmeyer *et al.* 2002).



**Figure 1.2** Partial amino acid sequence of the wheat protein encoded by the Rht-B1a and Rht-B1b loci with amino acid internal deletions of the allele causing the semi-dwarf phenotype.

Dwarfing genes are grouped on their response to applied GA. Mutants of the biosynthetic pathway, such as rice *sd-1*, are GA sensitive and the phenotype of the wild-type can be restored by exogenous application of GA. Dwarf mutants of the GA response, such as wheat *Rht-B1b* and *Rht-D1b*, the maize *dwarf 8 (D8)* or its ortholog in sorghum *dwarf3 (dw3)* and the *Arabidopsis* GAI, are insensitive to applied GA. In recent years more mutants related to either *sd-1*, *Rht* or other genes in GA metabolism have been identified causing dwarfism in wheat and rice (Milach *et al.*, 2002; Xu *et al.*, 1995; Carrera *et al.*, 2000; Hedden *et al.*, 1998).

In gibberellin response mutants, three main classes of mutations have been identified affecting plant height. These are (a) gibberellin-insensitive dwarfs, (b) gibberellin-deficient mutants in which the plants can be reversed closer to normal by co-expression of a second “suppressor” mutation, and (c) mutants with a constitutive gibberellin response also called “slender” mutants (Taiz and Zeiger, 2006). Examples for gibberellin-insensitive dwarfs are the wheat “green revolution” mutants with a mutation in the *Rht-1* and *Rht-2* genes and their orthologs in maize, *Dwarf8 (D8)*, and in *Arabidopsis*, *Gibberellic acid Insensitive – GAI*, were also found to confer a semi-dwarf phenotype. The *Rht* locus encodes a repressor protein (GAI) in *Arabidopsis* inhibiting stem elongation in the absence of GA. The *Arabidopsis* dwarf mutant (*gai*) protein has a 17 amino acids deletion rendering it insensitive to foliar application of GA (Fu *et al.*, 2001 and Peng *et al.*, 1999).

A mutation in the rice *sd-1 (GA20ox-2)* coding region (280 bp deletion) is a loss of function mutation causing a semi-dwarf phenotype when the mutated gene was expressed in other crops such as *Arabidopsis* and potato (Spielmeyer *et al.*, 2002). Rice plants with the mutation also had a greater harvest index allowing for increased use of nitrogen fertilizers. However,

the presence of multiple *sd-1* alleles prevented severe dwarfing due to a partial inhibition of GA production (Yamaguchi, 2008). In *Arabidopsis*, which carries three GA20 oxidases, such functional redundancy after mutation, and with GA still produced in other plant parts, caused a semi-dwarf phenotype (Spielmeyer *et al.* 2002).

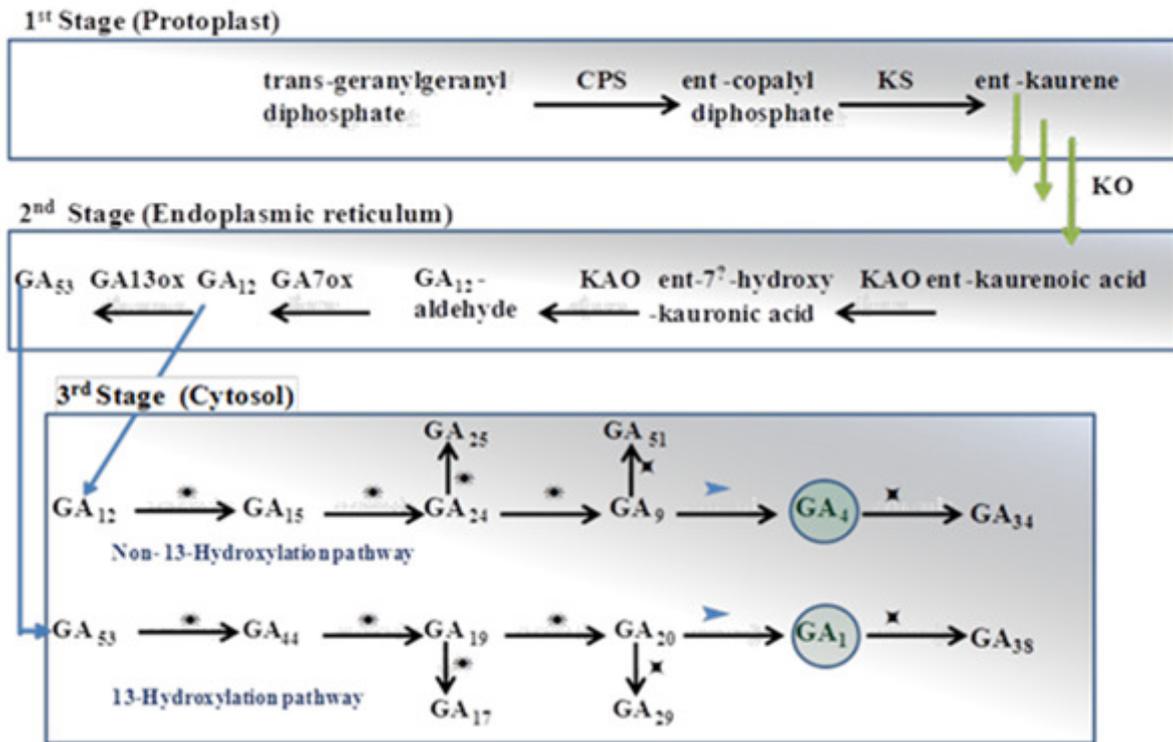
### **1.3.2 Manipulation of plant height using GA genes**

The key role shown above that GA metabolic genes play in plant architecture has made them prime targets for genetic manipulation. Characterization of these genes has paved the ground for geneticists and physiologists to target specific metabolic pathways in the production of higher yielding and hardier plants. However, from the agronomic point of view, not all the genes involved are of interest (Yamaguchi, 2008; Hedden and Phillips, 2000). Changing or manipulating the endogenous bioactive GA amount allowed the design of crops with a better morphological architecture. This approach offers an alternative strategy to introduce beneficial traits, such as dwarfism, into cereal varieties to improve grain yield.

Modifying the endogenous amount of bioactive GA might occur either through genes contributing to the production of the bioactive GA or through genes diverting GA forms to inactive molecules. This includes catabolic inactivation of bioactive GA forms or some of its precursors (Hedden and Phillips, 2000). Studies by Sun and Kamiya (1994) and Fleet *et al.* (2003) have shown that over-expression of the genes encoding enzymes that catalyse the early stages of GA biosynthesis, e.g. *ent*-copalyl pyrophosphate synthase (*AtCPS*) and *ent*-kaurene synthase (*AtKS*) in *Arabidopsis*, do not significantly increase amounts of bioactive GA with no effect on plant growth and development. Over-expression of genes downstream pathway, such as the GA 20-oxidases that are multifunctional and highly regulatory enzymes

(Figure 1.4), increased stem elongation, early flowering and decreased seed dormancy indicating that their activity limits GA biosynthesis (Lang, 1998). Suppression of the different GA 20-oxidase homologous genes in *Arabidopsis* through RNAi expression produced changes in the different parts of the plant showing their tissue specific role (Coles *et al.*, 1999). Over-expression of its own GA 20-oxidase in *Arabidopsis* resulted in the elongation of seedling hypocotyls, increased shoot growth, induced early flowering, and increased GA<sub>4</sub> level (Huang *et al.* 1998; Coles *et al.* 1999). However, over-expression of the same gene from citrus or *Arabidopsis* caused an increased amount of bioactive GA and elongated phenotypes in hybrid aspen and tobacco plants (Eriksson *et al.*, 2000; Vidal *et al.*, 2001; Biemelt *et al.*, 2004). In potato, Carrera *et al.* (2000) showed that antisense mRNA expression of a *GA20ox* gene reduced stem elongation and increased both tuberization and tuber yield. Studies by Israelsson *et al.*, (2004) and Phillips (2004) further indicated no difference in the morphology of transgenic plants following GA 3-oxidase over-expression in hybrid aspen and *Arabidopsis*. In rice, antisense copies of *GA3ox2 (D18)* reduced the final GA amount and caused semi-dwarf phenotypes in some of the transformants (Itoh *et al.*, 2002). This phenotype, however, was not stably transferred to the progeny possibly due to gene silencing.

Lowering of the endogenous GA amount is also possible through increasing the expression of GA 2-oxidase, a positive feed forward regulation enzyme, which catabolises bioactive GAs and some precursors. After its first isolation using cDNA from runner bean (*Phaseolus coccineus*) by a functional screening method (Thomas *et al.*, 1999), an ectopic expression of *OsGA2ox1* gene in rice resulted in reduced stem growth with small, dark green leaves with reproduction organs severely defective (Sakamoto *et al.*, 2001). However, expression of the same gene under the control of the shoot-specific *OsGA3ox2* promoter induced only a semi-dwarf phenotype with normal flower and grain development (Sakamoto *et al.*, 2003).



**Figure 1.3** Simplified pathway of the gibberellin (GA) biosynthesis and deactivation in plants. Most targeted genes in inducing dwarfism are the 2-ODD family multifunctional genes catalyzing several steps at the intermediate ( \* ) and late ( × ) steps of the biosynthesis pathway catalyzed respectively by GA20ox and GA2ox. In addition to inactivating the bioactive GAs (GA<sub>1</sub> and GA<sub>4</sub>), the GA 2-oxidase also deactivates intermediates GA<sub>9</sub> and GA<sub>29</sub>. (Adapted from Oikawa *et al.*, 2004)

Severe dwarf phenotypes were obtained in *Arabidopsis*, tobacco and poplar through over-expression of the rice GA 2-oxidase gene (Schomburg *et al.*, 2003; Biemelt *et al.*, 2004; Busov *et al.*, 2003). Plants with a dwarf phenotype were also produced by over-expression of a runner bean GA 2-oxidase in *Arabidopsis* and wheat and Hedden and Phillips (2000) suggested the superiority of this approach for the breeding of dwarf plants.

#### 1.4 **Brassinosteroid genes and lodging**

Mutated genes of the brassinosteroid metabolism also causes a dwarf phenotype. The *brachytic2* (*br2*) mutant in maize and its ortholog in sorghum, *dwarf3* (*dw3*), induce short internodes (Multani *et al.*, 2003). In barley, *uzu* dwarfism caused by the missense mutation in the *HvBR11* gene is a mutation in the brassinosteroid receptor protein resulting in a semi-dwarf phenotype (Chono *et al.*, 2003). Several other BR deficient and BR-insensitive mutants have been identified with phenotypic changes including dwarfism, small dark-green leaves, a compact rosette structure, delayed flowering and senescence and reduced fertility (Sasse, 2002). The practical use of the BR has been limited due to BR deficiency leading to severe and defected dwarfism and reduced fertility (Divi and Krishna, 2009). However, controlled changes i.e. slight decrease in BR levels or in BR signaling was found causing significant increase in yield as a result of change in plant architecture (Divi and Krishna, 2009).

In monocots, only BR-insensitive mutants have been identified (Yamamuro *et al.*, 2000.). A rice mutation in the C-22 hydroxylase, a BR enzyme involved in leaf inclination, resulted in a semi-dwarf phenotype that increased above ground biomass by 40% (Sakamoto, 2006). In tomato, a BR-responsive *dwarf* (*d*) mutant was found caused by inactivation of a cytochrome P450 enzyme (CYP85A1) (Bishop *et al.*, 1996). In *Arabidopsis*, overexpression of *DWF4*, a

gene that encodes a cytochrome P450 monooxygenase (CYP90B1) (Choe *et al.*, 1998) resulted in a dramatic promotion of vegetative growth and enhanced seed yields (Fujioka and Yokota, 2003). In barley, an ortholog of BRI1, a BR-insensitive mutant due to single nucleotide change in the BR-receptor gene produced a semi-dwarf phenotype with increase in yield and lodging resistance. Generally, the current knowledge on BR regulation of growth and development through altered BR activity is growing rapidly through characterization of a wide variety of BR-deficient and BR-insensitive mutants (Fujioka and Yokota, 2003).

## 1.5 Induced mutations

Inducing mutation in crops has been long exercised to create variability in germplasm for species and traits where there is little known variation for a trait of interest. In *E. tef* the genetic diversity for lodging resistant traits has not been found to exploit through genetic introgression into modern cultivars. In other cereal crops, however, inducing mutation to improve genetic diversity have brought about renewed interest because it mimics natural variation with relatively high frequency in the selected germplasm with desirable genetic backgrounds (Maluszynski and Szarejko, 2003). The technique provides novel genes or alleles (Table 1.1) of known phenotypes (Maluszynski and Szarejko, 2003) in better adapted or a more desirable background (Konzak, *et al.*, 1984). Through induced mutations, many genes involved in metabolic pathways responsible for plant development and growth, response to growth regulators and various biotic and abiotic stresses have been identified (Barkley and Wang, 2008; Maluszynski and Szarejko, 2003). The most obvious and attractive feature in inducing mutants is ease of inducing the genetic variation with relatively high frequency, and very often the mutations mimic natural variations. The genetic changes are made in advanced genotypes or in genotypes adapted to local environmental conditions or

having desirable background traits. Among economic traits largely targeted for improvement using this technique include semi-dwarfness for conferring lodging resistance in cereal crops (Barkley and Wang, 2008; Hu, 1973; Ullrich and Aydin, 1985; Maluszynski *et al.* 2003).

Semi-dwarfness is one of the most desirable traits targeted by induced mutations due to lack of diversity in desirable phenotypic traits in the genetic pool of many important crops. In rice, several semi-dwarf mutants, including stiff and lodging resistant, have been selected from mutated populations and led to the release of important new varieties (Maluszynski and Szarejko, 2003). Developing and selecting useful mutations involve random mutations for qualitative traits coupled with large screens of the mutated plants. This requires sufficient time (1- 2 years) and development of a high quality population. Such technical challenges in mutation technologies render the method less attractive to many scientists (Barkley and Wang, 2008; Baenzinger, 1988). In barley, a new semi-dwarf lodging resistance mutant variety has been selected with an average mature height reduced to 87 cm from a tall, 120-130 cm, phenotype. This increased yield by about 15% and with high input about 25% (Maluszynski and Sigurbjörnsson, 1988; Rutger, 1981). Inducing dwarfism or reduced plant height without losing the potential yield has been reported for wheat and other cereals (Table 1.1) (Barabäs and Kertész, 1988; Narahari, 1988).

Spontaneous mutation occur with an extremely low frequency, often unnoticed being difficult to detect in species like the tetraploid *E. tef*. Thus genetic manipulation such as inducing mutation in target genes using various mutagens can provide rapid generation and enhancement of genetic variability. Inducing short stature mutants without changing the background character of important traits will be extremely beneficial for developing lodging resistance in *E. tef*.

**Table 1.1** Semi-dwarf sources in *Rht* wheat induced by chemical or physical mutagens (Maluszynski *et al.* 2001).

Gene	Source	Parent variety	Mutagen	Breeding value
Rht4	Burt	M937	Gamma rays	
Rht5	Marfed M1	Marfed	EMS	
Rht7	Bersee Mut	Bersee	EMS	
Rht11	Krasnodarskii karlik	Bezostaja	MNH	***
Rht12	Karcag 522M7K	Karcag 522	Gamma rays	
Rht13	Magnif 41M1	Magnif 41	MNH	
Rht14	Cp B144	Cappelli	thN	***
Rht15	Durox	K6800707	EMS	*
Rht16	Edmore M1	Edmore	EMS	*
Rht17	Chris M1	Chris	DES	
Rht18	Icaro	Anhinga	fN	*
Rht19	Vic M1	Vic	EMS	*
Rht20	Burt M860	Burt	Gamma rays	*

\*\*\* high breeding value.

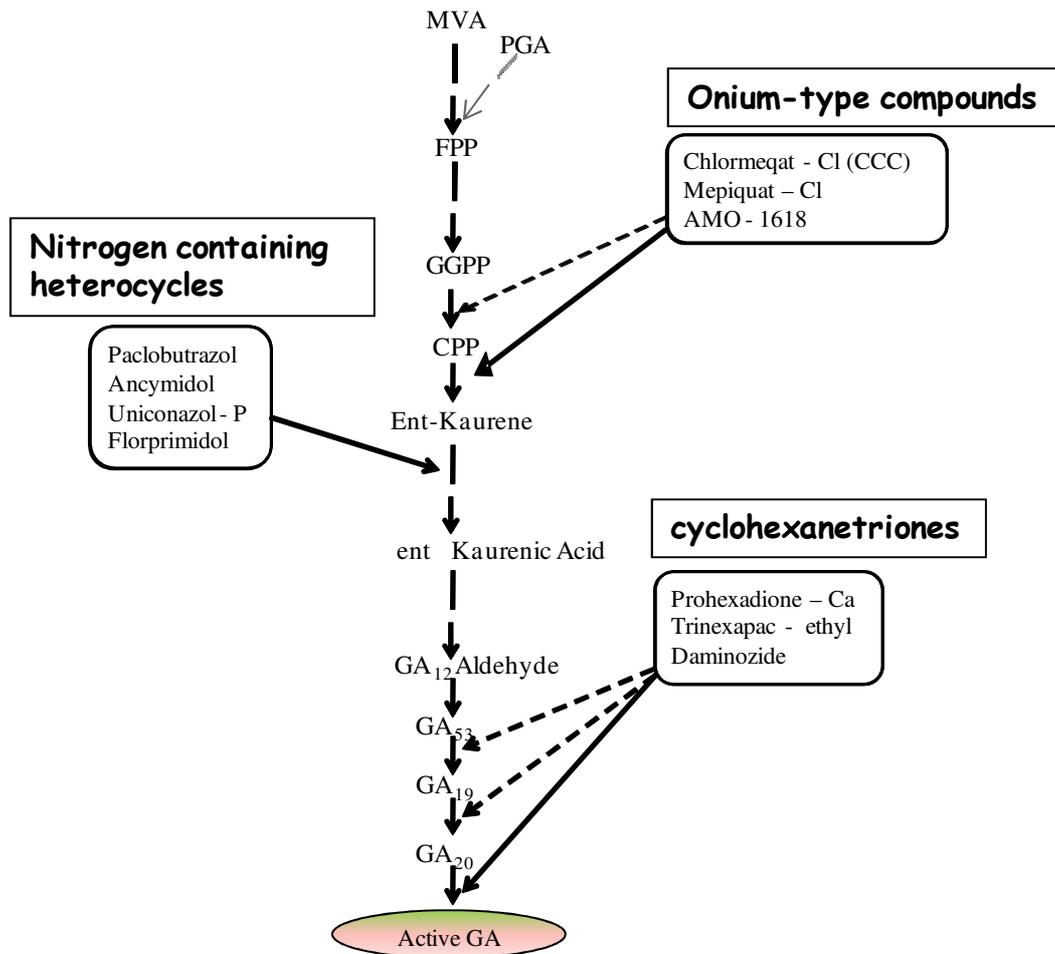
EMS= ethylmethane sulfonate; DES = ; MNH =  
Source: Maluszynski and Szarejko, 2003

## 1.6 Plant growth regulators for plant height control

Plant height, particularly culm length, is considered to be among the major factors associated with lodging sensitivity (Pinthus, 1973; Crook and Ennos, 1994; Berry *et al.*, 2000). Control of plant growth, such as plant or culm height, can be achieved chemically by using plant growth regulators (PGRs). Many chemical growth promoters or retardants have been used to treat crops and plants for controlling growth and development of vegetative or reproductive parts. PGRs that inhibit gibberellins (GA) biosynthesis are used in high input cereal management to shorten straw and thereby increasing lodging resistance. They have been extensively used in many crops to reduce lodging through shortening of the stem and to maintain a steady improvement in grain yield (Berry *et al.*, 2004; Rajala, 2003). Among the GA inhibitors that are used to control plant growth are the onium-type compounds, such as chlormequate chloride (2-chloroethyl-N,N,N-trimethyl-ammonium chloride, CCC) and mepiquat-Cl, interfering with *ent*-kaurene synthesis at the early stages of gibberellin biosynthesis (Rademacher, 2000) (Figure 1.1). Inhibition of the cyclization of geranylgeranyl diphosphate synthase (GGPP) into copalyl diphosphate synthase (CPP) due to CCC binding to the enzyme CPP-synthase reduces the availability of bioactive GA (Hedden and Philips, 2000; Graebe *et al.*, 1992; Rademacher, 2000).

A further group are nitrogen containing heterocycles such as *triazoles* and *imidazoles*. This includes paclobutrazol (PBZ) and the closely related uniconazole-P. Both compounds interfere in the oxidation of *ent*-kaurene to *ent*-kaurenoic acid (Rademacher, 2000). Inhibition of oxidation of mono-oxygenases occurs by sharing lone pair electrons to displace oxygen from the enzyme binding site at the proto-heme iron, this renders the oxygenase non-functional (Rademacher, 2000) (Figure 1.1). Commercially available PBZ in the (2*S*,3*S*)-

enantiomer is structurally similar to *ent*-kaurene. More recently developed GA inhibitors include cimectacarps (trinexapac-ethyl), interfering with the late stages of GA metabolic reactions mainly by inhibiting 3 $\beta$ -hydroxylation of GA<sub>20</sub> to produce the bioactive GA<sub>1</sub> (Rajala, 2003). In general, the stem growth inhibition due to PGRs can be variable, depending on species and genotypes (Rajala, 2003), and is further based on GA inhibitor mediated stem shortening by interfering with synthesis of an intermediate precursors, *ent*-kaurene or *ent*-kauronic acid, or by inhibiting 3 $\beta$ - hydroxylation of GA<sub>20</sub> to bioactive GA<sub>1</sub> (Graebe *et al.*, 1992; Rajala, 2003; Hedden *et al.*, 2010).



**Figure 1.4** Simplified scheme of GA biosynthesis steps and points of inhibition by plant growth regulators. Broken line represents minor inhibitor activities (Rademacher, 2000).

Reduction in plant height following application of PGRs is associated with reduced endogenous bioactive GA amounts and reduced elongation of internodes particularly of the uppermost internodes and peduncle (Sanvicente *et al.*, 1999; Rajala, 2003). CCC inhibits stem elongation in wheat by reducing up to 40% plant height (Humbries *et al.*, 1965). In oilseed rape, foliar treatment with a combination of CCC, ethephon and imazaquin reduced main stem length by 7% in the field and 16% under greenhouse conditions. The uppermost three internodes contributed significantly to the reduction (Sanvicente *et al.*, 1999). In barley,

responses to CCC were genotype-dependent (Rajala, 2003), but when wheat or barley plants were treated with CCC, the PGR had no effect when plants already contained dwarfing genes such as *Rht1*, *Rht2*, or *Dw6* (Abbo *et al.*, 1987; Peltonen-Sainio and Rajala, 2001). PBZ application reduced stem length in some rice cultivars by 90%, lodging from 60% (in controls) to 0% and increased yields up to 15% when compared to controls (French *et al.*, 1990). PBZ and the closely related uniconazole-P are highly active PGRs with practical uses in rice, fruit trees and ornamentals (Rademacher, 2000) and about 84% of the winter wheat in UK is treated with PGRs (Berry *et al.*, 2004).

PGR application (CCC treatment) has been found to increase lodging resistance due to an increase in stem diameter (Tolbert, 1960). However, other research groups showed no change in the content of structural compounds (cellulose, lignin and hemicelluloses) in the plant stem following CCC and ethephon treatment (Clark and Fedak, 1977; Knapp *et al.*, 1987). In barley, no clear relationship was found between stem diameter and cell wall thickness of the two basal internodes with lodging susceptibility (Stanca *et al.*, 1979). Shortening of stem after PGR treatment may also not necessarily result in reduced lodging as reported for wheat and barley (Knapp *et al.*, 1987; Ma and Smith, 1992).

Information about the use of PGRs in *E. tef* is very limited but increased yield following CCC application at 0.7 - 2.0 l a.i. ha<sup>-1</sup> has been reported but lodging was not prevented (Alkamper, 1970). This early *E. tef* result has been further supported by Berry *et al* (1998) applying CCC to a lodging-prone crop and reduced lodging area only from 88 to 83% at harvest.

## 1.7 Lodging in *E. tef*

### 1.7.1 *E. tef* growth

Tef, *Eragrostis tef* (Zucc.) Trotter, is a small-seeded full grain cereal with high economic importance in Ethiopia. It is the most resilient crop with low risk of failure (Tefera and Ketema, 2001) grown under very diverse environments and exhibits high diversity in most pheno-morphic and agronomic traits (Assefa, 2003). In Ethiopia, *E. tef* is grown on over 2.56 million ha<sup>1</sup>, accounting for about 28% of the total acreage and 19% of the gross grain production of the major cereals (CSA, 2008). The lives of estimated over 50 million people depend directly on *E. tef* as a staple food. *E. tef* grows on water-logged vertisol in the highlands as well as water-stressed areas in the semi-arid regions (Takele *et al.*, 2000). Suitable growing rain-fed areas are reported to be those with a growing period of 100 - 150 days, rainfall of 375 - 700 mm and a mean temperature of 12 - 22°C (Takele *et al.*, 2000). However, Kebede *et al.* (1989) reported that higher dry matter accumulation occurs at 35°C than at 25°C with the highest leaf carbon exchange rate, 31.8  $\mu\text{-mol m}^{-2} \text{s}^{-1}$ , occurs at this temperature.

Grain yield varies from 1-2.5 t ha<sup>-1</sup> with a national average yield of about 1.0 t ha<sup>-1</sup> and grain yield potential which might be elevated to 4.5 t ha<sup>-1</sup> (Tefera and Belay, 2008; Teklu and Tefera, 2005). Yield ranges between 2.5- 4.5 t ha<sup>-1</sup> have been reported for research plots using improved varieties and with support of a net to prevent lodging (Tefera *et al.*, 2001; Mamo and Parsons, 1987; Delden *et al.*, 2010). Early local varieties maturing in less than 85 days, such as Gea-Lamie, Dabi, Shewa-Gimira, Beten and Bunign, are widely used under short growing conditions experiencing low moisture stress in the mid and low altitude or low

temperature at high altitudes. Under a suitable growing environment, local cultivars, such as Alba, Ada and Enatit, are used. Modern varieties, such as DZ-01-354, DZ-01-196, DZ-01-787, are widely grown by farmers in areas with optimal rainfall and DZ-Cr-37 is grown in low-moisture stress areas. These varieties give mean grain yields ranging from 1.4 to 2.7 t ha<sup>-1</sup> (Assefa, 2010; Ketema, 1997; CSA 2008).

### 1.7.2 Pheno-morphic features related to lodging

Lodging is a key agronomic problem in *E. tef* production (Yu *et al.*, 2007) and up to 23% yield loss is accountable to lodging under natural conditions (Ketema, 1983) i.e. with minimal or no fertilizer condition. Even with good crop management practices, lodging is a major limitation to sustainable improvement of the crop. *E. tef* generally has a tall culm height up to 155 cm and a fine or slender stem with first and second basal culm internode diameter range from 1.2-4.5 mm (Aseffa *et al.*, 2010; Teklu and Teferea, 2005; Ketema, 1983). Thus *E. tef* is characterized by a low root-collar diameter to plant-height ratio. Nearly all improved varieties have a tall phenotype with culm height reaching up to 150 cm and with a basal internode diameter of <4.5 mm (Figure 1.5). The root system is fibrous and shallow emerging from nodes above the base, and growing 4 - 8 cm deep under field conditions. The panicle forms about a third of the culm length (Ketema, 1997; Kebede *et al.*, 1989). Most of the above characteristics appear to be typical making the crop very susceptible to lodging (Figure 1.5) due to weak stem-base having insufficient strength to hold the shoot up against leverage.

**A**



**B**



**Figure 1.5** *E. tef* plant stand in the field at (A) grain filling and (B) at maturity when almost all plants lodged ( Source: Dr. Likyelesh Gugssa (Holetta Agricultural Research Center)).

Most studies showed stem lodging due to bending at the basal internodes to be the major problem in *E. tef* (Ketema, 1983; Asefa *et al.*, 2000)). In a modelling work for the lodging character in *E. tef* Mark (1985) took into account node diameter, 1<sup>st</sup> and 2<sup>nd</sup> internode length, biomass and wind among external forces acting on the plant. According to him the lodging score (S) is computed as:

$$S = \frac{b_0 + b_1 h (W + Q)}{D^3 (1-t^4)}, \text{ where:}$$

**S** = lodging score; **b<sub>0</sub>**, **b<sub>1</sub>** = empirical constant; **W** = tiller weight, **Q** = drag force due to wind, **t** = node diameter ratio (inner: outer); **D** = mean internode length (1<sup>st</sup> & 2<sup>nd</sup>). Because of a high correlation of panicle length with yield and hence tiller weight (Mengesha, 1965), panicle length has been substituted for (W + Q) and mean diameter of the 1<sup>st</sup> and 2<sup>nd</sup> for internode length. However, this model could predict only about 33% of the variance recorded for lodging implicating possible involvement of other factors not accounted for. Further development of models to predict and improve the lodging character in *E. tef* has not been made except in a recent study to examine applicability of other crops' models developed for wheat and barley (Delden, 2010).

### 1.7.3 *E. tef* breeding for lodging resistance

*E. tef* breeding has mainly resulted in tall phenotypes with low root collar diameter to plant height ratio (Ketema, 1983) and most varieties so far developed for high yield have this tall phenotype (Teklu and Teferea, 2005). No genotype has been found so far to be lodging resistant and there is no clear agreement on the important trait to look for. Berhe (1981) regarded short, stiff-strawed genotypes as important, others suggest short plants (possibly

straw + panicle) (Ketema, 1983; Mengesha *et al.* 1965) and they reported a high correlation for lodging with stem diameter, plant height, panicle length and yield. Hundera *et al.* (1999) reported days to heading and maturity to be negatively associated with lodging while plant height, culm length, panicle length, culm diameter, panicle weight, and shoot biomass were highly significant and positively associated with lodging resistance. Asefa *et al.*, (2000) recommended stem morphology related characters, such as total height, number of nodes, thickness and length of basal internodes, to be important. Teferra *et al.* (2003) reported that high yielding lines tend to lodge more severely because of failure to bear the heavy panicles, indicating that lodging also imposes limitation on genetic improvement in *E. tef* for further yield increase. Recent studies in *E. tef* have shown strong correlations between lodging, panicle type, culm thickness, and grain yield (Yu *et al.*, 2007). Lodging index showed positive and highly significant correlations with primary shoot weight, 100 seed weight, grain yield, shoot biomass and negative correlations with peduncle length thus, high yielding lines tended to lodge. The positive and strong relationship of lodging with plant height and plant height with yield and other important yield component traits indicates lodging resistance improvement will remain challenging in *E. tef* until it is possible to uncouple plant height and yield traits. Overall, lack of knowledge of exact traits to look for is still the most critical drawback in modern *E. tef* cultivation.

Van Delden *et al.*, (2010) also reported that *E. tef* has the lowest value for plant base diameter, the diameter of tillers at the soil surface, and the average root plate diameter compared to other cereals like wheat and rice and emphasized the significance of root failure as yet another serious factor in *E. tef* lodging. However, it is not yet clear if root lodging could well be associated with *E. tef* root morphological attributes such as root strength and rigidity, root number and length, or stem characters like thicker stem base. Moreover, how

these factors interact with different soil characteristics to cause the lodging problem needs to be investigated.

## **1.8 Working hypothesis and aim of study**

In this PhD study the problem of reducing plant height in *E. tef* was addressed to improve lodging resistance in the crop. Since the GA metabolism plays a significant role in plant height control, it was hypothesized that regulation of the GA amount in *E. tef* will change pheno-morphic and also agronomic characteristics that will affect lodging and also decoupling plant height from yield. This study had therefore the aim to reduce plant height by either chemical PGR treatment or manipulation of gene expression to reduce plant height and also to study in more detail the expression of height regulating GA genes in *E. tef*. The objectives of the study were (i) to study the *in vivo* response of two *E. tef* genotypes (short variety: Gea Lammie and long variety: DZ-01-196) to treatment with PGRs to confirm a role of GA in *E. tef* plant height control, (ii) to optimize *E. tef* plant transformation and regeneration for the production of transformed *E. tef* plants with reduced GA content (iii) to characterize transformed *E. tef* plants over-expressing GA2-oxidase (*PcGA2ox1*) under the control of a CaMv3x35S promoter to decrease bioactive GA amounts (iv) to identify and characterize the genes involved in plant height control (rice *sd-1* and wheat *Rht* orthologous genes) in *E. tef* and (v) to characterize morphologically and physiologically existing semi-dwarf *E. tef* mutants derived from a TILLING process.



## **CHAPTER 2**

**CONTROLLING PLANT HEIGHT AND LODGING IN TEF  
(*Eragrostis tef*, Zucc.) USING GIBBERELLIN BIOSYNTHESIS  
INHIBITORS**

## 2.1 Abstract

Tef (*E. tef*) is a small seeded nutritious cereal and a primary food source in Ethiopia grown on over 2.56 million ha in Ethiopia. Tef productivity is low, 1.0 t per ha, due to several factors, among which lodging is the most critical causing direct losses of about 23% under natural condition. High yielding cultivars are usually tall and more susceptible to lodging and breeding effort has not yet succeeded to decouple height from yield. Inhibitors of gibberellin (GA) biosynthesis such as chlormequat chloride (CCC) are used extensively to restrict growth and improve lodging resistance in cereals. First, responsiveness of tef plants to GA<sub>3</sub> and CCC application was determined using two tef varieties Gea Lammie (short) and DZ-01-196 (tall). At 10<sup>-2</sup>M CCC plant height was reduced by 43% and 21% in the tall and short variety, respectively, within six weeks after plant emergence. CCC at 10<sup>-1</sup>M reduced tiller number in both varieties. More detailed analysis of growth regulator application by including Paclobutrazol (PBZ) on the tall tef variety DZ-01-196 revealed that, both CCC and PBZ reduced culm length, with a much stronger reduction from paclobutrazol. Grain yield on the other hand was not affected by CCC treatment. CCC-treatment reduced culm length by affecting all internodes, with the 1<sup>st</sup>- 3<sup>rd</sup> internodes, followed by the 6<sup>th</sup> and 7<sup>th</sup> most severely affected, whereas paclobutrazol treatment strongly affected all internodes, with greatest effect on the uppermost 4 internodes. Internode diameter was unaffected by both CCC- and paclobutrazol-treatments. A steady increase in mean internode diameter until the 6<sup>th</sup> internode was found for CCC-treated and also control plants revealing a poor tapering in tef plants. Reduction of GA amount in tef might be a target for improving lodging resistance allowing uncoupling of plant height and yield.

## **2.2 Introduction**

Tef (*Eragrostis tef* (Zuccagni) Trotter) is a panicle bearing, small-seeded nutritious cereal grown extensively in Ethiopia in diverse climatic and soil conditions with low risk of failure (Assefa *et al.*, 2010). Tef is grown on about 2.6 million ha and accounts annually for about 28% of the total acreage of cereal production in Ethiopia. However, tef suffers from low productivity with average yields of only 1.0 t ha<sup>-1</sup>. Among the factors contributing to low yield, lodging is the most important (Assefa *et al.*, 2010; Tefera *et al.*, 2003; Yu *et al.*, 2007).

In general, lodging interferes with water and nutrient transport, reduces light interception, provides a favourable environment for disease, increases harvesting cost and losses and decreases grain yield and quality (Tripathi *et al.*, 2003). It occurs either by buckling / bending at the basal culm internodes, or due to root lodging or failure of the anchorage system of the plant (Assefa *et al.*, 2000; Ketema, 1983; Pinthus, 1973). Culm length and the strength of the basal part of the culm are considered major factors associated with lodging sensitivity (Rajala, 2003; Tripathi *et al.*, 2003).

In cereals, improvement of lodging resistance has been predominantly achieved by reducing plant height, in particular by chemical inhibition of gibberellin (GA) production (Rademacher, 2000) or by the use of semi-dwarf varieties with reduced GA biosynthesis or signal transduction (Hedden, 2003). Chlormequat chloride (CCC), the most commonly used plant growth retardant (PGR), blocks GA biosynthesis by inhibition of the cyclization of geranylgeranyl diphosphate (GGPP) to *ent*-copalyl diphosphate (CPP) by CPP synthase (Rademacher, 2000). Triazole PGRs, such as paclobutrazol (PBZ), inhibit the conversion of the GA precursor *ent*-kaurene to *ent*-kaurenoic acid (Rajala, 2003; Hedden and Graebe,

1985). In general, PGRs have been extensively used in many crops to reduce lodging through shortening of the stem and to maintain a steady improvement in grain yield (Berry *et al.*, 2004; Rajala, 2003).

Reduction in plant height due to PGR treatment, is associated with reduced elongation of internodes particularly of the uppermost internodes and peduncle (Sanvicente *et al.*, 1999; Rajala, 2003). CCC inhibits stem elongation in wheat (Humbries *et al.*, 1965) and in oilseed rape. Foliar treatment with a combination of CCC, ethephon and imazaquin reduced main stem length in barley where shortening of the uppermost three internodes contributed significantly to the reduction (Sanvicente *et al.*, 1999). PBZ application was found to reduce stem length and lodging in rice and increase yield by up to 15% compared to controls (French *et al.*, 1990).

In tef, cultivars bred for improved grain yield possess a tall phenotype and are highly susceptible to lodging (Assefa *et al.*, 2010; Yu *et al.*, 2007). Thus, lodging susceptibility has prevented the introduction of higher yielding varieties with good grain quality, and also hampered the use of input-intensive husbandry. Currently, there is no detailed information available for tef on the effect of PGR treatment on lodging and yield responses. The objective of this study was therefore to investigate morphological and yield changes in tef following GA biosynthesis inhibitor treatment under controlled environmental conditions. Results obtained demonstrate that CCC treatment of tef plants significantly reduces plant height without affecting yield.

## **2.3 Materials and Methods**

Two experiments one with preliminary observation (also referred in this chapter as Experiment I) involving two growth regulators and two varieties and a second one (also referred to as Experiment II) involving one variety and two growth regulators have been carried out.

### **2.3.1 Plant material**

Seed material of the tef (*Eragrostis tef*) varieties DZ-01-196 and Gea Lammie used for the experiments was obtained from the Ethiopian Institute of Agricultural Research, Holetta Agricultural Research Center, Ethiopia. Plants of variety DZ-01-196 have a tall phenotype, derived from a conventional breeding program and is widely used for cultivation while Gea Lammie is a landrace grown by farmers for its earliness.

### **2.3.2 Plant growth**

Experiment I was done using varieties DZ-01-196 and Gea Lammie grown in a greenhouse at University of Pretoria. Plants were grown on a germination soil mix in a pot supplemented with a Hoagland fertilizer solution (Kebede *et al.*, 2008). With the second set of experiments, plants were grown at Rothamsted Research, UK from May to August using pre-germinated seeds in moist soil in pots. In both experiments a total of 12-16 plants were maintained in 3 to 4 per treatment. About 7-10 days after emergence thinning was done or transplanting of uniform seedlings was carried out to new pots [15 cm diameter (top) x 12.5 cm (height) and 10 cm diameter (bottom)]. Seedlings were grown on either a commercial germination mix

soil supplemented with half strength Hoagland solution in the observation trial or in a compost mix consisting of peat (75%), sterilized loam (12%), vermiculite (3%) and grit (10%), which was supplemented with a slow release fertilizer. Plants were well-watered every other day and the temperature was maintained at 23-27°C (day) and 15-18°C (night). Seedlings were grown for 14 weeks until plant maturity in an environmentally controlled greenhouse using a 16-h photoperiod provided by natural light supplemented with light from sodium lamps to maintain a minimum PAR of 350  $\mu\text{mol m}^{-2} \text{s}^{-1}$ .

### 2.3.3 PGRs treatment

In the first experiment, the above two genotypes were considered for the investigation. Gibberellic acid ( $\text{GA}_3$ ) and a GA-biosynthesis inhibitor, chlormequate chloride (2-chloroethyl-N,N,N-trimethyl-ammonium chloride, CCC) were applied by rubbing the underside of the leaf lamina. Application began after plant thinning and continued for six weeks every week. Both compounds were applied (10 $\mu\text{l}$ ) to the lower surface of the uppermost expanding young leaf per week, at concentrations ranging from  $10^{-1}\text{M}$  to  $10^{-6}\text{M}$ . In the second experiment, only one variety, DZ-01-196 was considered for a more detailed study. Plants were treated with CCC and a potent GA- biosynthesis inhibitor, paclobutrazol (PBZ), (2RS, 3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl) pentan-3-ol). CCC was applied at 10 mM and 100 mM and PBZ at 10  $\mu\text{M}$  and 100  $\mu\text{M}$ . For both inhibitors, individual solutions were prepared in  $\text{dsH}_2\text{O}$  and 100 ml of individual solution were applied every two weeks to the base of the pot when watering of plants was carried out. Treatment with inhibitors started 3 wks after seed germination.

#### 2.3.4 Growth measurements

Culm, individual internodes and panicle lengths of tillers were measured from the subtending nodes using a ruler. The internode and tiller number were also recorded for each plant. Primary tiller refers to the main tiller that emerges first as the seed germinates. Secondary tillers refer to shoots that emerge at a later stage during seedling growth. The internode diameter was measured at 3 mm above the node using a Standard Digital Caliper. Dry weight was determined from above-ground plant material by drying fresh material at 80°C for 2 days in an oven. Grain yield was determined by measuring the weight of seeds from all tillers.

#### 2.3.5 Analysis of endogenous GA content

The upper most two internodes including their nodes at shoot elongation stages and just before panicle initiation were harvested and stored at -80°C 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 40-45°C 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°C 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 HPLC column for fractionation. Recovery of fractions was monitored using tritiated

(3H) internal standards and GAs were quantified using gas chromatography- mass spectrometry (GC-MS) system using selective ion monitoring.

### 2.3.6 Data analysis

Growth and yield data were collected after six weeks and at plant maturity for the first and second sets of the experiments from 12 individual plants and their tillers per treatment. Analysis of variance (ANOVA) and Pearson Correlation Coefficients were performed for data analysis using the SAS statistical package (SAS Institute Inc., Cary, NC, USA). Statistical significance of difference between treatment means was determined using the Tukey's Studentized Range (HSD) Test. A *P*-value of <0.05 was considered as significant.

## 2.4 Results

### 2.4.1 Experiment I

The effect of exogenous application of gibberellic acid (GA<sub>3</sub>) and the GA inhibitor (2-chloroethyl-N, N, N-trimethyl-ammonium chloride (CCC) on two tef varieties, Gea Lammie and DZ-01-196, was investigated. In this preliminary observation, the two genotypes showed, to a considerable degree, contrasting response to the application of exogenous GA<sub>3</sub> and CCC. Different GA<sub>3</sub> or CCC amounts affected plant height beginning in the first week of its application in both varieties when compared to untreated control plants. In general, GA<sub>3</sub> application increased plant height whereas CCC reduced the plant height in both varieties (Fig. 2.1A and B).

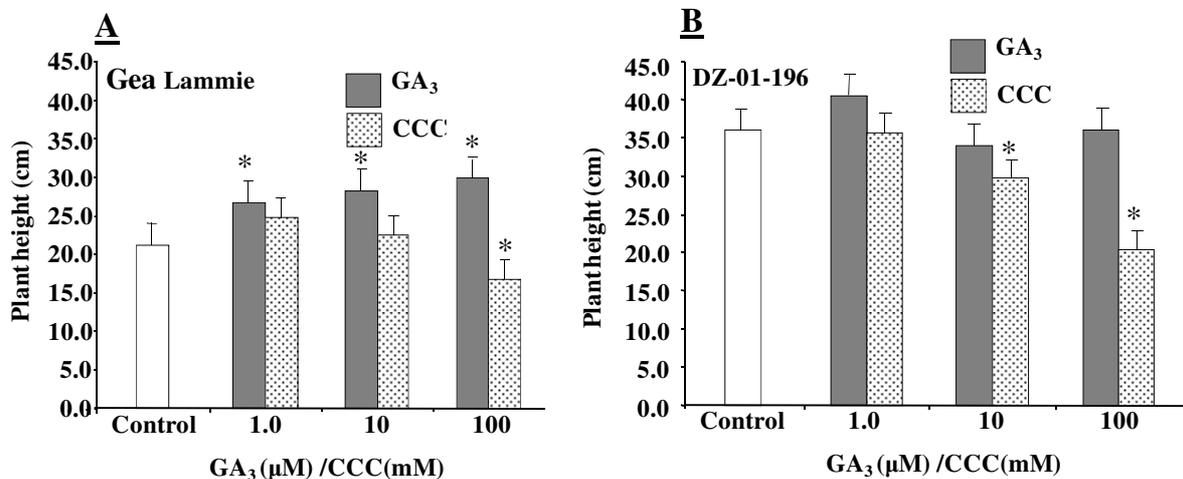


Figure. 2.1A and B. Growth (plant ht) response after six weeks of Gea Lammie and DZ-01-196 grown in greenhouse to exogenous application of GA and CCC. Treatments: C=Control; GA<sub>3</sub>/CCC: 1= 1μM/1mM, 10=10μM/10mM and 100=100μM/100mM

Application of GA<sub>3</sub> (10<sup>-2</sup>M) significantly increased ( $p < 0.01$ ) plant height by 41% in plants of the short genotype and 13% of the tall genotype under greenhouse conditions. In contrast, applying CCC (10<sup>-2</sup>M) significantly reduced ( $p < 0.01$ ) plant height by 46% in the tall genotype compared to a 27% reduction in the short genotype. GA<sub>3</sub> treated plants were tall and had slender stems compared with plants treated with CCC (data not shown). The above results led to further study in more detailed (second experiment) only one variety, DZ-01-196, using CCC and PBZ. Results show that both fresh and dry weight of DZ-01-196 plants were affected by CCC treatment. At a CCC concentration of (10<sup>-1</sup>M) fresh weight of plants was significantly reduced ( $p < 0.001$ ) in the taller genotype when compared to the untreated control (Fig. 2.3). Number of emerging tillers was also significantly ( $p < 0.05$ ) reduced by the highest CCC level (10<sup>-1</sup>M) in the taller genotype. There was at most one tiller per plant at elongation stage (in addition to the main tiller) in those treated with CCC ( $\geq 10^{-1}$ M) when compared to the untreated control (Fig. 2.2).

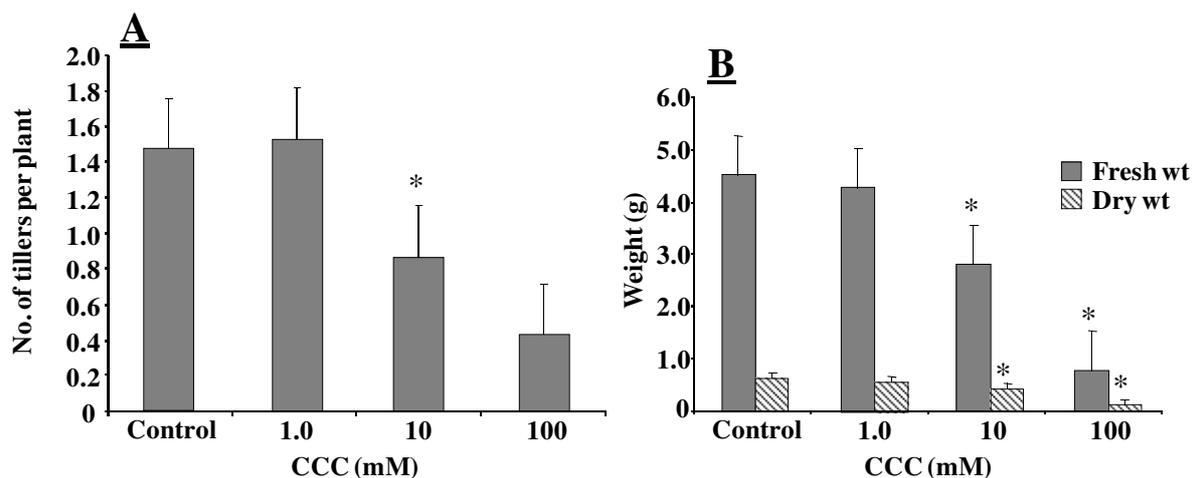


Figure 2.2. Tillering (A), fresh and dry weight (B) responses of six weeks old DZ- 01-196 seedlings to CCC treatment.

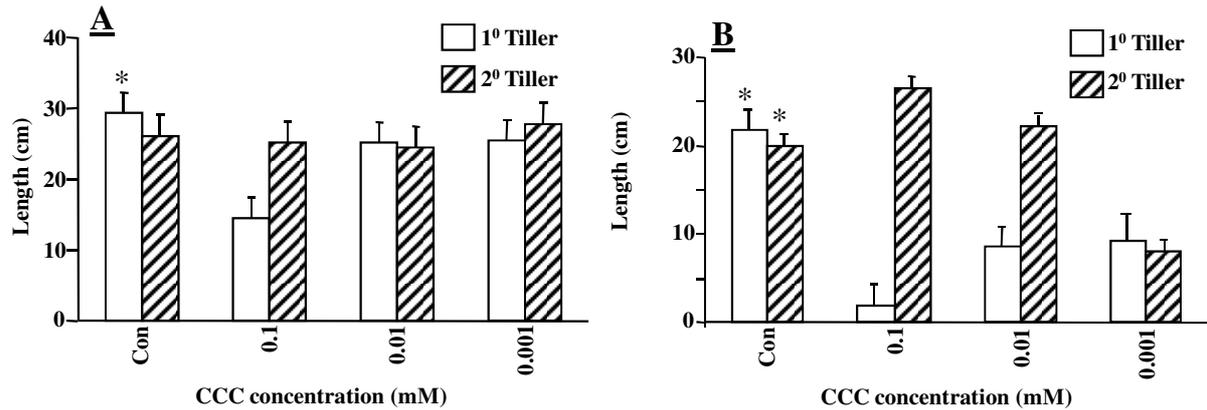


Figure 2.3 Effect of foliar applied CCC at different concentrations of foliar applied CCC on the length of the 7<sup>th</sup> internode (A) and 8<sup>th</sup> internode (B) of primary (1<sup>0</sup>) and secondary (2<sup>0</sup>) tillers in comparison to the untreated control. Data represent the mean  $\pm$  SE of tiller length of 12 individual plants.

Most of the reduction in the culm length was due to a significant reduction in internode elongation particularly in the upper-most two internodes following CCC treatment (Fig. 2.3 A and B). However, in the uppermost (8th) internode, it has been observed that the primary tiller has been more sensitive to CCC and the secondary tiller. Length of the secondary tillers was reduced at lower CCC levels than at higher level compared to the main tiller. Such contrasting response was not observed between the main and secondary tillers for the 7<sup>th</sup> internode. The reason for this is not clear.

#### 2.4.1.1 Analysis of endogenous GA content

The two genotypes of tef, Gea Lammie and DZ-01-196 were characterized to determine the state of GA concentrations in them and reveal the existence and nature of the relationship between GA and plant height.

The predominant GA pathway and the primary biologically active product in vegetative shoot of *tef* was previously unknown. On the other hand, endogenous GA content determination in plant material is a challenging task because of GA's extremely low concentration in plants and its complex biological matrices (Ge *et al.*, 2007). Other characteristics such factors as low ultraviolet (UV) absorption, absence of fluorescence, and distinguishing chemical characteristics as well as the need for specific chemical assay makes GA analysis complicated (Ge *et al.*, 2007). Therefore, the determination of GA levels in plants had to be carried out at Rothamsted Research/UK where expertise and facility were found.

Results from the endogenous GA analyses of plant tissues taken from the upper most two internodes including their nodes using analytical HPLC column fractionation and GC-MS monitoring shows that the shorter genotype Gea Lammie has generally lower level of bioactive GA and most of the precursors than the tall genotype (Table 2.1). The content of the most abundant bioactive GA<sub>3</sub> was about three fold less in the shorter genotype Gea Lammie compared to DZ-196-01. The bioactive GA level corresponds with plant heights, and associated other growth characteristics of the two genotypes. Moreover, the concentration of most of the immediate precursors in Gea Lammie were about half the amount in DZ-01-196. The amount of endogenous GA<sub>1</sub> concentration was moderately related with plant height than biomass or number of tillers. Concentrations of precursors for the Non- 13-Hydroxylation pathway such as GA<sub>15</sub>, GA<sub>9</sub>, and GA<sub>34</sub> were extremely low or nil in most cases. The amount of and the bioactive final product, GA<sub>4</sub>, of this pathway was nil in both genotypes (Table 2.1). Therefore, the analysis showed that the early 13-hydroxylation is the major or preferred pathway in *E. tef* and the most abundant bioactive form was GA<sub>1</sub> (Table 2.1).

**Table 2.1** Quantification of GA intermediates and bioactive forms from internode sample analysis at stem elongation stages of two tef varieties, DZ-01-196 (tall) and Gea Lammie (short).

Genotype	GA <sub>1</sub> <sup>§</sup>	GA <sub>29</sub>	GA <sub>3</sub> <sup>§</sup>	GA <sub>15</sub>	GA <sub>4</sub> <sup>§</sup>	GA <sub>8</sub>	GA <sub>20</sub>	GA <sub>19</sub>	GA <sub>34</sub>	GA <sub>9</sub>	GA <sub>53</sub>
<b>DZ-01-196</b>	5.71 <sup>**</sup>	2.25	3.17	0.0	0.0	9.36	6.65	19.22	0.18	ND	10.68
<b>Gea Lammie</b>	4.22	1.12	1.07	0.0	0.0	5.35	4.63	10.97	0.3	0	4.28
<b>SE</b>	0.25	0.71	1.39			0.53	0.31	0.53	0.28	1.06	0.25

ND =not determined; <sup>§</sup>Bioactive forms of GA; \**E. tef* has the 13 $\beta$ -hydroxylation as a major pathway for GA biosynthesis hence GA<sub>1</sub> is a major bioactive product.

<sup>\*\*</sup>Values are in ng per g dry weight and a data point represents average of three samples

## 2.4.2 Experiment II

### 2.4.2.1 Culm and panicle length

In the second experiment treatment of tef plants with PBZ using soil application of the growth regulator significantly reduced culm and panicle length of plants by internode shortening when compared to CCC treatment or to the untreated control (Fig. 2.4; Table 2.2). PBZ also showed significant effects on growth parameters at a much lower concentration than CCC (Table 2.1). When plants were treated with PBZ at 10  $\mu$ M and 100  $\mu$ M, culm length was reduced by 92% and 98%, respectively. However, CCC treatment only reduced

culm length by 9.3% (10 mM) and 22.3% (100 mM). Further, PBZ treatment significantly reduced also panicle length (Table 2.2). In contrast to PBZ treatment, CCC treatment increased panicle length at both concentrations applied (10 mM and 100 mM) with a significant increase at 100 mM when compared to the untreated control (Table 2.2). In addition, the panicle length to culm length ratio of 1.61 (10  $\mu$ M PBZ) and 1.51 (100  $\mu$ M PBZ) decreased to 0.37 and 0.49 when plants were treated with either 10 mM CCC or 100 mM CCC, respectively. This indicates that both inhibitor treatments had a stronger effect on culm length than on panicle length (Fig. 2.5), with CCC treatment resulting in an increased panicle length at both concentrations. Furthermore, after PBZ and CCC treatment panicle length was strongly correlated with culm length, culm dry weight and total above ground shoot dry weight and also negatively correlated with tiller number per plant and dry weight per height ratio (Table 2.5).

#### 2.4.2.2 *Internode growth*

Both internode length and diameter were significantly reduced by PBZ treatment as a soil application when compared to CCC treatment or to the untreated control (Tables 2.2 and 2.3). In PBZ-treated plants, internode length was reduced in all internodes. However, the two upper most internodes of plants treated with 10  $\mu$ M PBZ and the four uppermost internodes of plants treated with 100  $\mu$ M PBZ completely failed to elongate (Table 2.3). In CCC-treated plants, reduction in internode length also varied between the different internodes. The first three internodes (internodes 1-3) contributed 48%, whereas the last three internodes (internodes 5-7) contributed 46% to the total internode length reduction. In the preliminary study when CCC was used as a foliar application and not as a soil application, the upper-most two internodes contributed most of reduction in internode length (See Fig. 2.3: A and B).

However, for the 8<sup>th</sup> internode, length of the secondary tillers was less at lower CCC levels than at higher level.

The lowermost two internodes (I1+I2) of CCC-treated plants were further more positively correlated ( $r = 0.70$ ,  $P < 0.05$ ) to culm length when compared to the uppermost two internodes (I7+I8) ( $r = -0.04$ ,  $P < 0.05$ ) (data not shown).

**Table 2.2** Effect of CCC and PBZ on culm and panicle length, number of tillers and seed weight per plant of tef cv. DZ-01-196.

<b>Treatment</b>	<b>Culm length (cm)</b>	<b>Panicle length (cm)</b>	<b>No. of Tillers</b>	<b>Seed wt (g)</b>
<b>Control</b>	159.9± 3.7a	50.26±1.9c	5.0±0.6b	3.79±0.5a
<b><u>CCC</u></b>				
<b>10mM</b>	145.0±2.2b	53.93±0.9b	6.0±0.6b	3.63±0.7a
<b>100mM</b>	124.2±3.3c	61.50±0.7a	7.3±1.1b	3.78±0.3a
<b><u>PBZ</u></b>				
<b>10µM</b>	11.5±1.1d	18.57±0.8d	17.3±2.8a	1.11±0.3b
<b>100µM</b>	3.9±0.3e	5.87±0.3e	15.0±2.1a	0.05±0.03b
<b>Significance</b>	***	***	***	***

Letters within the column denote significance as determined using the Student's *t*-test. Data shown represent mean values ±SE of 12 individual plants. Significance level was determined using ANOVA (\*\*\*)  $P < 0.001$ ) and difference between treatment means was determined using the Tukey's Studentized Range (HSD) Test. Means followed by the same letter are not significantly different.

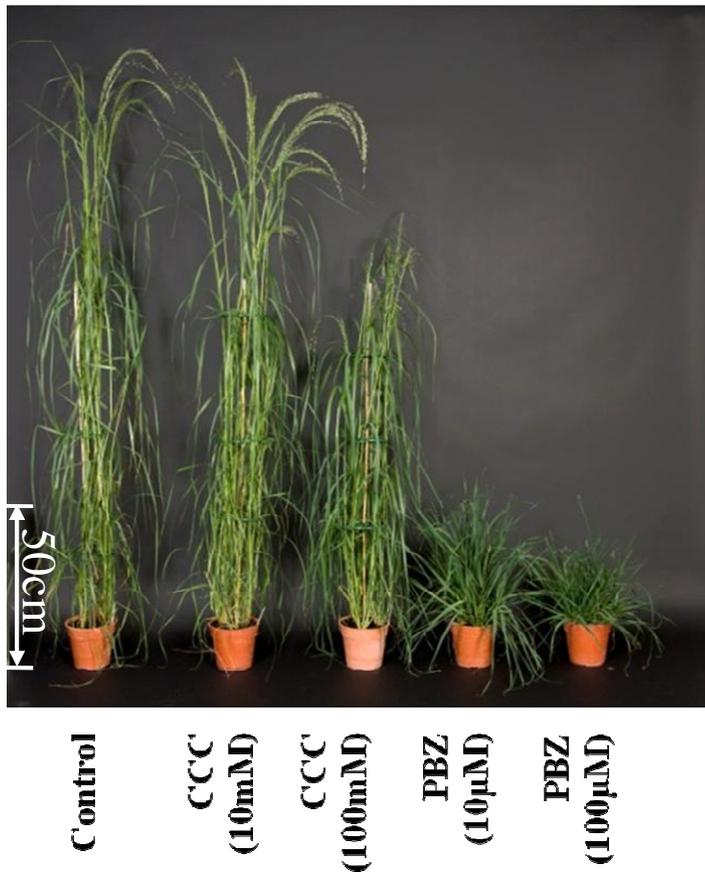


Figure 2.4 Effect of CCC (100mM) and PBZ (100µM) on plant height near plant maturity in comparison to the untreated control.

The internode diameter was, however, unaffected by either PBZ or CCC treatment except for the first internode after 10 mM CCC treatment, internodes 5 and 6 after 10 µM PBZ treatment and internode 4 after 100 µM PBZ treatment. In CCC-treated and control plants, the internode diameter steadily increased from the base up to internode 6. This indicated a poor tapering characteristic of tef plants under the selected growth conditions (Table 2.4).



Figure 2.5 Comparison of plant height and panicle growth at plant maturity as affected by PGRs (CCC: 100mM and PBZ: 10µM) application.

#### 2.4.2.3 *Tillering, above ground biomass and yield*

PBZ-treated plants had a three-fold increase in the number of tillers per plant whereas CCC-treated plants had no significant increase in the number of tillers when compared to the untreated control (Table 2.2). PBZ treatment also significantly reduced culm and panicle dry

**Table 2.3** Effects of CCC and PBZ on length of different internodes of tef var. DZ-01-196.

Treatment	Internode length (cm)							
	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8
<b>Control</b>	8.6±0.9a	14.3±0.85a	18.7±0.5a	18.3±1.1a	21.9±0.9a	23.0±2.0a	27.2±1.1a	28.4±3.3a
<b><u>CCC</u></b>								
<b>10mM</b>	8.1±0.9a	12.3±0.5b	15.1±0.9b	17.3±0.8a	20.4±0.1b	22.7±0.9a	24.1±0.9b	25.0±2.7a
<b>100mM</b>	3.3±0.6b	8.9±0.6c	12.8±0.7c	15.8±0.5b	17.0±0.6c	18.3±0.8b	20.5±0.7c	27.6±1.3a
<b><u>PBZ</u></b>								
<b>10µM</b>	1.3±0.5c	2.0±0.5d	3.0±0.5d	4.0±0.7c	1.7±0.8d	0.8±0.1c		
<b>100µM</b>	0.45±0.1d	0.9±0.3e	1.0±0.3e	0.8±0.2d				
<b>Significance</b>	***	***	***	***	***	**	**	NS

Letters within the column denote significance as determined using the Student's *t*-test. Data shown represent mean values ±SE from 12 individual plants. Significance level was determined using ANOVA (\*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; NS = not significant) and difference between treatment means was determined using the Tukey's Studentized Range (HSD) Test. Means followed by the same letter are not significantly different.

**Table 2.4** Effect of CCC and PBZ on diameter of different internodes of tef var. DZ-01-196.

Treatment	Internode diameter (mm)							
	I-1	I-2	I-3	I-4	I-5	I-6	I-7	I-8
<b>Control</b>	3.2±0.1a	3.7±0.1a	4.2±0.2a	4.34±0.2a	4.6±0.3a	4.8±0.3a	4.7±0.3a	4.0±0.3a
<b>CCC</b>								
<b>10mM</b>	2.8±0.1b	3.5±0.1a	3.9±0.1a	4.25±0.1a	4.6±0.1a	4.9±0.1a	4.9±0.1a	4.2±0.4a
<b>100mM</b>	3.2±0.1a	3.9±0.12	4.1±0.1a	4.22±0.1a	4.4±0.1a	4.4±0.1b	4.4±0.2a	3.8±0.2a
<b>PBZ</b>								
<b>10µM</b>	3.7±0.5a	3.9±0.4a	3.7±0.6a	3.66±0.6a	2.0±0.8b	0.8±0.6c		
<b>100µM</b>	3.8±0.5a	4.1±0.4a	3.7±0.6a	0.56±0.4b				
<b>Signif.</b>	***	NS	NS	**	*	**	NS	NS

Letters within the column denote significance as determined using the Student's *t*-test. Data shown represent mean values ±SE from 12 individual plants. Significance level was determined using ANOVA (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; NS = not significant) and difference between treatment means was determined using the Tukey's Studentized Range (HSD) Test. Means followed by the same letter are not significantly different.

weight in both the primary and secondary tillers when compared to CCC treated plants or to the untreated control (Table 2.6). PBZ also significantly suppressed above-ground shoot dry weight by 43.2% and 75.9% at 10  $\mu$ M and 100  $\mu$ M PBZ, respectively (Table 2.5; Fig. 2.6). In plants treated with 10 mM CCC secondary tiller culm and panicle dry weights were increased significantly as was total above-ground shoot dry weight when compared to untreated plants and those treated with PBZ 1. In CCC-treated plants, the change in the above-ground shoot dry weight was all due either to the increase (at 10 mM) or the decrease (at 100 mM) in secondary tiller growth.

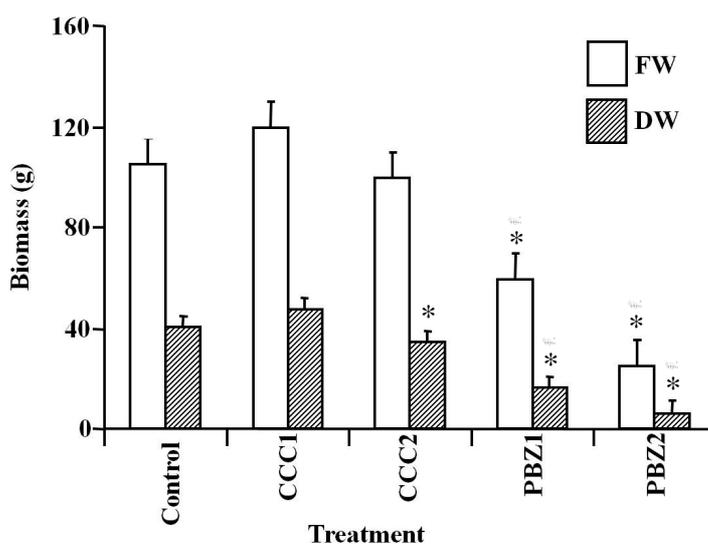


Figure 2.6 Effect of different concentrations of CCC and PBZ on biomass: fresh weight (FW) or dry weight (DW) per plant in comparison to biomass of untreated control plants. Data represent the mean  $\pm$  SE of 12 individual plants. (CCC1 = 10 mM CCC, CCC2 = 100 mM CCC, PBZ1 = 10 mM PBZ and PBZ2 = 100 mM PBZ).

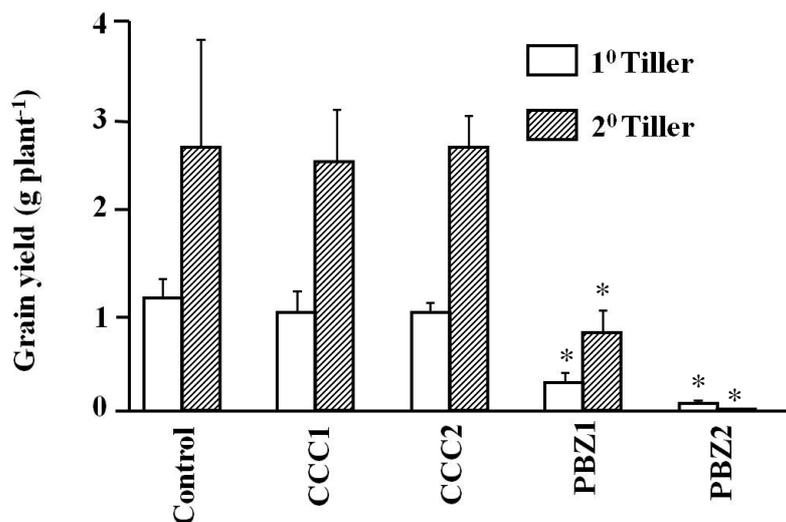


Figure 2.7 Effect of different concentrations of CCC and PBZ on primary (1<sup>o</sup>) and secondary (2<sup>o</sup>) tiller grain yield per plant in comparison to the untreated control. Data represent the mean  $\pm$  SE of tiller grain yield of 12 individual plants. (CCC1 = 10 mM CCC, CCC2 = 100 mM CCC, PBZ1 = 10 mM PBZ and PBZ2 = 100 mM PBZ).

Further, in GA inhibitor-treated plants, panicle dry weight contributed less (by 8.4 - 14.5%) to above ground shoot dry weight while for panicle in the untreated control was higher (12.0%). Also, in all GA inhibitor-treated plants the tiller number per plant was negatively correlated with above-ground shoot dry weight but positively correlated with the ratio of dry matter to shoot height (Table 5). PBZ treatment (10  $\mu$ M and 100  $\mu$ M) significantly reduced the seed weight per plant when compared to the untreated control. Such a significant reduction was not found after CCC treatment. In CCC treated plants panicle bearing secondary tillers further contributed 63.4% to the total yield per plant (Fig. 2.7). This indicates that PBZ-induced profuse tillering, resulting mainly in non-

panicle bearing tillers, which did not contribute to grain production. Nevertheless PBZ had a stronger effect on culm than on panicle length and also clearly demonstrated that the responsiveness of *E. tef* to GA inhibition has been different between culm and panicle (Fig. 2.8).

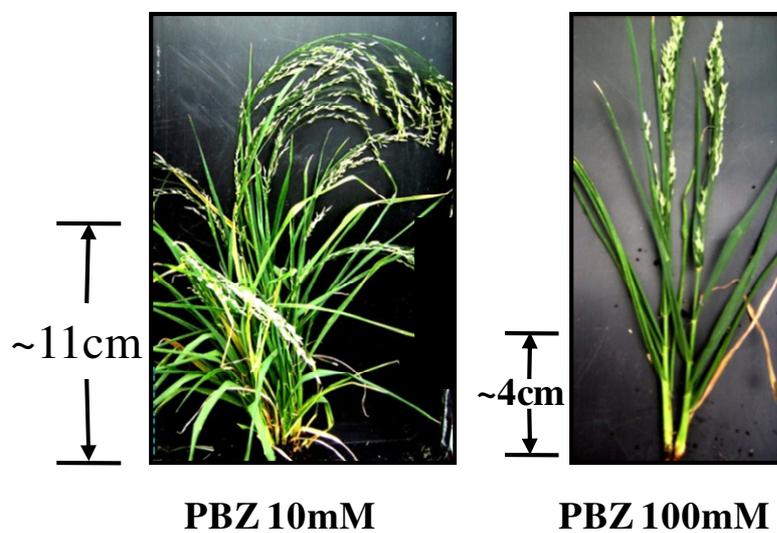


Figure 2.8 Comparison of panicle elongation with PBZ treatments in proportion to culm reduction in DZ-01-196.

**Table 2.5** Effect of CCC and PBZ on dry weight of culm and panicle tillers of tef var. DZ-01-196.

Treatment	Primary tiller (g)		Secondary tiller (g)		Total shoot (g)
	Culm	Panicle	Culm	Panicle	
<b>Control</b>	7.11±0.3a	2.01±0.2a	25.67±1.8b	5.52±0.6b	40.30±1.9b
<b><u>CCC</u></b>					
<b>10mM</b>	6.70±0.3a	2.17±0.2a	31.10±1.3a	6.93±0.6a	46.89±1.7a
<b>100mM</b>	4.60±0.3b	1.87±0.1a	19.89±1.6c	6.51±0.6ab	34.53±1.9c
<b><u>PBZ</u></b>					
<b>10µM</b>	0.72±0.1c	0.73±0.2b	11.81±1.2d	2.82±0.6c	16.10±1.8d
<b>100µM</b>	0.41±0.1d	0.31±0.1c	5.63±0.3e	0.29±0.1d	6.41±0.4e
<b>Significance</b>	**	**	**	**	**

Letters within the row denote significance as determined using the Student's *t*-test. Data shown represent mean values ±SE from 12 individual plants. Significance level was determined using ANOVA (\*\* *P* < 0.01) and difference between treatment means was determined using the Tukey's Studentized Range (HSD) Test. Means within a column followed by the same letter are not significantly different.

**Table 2.6** Correlation coefficients for morphological and yield components of tef var.

DZ-01-196.

	PL	PaL	TH	IN	NT	CDW	PDW	TSDW	DW/Ht	S/P
<b>CL</b>	0.5352	0.9281	0.9872	0.9291	-0.6415	0.8872	0.7779	0.8966	-0.8687	0.7191
<b>PL</b>		0.5555	0.5663	0.5579	-0.2571	0.5012	0.4383	0.5065	-0.4561	0.4557
<b>PaL</b>			0.9511	0.9276	-0.6028	0.8356	0.8251	0.8748	-0.8585	0.7587
<b>TH</b>				0.9321	-0.6145	0.8609	0.7859	0.8833	-0.8848	0.7538
<b>IN</b>					-0.6252	0.8486	0.7447	0.8634	-0.8532	0.6522
<b>NT</b>						-0.4258	-0.4248	-0.4307	0.8029	-0.4401
<b>CDW</b>							0.7904	0.9803	-0.7199	0.6682
<b>PDW</b>								0.8809	-0.6543	0.8923
<b>TSDW</b>									-0.7372	0.7511
<b>DW/Ht</b>										0.9869

CL = Culm length; PL = Peduncle length; PaL = Panicle length; TH= Total height; IN= Internode number; NT = No. of tillers; SFW= shoot fresh weight; CDW = Culm dry weight; PDW = Panicle dry weight; SDW = Shoot dry weight; DW/Ht = shoot dry weight per height ratio; S/P = seed weight per plant. Significance level for the Pearson Correlation Coefficients was determined using the SAS statistical package and all values are significant at  $P < 0.001$ .

## 2.5 Discussion

Results have shown a GA-dependent control of plant height in tef. Application of GA<sub>3</sub> (10<sup>-2</sup>M) increased plant height by 41% in plants of the short genotype and by 15% in the tall genotype under greenhouse conditions. Applying CCC at 10<sup>-2</sup>M reduced plant height by about 43% in the tall genotype DZ-01-196 when compared to a 21% reduction in the short genotype Gea Lammie during the six weeks of plant growth. Application of the GA inhibitor CCC reduced the plant height in plants of DZ-01-196 (tall phenotype). This effect was more dramatic than the effect on plants height in Gea Lammie. Since GA increased height in Gea Lammie and CCC reduced height in both, the short phenotype in Gea Lammie is not considered to be either due to interference in the GA-biosynthesis or GA- response but could be associated to other factors not relevant to GA metabolic genes. Studies in wheat has shown that tall and intermediate varieties showed a greater response to CCC than the short or semi-dwarf varieties containing mutant GA genes like *Rht1/Rht1* (Börner & Meinel, 2006; Abbo *et al.*, 2004).

The content of the bioactive GA<sub>1</sub> and GA<sub>3</sub> and all the precursors were higher in the tall genotype, DZ-01-196, than in Gea Lammie showing in most cases about a two fold increase. Thus the bioactive GA concentration was a good reflection of the difference in plant height between the two genotypes. This also explains the differential responses to exogenous GA<sub>3</sub> application where DZ-01-196 was less responsive to exogenous GA treatment compared with Gea Lammie because of higher bioactive GA amount in the plant tissue.

Further study using only the tall variety (Experiment II) has demonstrated that the GA inhibitors CCC and PBZ reduce stem growth in *tef* but with a much stronger effect of PBZ when compared to CCC. Culm length was the most responsive plant part to inhibitor action and CCC reduced culm length by one quarter but without reducing panicle growth and grain yield.

Based on our finding that CCC treatment reduces plant height in *E. tef*, the PGR effect on DZ-01-196 was investigated in greater detail because this variety has been widely grown for its high yield and grain quality. It is also used as a parental line in *E. tef* breeding program but suffered from lodging losses (Yu *et al.* 2007; Assefa *et al.* 2010). Therefore, effect of GA inhibitors CCC and PBZ on DZ-01-196 was specifically studied further. Both GA inhibitors CCC and PBZ significantly reduced stem growth in DZ-01-196 but with a much stronger effect of PBZ at a much lower concentration when compared with CCC because of its stronger inhibitory effect on GA biosynthesis than CCC (Lurie *et al.*, 1997)). Culm length was most responsive and CCC reduced culm length almost by a quarter without reducing panicle growth and grain yield. A further reduction of culm height might be achieved by increasing CCC concentration, but such increased concentration should not affect panicle growth. Yield increase associated with CCC application, reported for other crops (Berry *et al.*, 2004), was not found for *E. tef*. This could be because of absence of increased head-bearing tillers following CCC application.

PBZ, greatly reduced culm elongation particularly affecting elongation of the uppermost four internodes which in most cases was completely inhibited. Also, a lower PBZ

concentration might be applied so that a less drastic effect is achieved in reducing the plant height and with minimal effect on panicle growth and yield.

Since GA inhibitor action greatly affected culm length, this indirectly imply that a higher demand for endogenous GA may exist by the elongating stem than by the panicle. This may be due to a higher meristematic activity and cell elongation in the intercalary meristem of the elongating stem rendering it more sensitive to changes in the GA amount. Shortening was more pronounced in the lowermost internodes with up to a 2.6-fold reduction when compared to the control. Shortening of basal internodes, if associated with stem wall thickening or increase in dry weight per unit of basal internodes, will further minimize the lodging risk. For example, the basal internode length and plant height in wheat are the two most important culm traits closely associated with lodging (Kelbert *et al.* 2004).

It was also observed that soil-applied CCC shortened all internodes, foliar application shortened the uppermost two internodes (Gebre, *et al.*, 2011). A similar effect for foliar-applied CCC has been previously also reported for barley (Sanvicente *et al.*, 1999) and hybrid rye (Froment and McDonald, 1997). The results also indicate a possible differential response to mode of application which could be related to the translocation of CCC to the site of the biochemical targets being more localized in leaf application. *E. tef* may benefit more from foliar than soil application. Irregularity of responses has been noted in other crops presumably for differences in the decomposition of the chemical in the soil, depending on prevailing temperature and humidity conditions (Radmacher, 2000; Pintus, 1973).

In our study CCC treatment even increased panicle length, but this increase was not related to any change in seed weight per plant. Whereas in a previous study with wheat improved grain set thus increased harvest index was obtained following shortening of stem (Rajala, 2003). Absence of a CCC effect on *E. tef* panicles possibly indicates a low demand for bioactive endogenous GA for panicle growth and therefore inhibition of GA biosynthesis does not greatly affect yield while reducing plant height. Agronomically, this would be advantageous allowing high productivity in tef plants while minimizing lodging.

In this study, we also investigated the effect of the GA-biosynthesis inhibitors on stem diameter. In PBZ-treated plants tapering stem morphology with a steadily increasing stem diameter was found. In contrast CCC-treated and control plants only had an increase in stem diameter but in the upper internodes only. Such steady acropetal increase in stem diameter might exacerbate lodging susceptibility and this problem might even become more serious with increasing N-fertilization allowing minimum wind speed or rain to cause lodging. This also indicates that *E. tef* plants have a weak transition from shoot to root with a smaller plant-base diameter causing poor tapering. Absence of an effect on stem-base diameter by CCC is not unique to *E. tef* as it has also been reported in wheat and barley (Gendy & Hofner 1989; Berry *et al.* 2000).

PBZ-treated plants had a higher tiller number compared to CCC-treated plants. A continuous application of a high PBZ dose strongly promoted tillering but inhibited tiller elongation. This limits leaf expansion and photo-assimilation thereby reducing carbohydrate reserves required for rapid growth (Stavang *et al.*, 2009). Promotion of

tillering by GA deficiency and inhibition of stem elongation has been recently also reported for other cereals (Rajala, 2003; Lo *et al.*, 2008). However, in contrast to wheat, barley and oats, where increased tillering was found after foliar and seed treatment with CCC (Naylor *et al.*, 1989; Craufurd and Cartwright, 1989; Peltonen and Peltonen-Sainio, 1997; Peltonen-Sainio *et al.*, 2003), no significant increase in tillers was found in tef when treated with CCC. It has been observed, in general, that gibberellins tend to cause less development of auxiliary buds while promoting the elongation of already growing (initiated) stem as well as tillers (Peltonen and Peltonen-Sainio, 2001). On the other hand the application of GA-inhibitors promote tiller initiation and stunting of the central stem. Tillering is generally considered an adaptation to environmental changes. Under a long-day condition, as applied in this study, CCC treatment has been found to produce in other cereals more tillers per main shoot at maturity (Rajala, 2003). Further, in wheat a high number of tillers reduces plant productivity in terms of grain production and lodging resistance and it has been further suggested that a low tiller number per m<sup>2</sup> is required for lodging resistance (Tripathi *et al.*, 2003). In tef breeding, however, the main focus has been on obtaining high tillering with tall plants for improved grain yield. It would be therefore important for developing a lodging resistance tef ideotype where plant height is differentially controlled (decoupling of plant height and yield) while maintaining optimum number of panicle bearing tillers that is yet to be determined from the lodging stand point.

In *E. tef* breeding, taller plants with higher number of tillers have been the main focus for grain yield improvement. In this study, it has been demonstrated that CCC can reduce plant height without affecting grain yield. It is therefore important to develop a lodging-

resistant *E. tef* ideotype differentially controlling plant height and yield. Generally, this study has demonstrated that GA biosynthesis is a prime target for plant height regulation. Since CCC reduces plant height without affecting grain yield, this compound might be suitable for lodging prevention providing the advantage of high productivity. Treatment with CCC did not increase stem diameter, the diameter to height ratio however increased and CCC treatment would therefore also improve plant standability. Although PBZ is probably useful at lower concentrations, its high cost and persistence in the soil would restrict its wider application. However, future fine-tuning might be required to optimize CCC use for commercial application in lodging prevention without compromising seed yield. However, extensive use of chemicals such as CCC may not be sustainable for environmental concerns. In which case long term solutions through genetic modification of GA metabolism and targeting genes, such as the rice *sd-1* and the wheat *Rht* orthologs in *E. tef*, could be a strategy for plant height control in *E. tef*.



# 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<sub>0</sub> 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<sub>1</sub> generation was inconsistent although phenotypic characterization of semi-dwarf T<sub>1</sub> 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<sub>1</sub> 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<sub>1</sub> 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 embryogenic 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  $26\pm 2 / 18^{\circ}\text{C}$  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

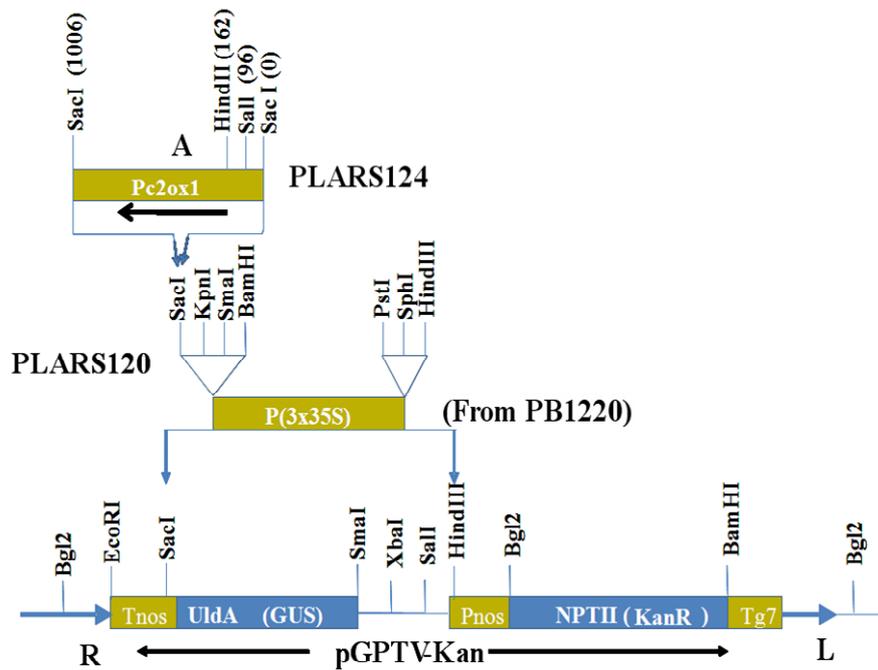
Immature embryos (IEs) from *E. tef* plants of cultivar DZ-01-196 were isolated and callus was induced according to the protocol reported by Gugssa (2008). Callus inoculation and co-cultivation with *Agrobacterium* during *E. tef* transformation and further regeneration was carried out following various 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 to 14 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°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<sub>2</sub>O (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°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 ( $50 \text{ mg l}^{-1}$ ) and rifampicin ( $25 \text{ mg l}^{-1}$ ) and plates were incubated for 2 days at  $28^\circ\text{C}$  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  $\mu$ l) were transferred into a 500 ml Erlenmeyer flask containing 250 ml LB/YEP medium and kanamycin (50 mg l<sup>-1</sup>). The culture was shaken at 200 rpm at 28°C until the OD<sub>660</sub> 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<sup>-1</sup> 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<sup>-1</sup> 2,4-D, 250 mg l<sup>-1</sup> cefotaxime (or 250 mg l<sup>-1</sup> timetin) and 100 mg l<sup>-1</sup> 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<sup>-1</sup> 2,4-D, 200 mg l<sup>-1</sup> cefotaxime (or 200 mg l<sup>-1</sup> timetin) and 100 mg l<sup>-1</sup> 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  $24\pm 2^{\circ}\text{C}$ . Developing embryos (sometimes turning green) transferred to K4NM regeneration medium were grown under a 16 h photoperiod maintaining a temperature of  $24\pm 2^{\circ}\text{C}$ .

### 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 ( $50\text{mg l}^{-1}$ ) and cefotaxime ( $125\text{ mg l}^{-1}$ ). 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  $350\ \mu\text{molm}^{-2}\text{s}^{-1}$ . The temperature was maintained at  $23\text{-}27^{\circ}\text{C}$  (day) and  $15\text{-}18^{\circ}\text{C}$  (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<sub>2</sub> (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<sup>0</sup>C, 30 sec.; primer annealing: 60<sup>0</sup>C, 30 sec.; DNA extension: 72<sup>0</sup>C, 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<sub>1</sub> generation

T<sub>1</sub> generation transformed plants were grown from seeds after selfing putative transformed plants (T<sub>0</sub> generation) that have shown *GA2ox1* amplification by PCR from isolated genomic DNA. T<sub>1</sub> 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°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<sub>1</sub> 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  $-80^{\circ}\text{C}$  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.

<b>K99EM* based callus induction medium</b>	
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 <sub>2</sub> O; 2.17 mg (10 μM) 2,4-D; pH 5.8
<b>MS based co-cultivation medium</b>	
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
<b>K99EM based selection medium</b>	
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 <sub>2</sub> O; 2.17 mg (10μM) 2,4-D; pH 5.8, and 250 mg cefotaxime; 100 mg kanamycin
<b>K4NB** based regeneration medium</b>	
PRE-RE	Medium ( 1 L) contains: 0.25 M glutamine, 10 mM CuSO <sub>4</sub> , 36 g (100 mM) maltose H <sub>2</sub> O; 1 mM BAP; 50 mg kanamycin; 125 mg cefotaxime; pH 5.8
RE	Medium ( 1 L) contains: 0.25 M glutamine; 10mM CuSO <sub>4</sub> , 100 mM maltose H <sub>2</sub> O; 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 <sup>-1</sup> )	Organics I	Conc. (mg l <sup>-1</sup> )	Organics II	Conc. (mg l <sup>-1</sup> )
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	80 (1 mM)	Retinol	1.01 (0.04 μM)	Malic acid	40 (0.3 μM)
KNO <sub>3</sub>	2,022 (20 mM)	Thiamine HCl	1.0 μM	Citric acid	40 (0.1 μM)
KH <sub>2</sub> PO <sub>4</sub>	340 (2.5 mM)	Riboflavin	0.2 (0.5 μM)	Fumaric acid	40 (0.3 μM)
CaCl <sub>2</sub> ·2H <sub>2</sub> O	441 (3 mM)	Ca-panthenate	1.0 (4.2 μM)	Na-pyrovate	20 (0.2 μM)
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	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 <sub>4</sub> ·4 H <sub>2</sub> O	11.2 (50 μM)	Cobalamine	0.02	MES	213.2 (0.1 M)
H <sub>3</sub> BO <sub>3</sub>	3.1 (50 μM)	Ascorbic acid	2.0 (11.4 μM)	Maltose H <sub>2</sub> O	90,000 (250 mM)
ZnSO <sub>4</sub> ·7 H <sub>2</sub> O	7.2 (25 μM)	Calciferol	0.01 (0.03 μM)	2,4-D	2.17 (10 μM)
Na <sub>2</sub> MoO <sub>4</sub> ·2 H <sub>2</sub> O	0.125 (5 μM)	Biotin	0.01 (0.04 μM)		
CuSO <sub>4</sub> ·5 H <sub>2</sub> O	0.025 (0.2 μM)	Cholin chloride	1.0 (7.1 μM)	Phytigel	0.3%
CoCl <sub>2</sub> ·6 H <sub>2</sub> O	0.025 (0.2 μM)	p-aminobenzoic acid	0.02 (0.1 μM)	pH	5.8
KI	0.17 (1 μM)	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.

<b>Components of K4NB regeneration medium</b>			
<b>Inorganic salts</b>	<b>Concentration (mg l<sup>-1</sup>)</b>	<b>Organics</b>	<b>Concentration (mg l<sup>-1</sup>)</b>
<b><u>Macro-nutrients</u></b>		<b><u>Organics I</u></b>	<b><u>T-vitamins (1000x)</u></b>
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	320 (4 mM)	Thiamine HCl	10.0
KNO <sub>3</sub>	3640 (36 mM)	Pyridoxine HCl	1.0
KH <sub>2</sub> PO <sub>4</sub>	340 (2.5 mM)		
CaCl <sub>2</sub> ·2H <sub>2</sub> O	441 (3 mM)	<b><u>Organics II</u></b>	
MgSO <sub>4</sub> ·7 H <sub>2</sub> O	246 (1 mM)	Glutamine	0.25 mM
Na-FeEDTA	27.5 (75 μM)	CuSO <sub>4</sub>	10.0 mM
<b><u>Micro</u></b>		MaltoseH <sub>2</sub> O	100 mM
MnSO <sub>4</sub> ·4 H <sub>2</sub> O	11.2 (50 μM)	2,4-D	1 μM
H <sub>3</sub> BO <sub>3</sub>	3.1 (50 μM)	Phytigel (Sigma-PB169)	0.3%
ZnSO <sub>4</sub> ·7 H <sub>2</sub> O	7.2 (25 μM)	pH	5.8
Na <sub>2</sub> MoO <sub>4</sub> ·2 H <sub>2</sub> O	0.12 (0.5 μM)		
CuSO <sub>4</sub> ·5 H <sub>2</sub> O	1.25 (5 μM)		
CoCl <sub>2</sub> ·6 H <sub>2</sub> O	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.

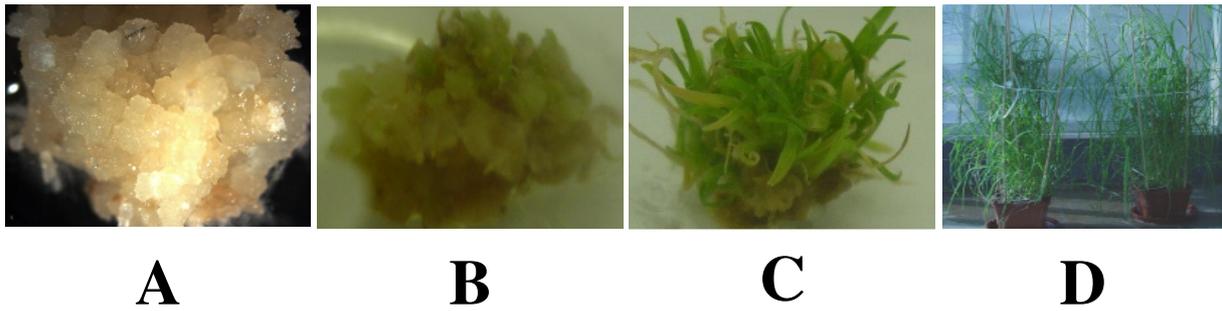
The antibiotic-containing selection medium was optimized for selecting kanamycin-resistant germinating mature embryos (Table 3.1). At 25 mg l<sup>-1</sup> G418, up to 75% of shoots derived from non-transformed embryos wilted after 12 days exposure to the antibiotic without completely collapsing. The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

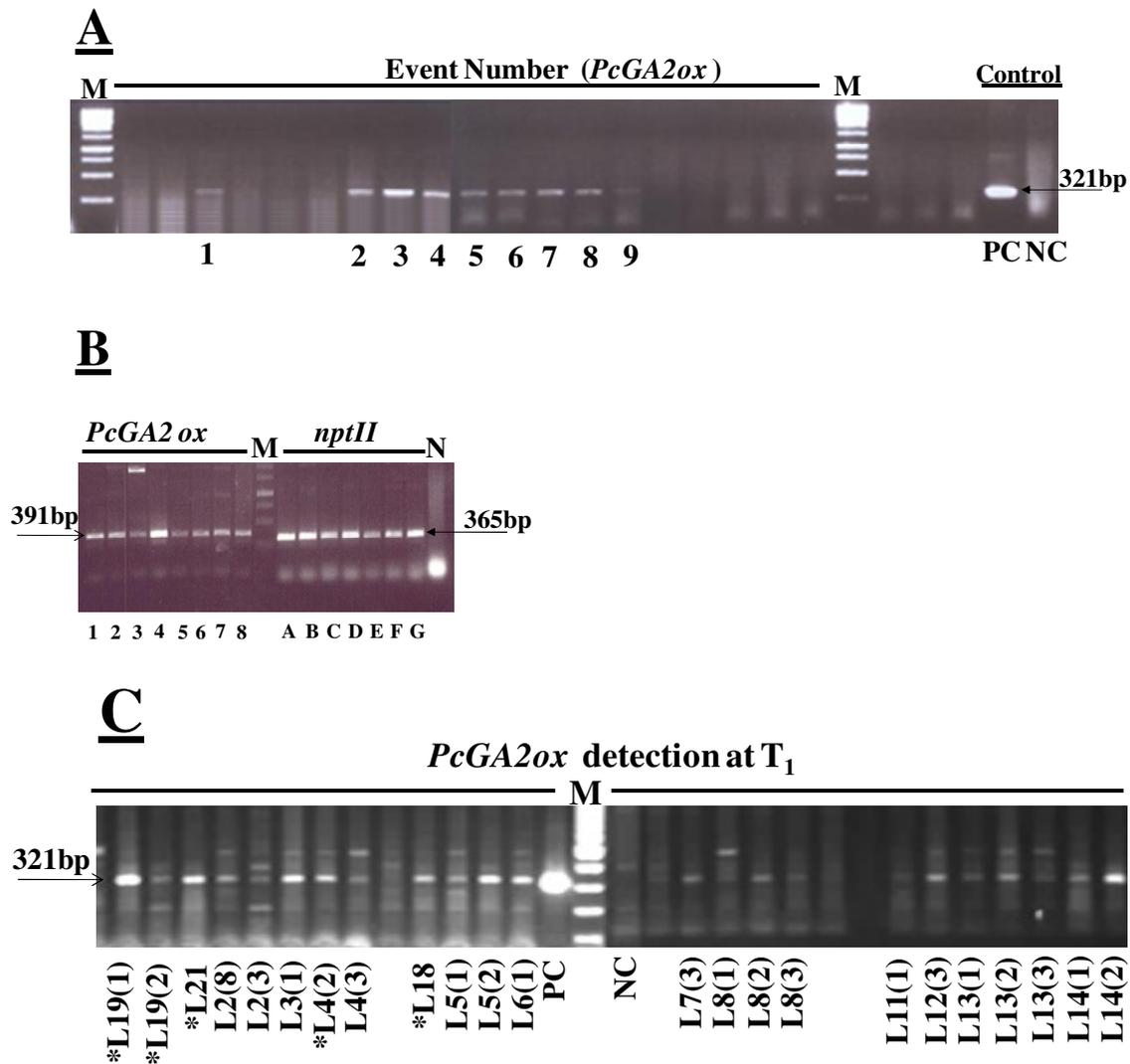
In 8 of the 55 putative transformed plants ( $T_0$  generation), which were regenerated and grown in the phytotron, the genome-inserted *PcGA2ox* or *nptII* sequences were detected by PCR in isolated genomic DNAs (Fig. 3.3). Sequence analysis of the amplified PCR product confirmed that amplified products 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.



**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 <sup>-1</sup> )	Survival (%)	Remarks
0	100	
20	100	<50% yellowing of leaf
25	100	<75% wilting and yellowing
30	100	100% yellowing of leaf with 1/3 <sup>rd</sup> 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<sub>0</sub> (A & B) and T<sub>1</sub> (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.

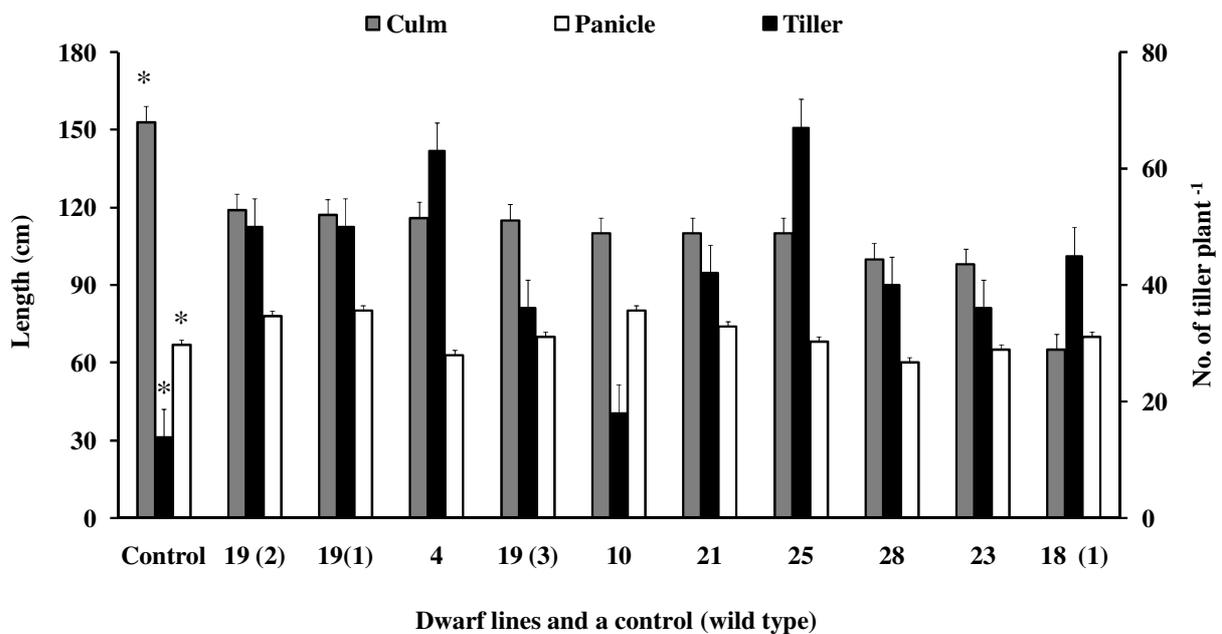
### **3.4.3 Phenotypic characterization**

#### **3.4.3.1 Culm, internode and panicle length**

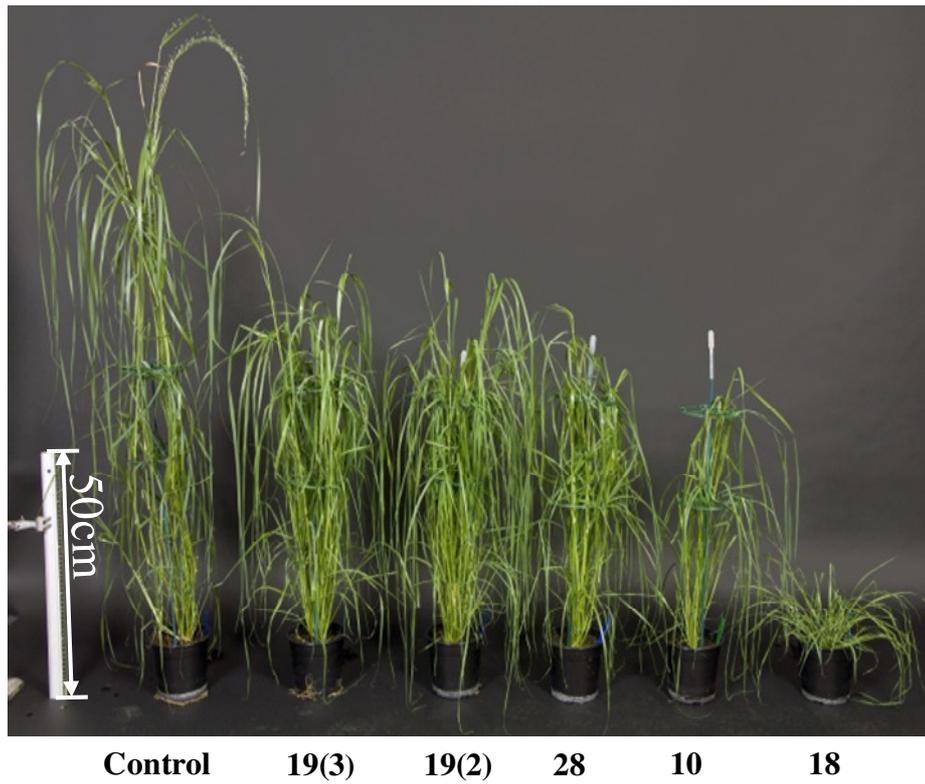
Despite inconsistent PCR results, significant ( $P < 0.001$ ) variation in mean culm height was found between T<sub>1</sub> plants, with a dwarf/ semi-dwarf phenotype, and wild-type non-transformed plants. Plants with a dwarf/semi-dwarf phenotype had a Culm height ranging from 65 cm to 117 cm whereas 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<sup>th</sup> and/or 8<sup>th</sup> 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<sup>rd</sup> 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<sup>th</sup> 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.

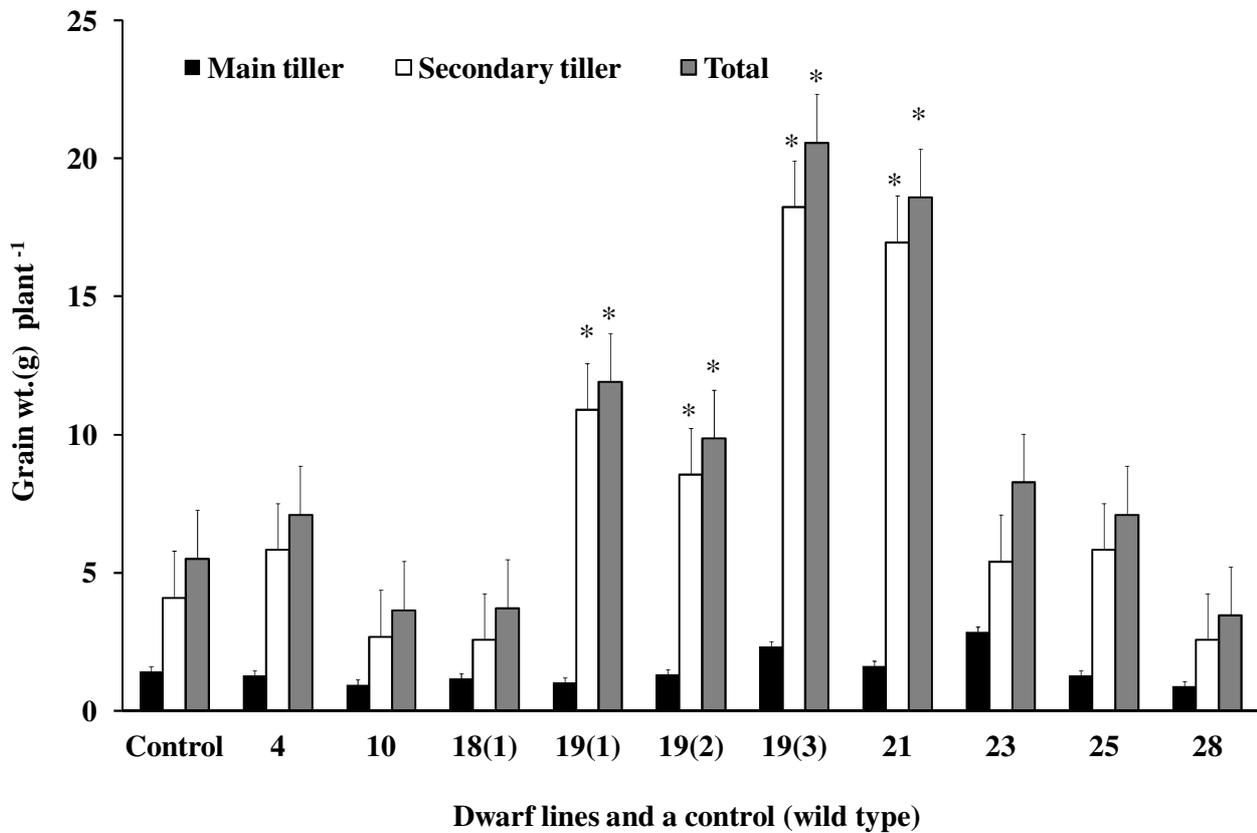


**Figure 3.4** Culm and panicle height (cm) and number of tillers per plant of putatively transformed dwarf *E.tef* plants. Standard error (SE) values and significance level was determined by Student's *t*-test using GenStat Discovery Edition (VSN International Ltd) ( $P \leq 0.001$ ).



**Figure 3.5** Selected *E. tef* dwarf (18) and semi-dwarf (19(3), 19(2), 28 and 10 T<sub>1</sub> 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

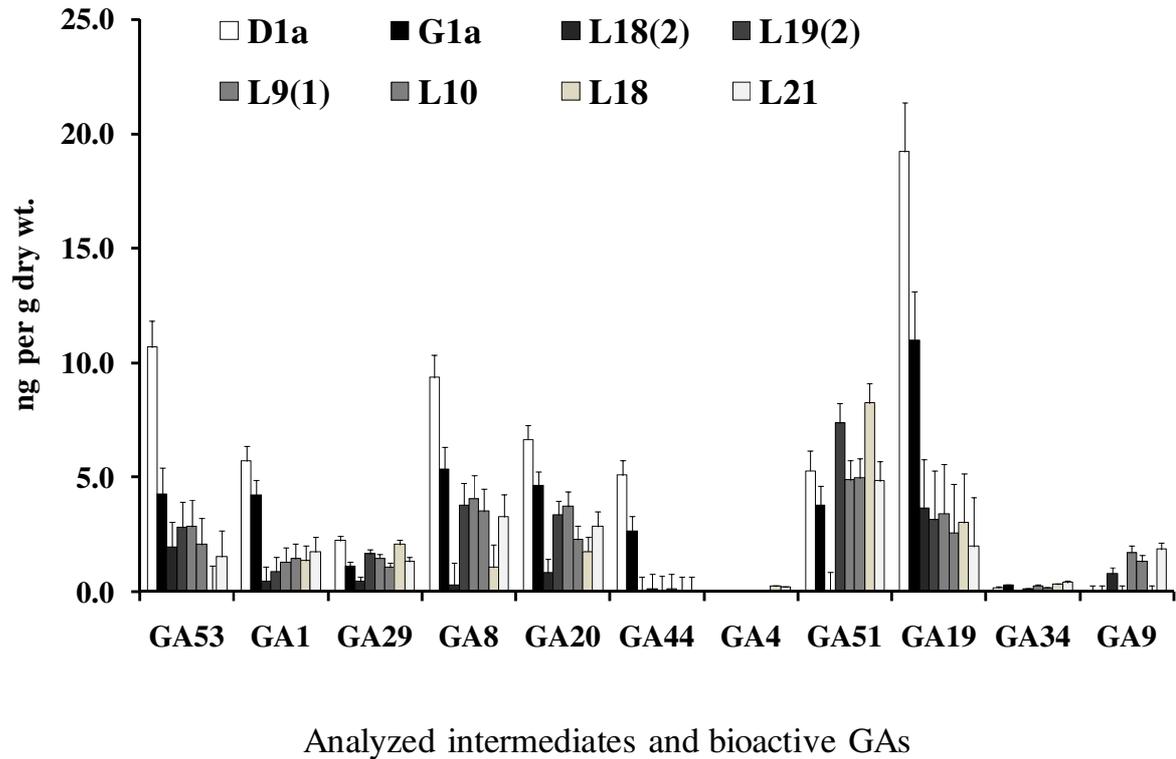
Lines	Shoot FW	Culm + Leaf DW	Panicle DW	Tillers Culm + Leaf DW	Tillers panicle DW	Total Shoot DW
<b>Control</b>	213.7	5.9	2.7	54.9	10.8	74.2
<b>4</b>	429.9	4.7	2.3	120.8	28.9	177.7
<b>10</b>	375.3	3.8	2.2	103.2	12.8	174.0
<b>18 (1)</b>	155.9	4.2	2.6	42.0	10.1	144.2
<b>19(1)</b>	493.3	4.5	2.3	134.4	36.5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

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<sub>1</sub>, 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<sub>1</sub> precursors, such as GA<sub>44</sub>, GA<sub>19</sub>, GA<sub>20</sub> and in particular GA<sub>19</sub>, were also reduced in the dwarf plants (Figure 3.7). An expected increase in GA<sub>29</sub> 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 13 $\beta$ -hydroxylation pathway (See Table 2.1) except in some cases like GA<sub>29</sub> and GA<sub>51</sub>) 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<sub>0</sub> 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<sup>-1</sup>. Gugssa (2008) previously found no inhibition of *E. tef* callus induction and somatic embryo formation from immature embryos with 50 mg l<sup>-1</sup> 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*.

*Agrobacterium* growth during inoculated callus development was suppressed using 250 mg l<sup>-1</sup> cefotaxime, but *Agrobacterium* growth was sometimes not completely blocked. A higher cefotaxime concentration (500 mg l<sup>-1</sup>), 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<sup>-1</sup>. 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<sub>1</sub>, a metabolite of 13 $\beta$ -hydroxylation and a dominant GA biosynthesis product in *E. tef*. However, accumulation of GA<sub>8</sub>, a deactivation product of the bioactive GA<sub>1</sub>, in the semi-dwarf plants was not proportional to the relative height differences or to deactivation of GA<sub>1</sub>.

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*.

## CHAPTER 4

**ISOLATION, CHARACTERIZATION AND EXPRESSION OF  
GA GENES WITH PARTICULAR EMPHASIS ON *GA20ox* IN  
*TEF* (*Eragrostis tef*)**

#### 4.1 Abstract

To isolate *GA20ox* and *Rht* genes and further characterize and monitor the expression of *GA20ox* in *E. tef*, degenerated primers derived from rice, wheat, maize and sorghum ortholog sequences were used of full or partial sequences (422 to 1500 bp) by normal, “anchored” and nested PCR. Three putative *GA20ox* genes orthologous to the rice *sd-1* (*semi-dwarf 1*) were identified. Sequences contained the characteristic domains KLPWKET and NYYPXCXXP and the conserved H and D amino acid residues. Three more sequence orthologous to the wheat *Rht* gene, the rice *Elongated Uppermost Internode* (*Eui*) and a Cytochrome P450 monooxygenase gene involved in brassinosteroid (BR) deactivation were also isolated. The *E. tef Rht* sequence had the conserved motifs DELLA, VHYNP, VHVVD, and a C-terminus of the GRAS domain. *E. coli* expressed *GA20ox1a* catalyzed the conversion of the [<sup>14</sup>C]-labelled gibberelline precursor GA<sub>12/</sub> to GA<sub>9</sub>. The three *GA20ox* were further differentially expressed in different plant tissues. *EtGA20ox1a* and *EtGA20ox1b* had highest transcription in the uppermost internodes whereas *EtGA20ox2* transcription was low in most tissues. Further, in *E. tef EtGA20ox1b* was possibly the functional equivalent to the rice *sd-1* gene. All sequences showed close homology with ortholog genes from sorghum, maize, rice barley.

## 4.2 Introduction

Among the genes of GA-biosynthesis, GA 20-oxidase is shown to be an important regulator in the pathway. It catalyses several late steps converting GA<sub>12</sub> and GA<sub>53</sub> in parallel pathways to respective products GA<sub>9</sub> and GA<sub>12</sub> which then are converted to bioactive forms, GA<sub>4</sub> and GA<sub>1</sub> by GA 3β-hydroxylase (see chapter 1 “Introduction”). Availability of genes encoding GA-biosynthetic enzymes has allowed understanding how these genes function. In many species the members of the dioxygenases family, GA 20-oxidases, are encoded by several genes. They show distinct spatial and temporal expression patterns with some overlapping function in plant development regulated by environmental signals and endogenous factors (Hedden and Phillips 2000; Lange, 1998; Hedden and Kamiya, 1997). Such functional redundancy in the GA 20-oxidases has been found for many crops and has been considered as the reason why null mutations in some of the genes exhibit a semi-dwarf phenotype. The existence of an overlap between expression patterns of isozymes and mobility of the GA products within a plant system prohibits severe dwarfing of the stem (Hedden and Phillip, 2000). Due to their regulatory role determining GA concentrations, members of this gene family have been the target for plant genetic manipulation and introduction of agronomically useful traits (Appleford *et al.*, 2006; Carrera *et al.* 2000; Sakamoto *et al.* 2003).

Genomic DNA amplification by PCR for isolation of gene sequences is a common procedure and has also been used in this part of the study to isolate GA biosynthesis and signalling genes. A genomic survey at the NCBI database (<http://www.ncbi.nlm.nih.gov>), as done for this study, is also a vital tool to provide information for the design of primers for gene amplification in PCR application for reverse transcription (e.g. RACE). The Rapid Amplification of cDNA Ends (RACE) is a method for amplifying DNA sequences from a

mRNA templates between a defined or identified internal site and unknown sequences at either to the 3' or the 5' -end of the mRNA. It requires two gene sequence-specific primers that flank the region of sequence to amplify relatively few target molecules in a complex mixture (Miao *et al.*, 2010). Therefore, 3' and 5' RACE methodologies offer possible solutions to the severe limitation impose on the PCR to amplify regions of unknown sequences. The 5' RACE, also known as “anchored” PCR, is a technique that facilitates the isolation of unknown 5' ends from low-copy transcriptions. Low level of concentration is expected for phytohormones and their corresponding related genes such as *GA20ox* that involve in the, GA, regulation (Hedden and Phillip, 2000).

No GA gene so far has been isolated from *E. tef* to allow studying the allelic diversity of GA genes in the genetic pool, explore their functional roles to identify a useful mutation in GA genes. Having verified the importance of GA in *tef* height regulation, therefore, the objectives of this part of the study was to first identify and clone full-length cDNAs from various GA genes in particular encoding the multifunctional GA20-oxidase. Secondly, the objective was to characterize these cloned GA gene sequences on the genomic level and study their expression in various plant parts during plant development.

### **4.3 Materials and Methods**

#### **4.3.1 Plant material and plant growth**

Seed material of tef (*Eragrostis tef*) variety DZ-01-196 for this experiment was obtained from the Ethiopian Institute of Agricultural Research, Holetta Agricultural Research Centre, Ethiopia. Seeds were germinated on germination mix soil and the seedlings were grown in pots (about 225 plants per m<sup>2</sup>) under a 26±2 / 18°C day/night temperature and a 14 hr day length. Plants were further supplemented with a half-strength Hoagland nutrient solution until all samples were collected.

#### **4.3.2 Genomic DNA isolation**

Young leaves were homogenized in liquid nitrogen and transferred to a reaction tube containing pre-heated (60<sup>0</sup>C) extraction buffer according to the method of Harini *et al.* (2008) with a modification omitting the NaCl addition. DNA spooling was further replaced by precipitating the DNA. Samples not immediately used were frozen and stored at -80<sup>0</sup>C. The mixture of fine-powdered plant material was immediately incubated after homogenization in extraction buffer at 60<sup>0</sup>C for 30 min with intermittent shaking. This was followed by adding an equal volume of chloroform: isoamyl alcohol (24:1) and subsequent centrifugation at 6000 g for 10 min. The aqueous phase was further extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 8000 g for 10 min. After centrifugation, the supernatant was transferred into 2.5 volumes of chilled ethanol and genomic DNA was precipitated. Further purification was made using isopropanol which was followed by centrifugation at 8000 g for 10 min at 4<sup>0</sup>C to collect precipitated DNA. Washing

of precipitated DNA was carried out with 70% ethanol twice followed by centrifugation. The genomic DNA was finally dissolved either in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or dsH<sub>2</sub>O and further treated with RNase and precipitated again as outlined above. DNA concentration of samples was determined using the nanodrop technique (Thermo Scientific NanoDrop 3300) and then visually by running 2 µl of sample DNA on a 1% agarose gel to check for purity.

#### 4.3.3 Gene identification and isolation

Identification and cloning of GA biosynthetic genes, the rice *SD-1* homologues (*GA20-oxidases*) in *E. tef* was carried out using degenerated oligonucleotide (sense and antisense) primers (Table 3.1). Primers for specific amplification of the genes sequences were designed based on the published sequences of the *GA20ox* genes of different monocot plants accessed from the GenBank. Primers were designed based on conserved amino acid domains of rice, sorghum, maize and wheat orthologous sequences obtained from the GenBank database. The sequences of these nucleotides were designed using the primer3 program (Rozen and Skaletsky, 2000). The PCR conditions were optimized using these primers to amplify either genomic DNA or cDNA.

**Table 4.1** Primers used in PCR to amplify *GA20ox* gene fragments using *E. tef* genomic DNA

Target gene	Primers	Degenerate/specific primers (5' – 3')
GA20 ox	F1	GCTGCCGTGGAAGGAGAC
	F2	CACCGATGATGATGATGATGATG
	F3	CTACGCGAGCAGCTTCACG
	R1	CGCCGATGTTGACGACGA

Primers for *GA20ox* were targeted to the region encoding the amino acid sequence KLPWKET (sense) and NYYPXCXXP (antisense). PCR amplification of *E. tef* genomic DNA (gDNA) and complementary DNA (cDNA) was done using either Dream Taq (Fermentas, Canada) or FastStart Taq (Roche, UK) DNA-polymerases. A 50 µl reaction mixture consisted of the following components: 5x PCR buffer, 10 µl MgCl<sub>2</sub> (25 mM), 2 µl dNTPs (10 mM each); 1 µl each for forward and reverse primers (10 µM), 1 µl DNA (100 ng/µl); 1 µl DMSO; 0.25 µl Dream Taq DNA-polymerase (5 U/µl); 33.75 µl H<sub>2</sub>O. PCR reactions were prepared on ice and run at the following standard cycling conditions: 94°C (2 min) followed by 35 cycles consisting of 94°C (30 sec); 55°C-65°C (30 sec); 72°C (1 min/kb). This was followed by DNA extension at 72°C for 7 min and then holding the reaction at 4°C.

When amplification of a high-fidelity PCR component was required, the “Phusion” polymerase proof reading enzyme was used (Finnzymes). The PCR reaction contained the following: 10 µl 5x Phusion “GC” buffer; 1 µl dNTPs (10 mM each); 1 µl each for forward

primer (10  $\mu$ M); 1  $\mu$ l DNA (100 ng/ $\mu$ l); 1  $\mu$ l DMSO; 0.5  $\mu$ l Phusion polymerase (2 U/ $\mu$ l); 34  $\mu$ l H<sub>2</sub>O. Preparation of the PCR reactions were carried out on ice with reactions running at the following standard cycling conditions: 98°C (30 sec) followed by 35 cycles with 98°C (10 sec); 55°C-70°C (30 sec); 72°C (1 min/1kb) and DNA extension after 35 cycles at 72°C for 7 min followed by holding the reaction at 4°C. Genomic DNA amplifications were done using GC-rich buffer and addition of DMSO (molecular grade) in reactions to improve amplification efficiency. PCR products were visualized on a 1% agarose gel. For cloning, bands were excised from the gel and purified with a commercially available PCR product purification kit (Qiagen, Germany). The extracted DNA was used as a template for cloning, or nested PCR using the conditions described above. Cloning was carried out using the pGEM-T Easy cloning vector (Invitrogen, USA).

#### 4.3.4 Isolation of complete *E. tef GA20ox* coding regions

For extension of *GA20ox* homologous (GA20 ox1-ox3) to their unknown 5' and 3', a total of 20 primers either as specific primers SPR1, SPR2 or SPR3 (nested primers for upstream extension in 5' direction) and SPF1 and SPF2 (nested primers for downstream extension in the 3' direction) were designed and used for nested PCR amplification (Table 5.2). cDNA synthesis was done using anchor primers (supplied with cDNA synthesis kit; Invitrogen, USA) and manually designed specific primers (SP primers) (Table 5.2). Primers were allowed to anneal identified polymorphic regions to also enable selective amplifications of homologous upstream and downstream regions of alleles using reverse PCR (RACE-PCR). cDNA synthesis. PCR amplification procedures were done using the 2<sup>nd</sup> Generation 5'/3' RACE Kit (Roche, Switzerland) following the manufacturers recommendations for full-length cDNA synthesis of genes of interest.

Primers for isolation of a full-length sequence of GA20 ox1 (1420 bp), but containing also the start and stop codon and restriction sites BamHI and HindIII (underlined), were designed as follows, sense: 5'-AGG GAT CCA GCC AGC TGC CCG TGA TG-3' and antisense: 5'-TGA AGC TTA ACA GAA CAG GCG GTC ATG GAT GAC-3'. A nucleotide ("A") was added to this primer to ensure that the start codon (ATG) in the GA20 ox1 is "in-frame" during after cloning into the plasmid pET-32a(+). In order to avoid error incorporated during the PCR-amplification, high fidelity "Phusion" polymerase proof reading enzyme (Finnzymes) was used and DNA sequences were determined from at least 3 -5 independent clones of the amplified product.

**Table 4.2** Primers used for GA20ox sequence RACE- PCR amplification

Target gene	Primers	Degenerate/specific primers (5' – 3')
GA20 ox1	GA20 ox1SPR3	GCGGCAGCGTGAAGAAGGCGTCCAT
	GA20 ox1SPR2	CACATCATCATCATCATCATCGGT
	GA20 ox1SPR1	CTCGGCGGGTAGTAGTTGAGGCGCAT
	GA20 ox1SPF1	CCTTCGTCGTCAACATCGGCG
	GA20 ox1SPF2	CGGAGACAACCAAAGGAGGCG
GA20 ox3	GA20 ox3SPR3	GTCTCCTTCCACGGCAGCAATCG
	GA20 ox3SPR2	CGATTGGCAGTAGTCTCGGAACA
	GA20 ox3SPR1	CGGGCACGGCGGGTAGTAGTTG
	GA20 ox3SPF1	GCAGGGACTTCTTCGCCGACG
	GA20 ox3SPF2	CGGGAGCCATCGTCGTCAACATC

#### 4.3.5 Cloning and sequencing of PCR products

Cloning of the purified PCR products was done using the pGEM-T Easy vector system (Promega, UK). Insert and plasmid DNA (pGEM-T Easy) was mixed at a 3:1 ratio in a 10 µl ligation mixture with 1 µl T4 DNA ligase (5 U/µl) and 1x T4 DNA ligase buffer (Invitrogen, USA). The mixture was incubated at room temperature overnight. Competent Top10 or DH5α *E. coli* cells were transformed with DNA fragments legated into the pGEM T-easy plasmid by heat shock treatment at 42°C for 45 sec followed by 2 min incubation of transformed cells on ice. *E. coli* cells were then incubated for 1-1.5 hr at 37°C with rotation

(150 rpm) on a shaker after adding 250  $\mu$ l SOC media (20 g/l bacto-tryptone, 5 g/l bacto-yeast extract, 0.5 g/l NaCl, 20 mM glucose, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, pH7.0). Transformed cells (200  $\mu$ l) were then plated onto 2YT agar plates (16 g/l bacto-tryptone, 10 g/l bacto-yeast extract, 5 g/l NaCl, 1.5% (w/v) agar, pH 7.0) supplemented with 0.5 mM IPTG, 0.01 mg/ml X-Gal and 0.1 mg/ml ampicillin allowing blue-white selection of transformed colonies.

Five white colonies were cultured overnight in 5 ml 2YT media supplemented with 0.1 mg/ml ampicillin. Plasmid DNA was purified from cells using the “QIAprep plasmid mini kit” (Qiagen, Germany) according to the manufacturer’s instructions. Isolated DNA was eluted in 50  $\mu$ l buffer EB (Qiagen, Germany). Positive cloned inserts were confirmed by *Eco*RI digestion followed by agarose gel electrophoresis. Confirmed DNA samples were further run for sequencing in a PCR reaction containing 2  $\mu$ l BigDye Terminator 3.1, 2  $\mu$ l Sequencing Buffer (5X), 1  $\mu$ l M13 or custom-designed sequencing forward or reverse primers, 2  $\mu$ l sdH<sub>2</sub>O and 3  $\mu$ l template DNA. The PCR reaction product was then purified using Sephadex G-50 Fine Grade Slurry (Sigma, UK) and Centri-Sep columns before sequencing.

#### 4.3.6 DNA sequence analysis and phylogenetic analysis

DNA sequences were analyzed using the BLAST program (<http://www.ncbi.nlm.nih.gov/blast/>) by comparing with sequences in the GenBank database. Candidate nucleotide sequences were translated using the ExPASy Translation Tool (<http://au.expasy.org/tools/dna.html>) before BLAST search for determining homology of deduced amino acid sequences of closely related plant species. Phylogenetic analysis was computed using all the

amino acid sequences of related genes from the different species using CLC Bio Mainbench (Version 5.5)/ MEGA 4.1 (beta).

#### 4.3.7 RNA isolation and cDNA synthesis

The frozen immature leaf sample of DZ-01-196 was homogenized in liquid nitrogen followed by total RNA extraction using the RNeasy kit (Qiagen, Germany) following the instructions by the manufacturer. Up to 2 µg of total RNA and oligo dT (18 - mer) was used for cDNA synthesis following first-strand cDNA which was reverse transcribed by using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, UK) according to the manufacturer's instruction.

#### 4.3.8 Isolation of the *Rht* and other genes

Using an identical PCR approaches stated above (Section 4.2.3 to 4.2.7) were employed for designing primers, running PCR, cloning and sequencing of PCR products for the isolation of from *E. tef* homologous sequences to the wheat *Rht* gene and other genes (the rice Elongated Uppermost Internode (EUI) and brassinosteroid deactivation (BR)). Primers used are shown in Table A1 and A2 (See Appendix).

#### 4.3.9 GA 20-oxidase expression in *E. tef*

The expression of the three *E. tef* GA20 oxidases in different *E. tef* tissues at different developmental stages was determined by a quantitative real time PCR (qRT-PCR) using purified total RNA from different tissues. The qRT-PCR analysis was done using the

LightCycler technique for quantitative reverse transcription (RT)-PCR of the mRNA levels of the gene of interest using SYBR Green (fluorophore that binds double-stranded DNA) to produce fluorescence for detection. Various internal control primers were designed (Table 4.1) based on constitutively expressed house-keeping *tef* Actin and 25S rRNA gene to optimize qRT-PCR measurements. The *E. tef* 25S rRNA gene was selected and amplification was optimized based on the LightCycler 480 (Roche Diagnostic, UK) result. The crossing point value (CP) was generated representing the fractional cycle number at which the amount of amplified target reaches a fixed threshold. The following protocol was used to determine the expression ratio: the  $C_t$  value difference between each triplicate was kept below 1.5 and outliers with  $SD > 2$  were removed from the analysis and the mean  $C_t$  and standard deviation (SD) for each sample were determined. In the first step of analysis ( $\Delta C_t$ ), all samples values were normalized with respect to the least expressed sample. The highest  $C_t$  value was subtracted from the  $C_t$  value of each sample where the least expressed sample will have a  $\Delta C_t$  value of 0. The difference of samples from the reference value was computed using the function ( $\Delta C_{t \text{ sample}} - \Delta C_{t \text{ reference}}$ ) to obtain a  $\Delta \Delta C_t$  value used to calculate  $2^{-\Delta \Delta C_t}$  along with calculating the means for each group, the standard deviation and standard error of the mean (SEM). This provides a relative expression ratio (in arbitrary units) and variation between ratios.

Expression of GA20ox sequences from *E. tef* was further analyzed using total RNA extracted from different *E. tef* tissues at the same time i.e. at rapid stem elongation stage (10-15 days before panicle emergence), except the young panicle (inflorescence), which was sampled three weeks later. The RNase Plant Mini Prep kit (Qiagen, Germany) was used for RNA isolation according to the manufacturer's guide followed by DNAase treatment. RNA (2.0  $\mu$ g) was converted into cDNA using the Superscript III First-Strand Synthesis System for RT-

PCR (Invitrogen, UK). The reaction was stopped by heat inactivation at 94 °C for 5 min. PCR amplification of tef GA20ox cDNA using 0.4 mM of the specific primers (Table 5.1) that have been designed on the basis of polymorphic regions of the three cloned homologous sequences of the EtGA 20-oxidase gene. PCR DNA amplification was carried out at 94 °C for 1 min followed by a 32 cycles for GA20ox1 and GA20ox1b and a 40 cycle for GA20ox2 of 94 °C for 1 min, 67 °C for 30 sec and 72 °C for 30 sec. The length of the expected amplification fragment ranged between 80-190 bp. Tef SDH gene was used as a control.

**Table 4.3** Primers used in quantitative and semi-quantitative PCR amplification of GA20ox sequences

Target gene	Primer	Primer sequence (5' – 3')
GA20 ox1	GA20 ox1 02F	ATGTGGTGGGCTACTACGTCAGCAAG
	GA20 ox1 01R	TCATCTCCGAGCAGTAGCGCCCGT
GA20 ox2	GA20 ox2 02F	CGGCCACACCCCTCTTGCTCCA
	GA20 ox2 02R	TTGACGACGATGGCTCCCGGCTT
GA20 ox3 01F	GA20 ox3 02F	GGACTACCTGGTGGGCCGC
	GA20 ox3 01R	TAGTAGTTGAGCCGCATGATGGAGTCG
SDH	SDH 01F	CACAGCTGAGCGCTACGTTCTC
	SDH 01R	CCCAATGCAACACCGAAAATACG
25S	25S 01F	ATAGGGGCGAAAGACTAATCGAACC
	25S 01R	GAACTCGTAATGGGCTCCAGCTATC

#### 4.3.10 Expression of *EtGA20ox1a* in *E. coli*

The activity of *EtGA20 ox1* was monitored using a gel-purified PCR product (1.2 kb) that was amplified using primers sense: 5'-AGG GAT CCA GCC AGC TGC CCG TGA TG-3' and antisense: 5'-TGA AGC TTA ACA GAA CAG GCG GTC ATG GAT GAC-3' from a cDNA template. The PCR amplification was done using "Phusion" polymerase proof reading enzyme (Finnzymes) and the blunt-end PCR product was gel-purified and cloned into the plasmid pET32a vector (Novagen, UK) using the BamHI and HindIII (underlined) restriction sites. Competent *E. coli* DH5- $\alpha$  cells were transformed with the *E. tef* GA20ox-1 containing plasmid. From transformed cells, recombinant plasmid DNA was isolated and *E. coli* BL21 cells were transformed with the pET32-a fusion by heat shock treatment. A transformed colony was selected and cells were cultured overnight in 5ml 2YT containing 0.1 mg/ml carbenicillin for selection. A cell suspension (500  $\mu$ l) was then added to 50ml 2YT containing 0.1 mg/ml carbenicillin and incubated at 37°C for 2 hr and then 50  $\mu$ l of 1M IPTG was added to the cell suspension. Cells were then cultivated at 25°C for 6 hr under shaking (200 rpm). The cell suspension was then centrifuged for 5 min and the cell pellet was frozen at -20°C overnight.

#### 4.3.11 HPLC analysis

Defrosted cells were re-suspended in 1.5 ml lysis buffer containing in 10 ml 1 ml 1 M Tris-HCl, pH 7.5, 50  $\mu$ l of 1M DTT, 200  $\mu$ l of 50 mg/ $\mu$ l lysozyme, 8.75 ml sdH<sub>2</sub>O. The cell suspension (1.5 ml) was incubated for 15 min at room temperature, treated with DNase and cell debris were removed by centrifugation for 10 min in an Eppendorf centrifuge at 13 000 g at 4°C and the cell lysate was kept for at -80°C. The lysate was thawed and the following

added to 90  $\mu$ l supernatant: 5  $\mu$ l dioxygenase co-factor mix (containing in 5 ml 80 mM 2-oxoglutarate, 80 mM ascorbate, 80 mM DTT, 10 mM FeSO<sub>4</sub>, 40 mg mL<sup>-1</sup> BSA, and 20 mg mL<sup>-1</sup> catalase in 100 mM Tris-HCl, pH 7.5; Williams *et al.*, 1998) and 5  $\mu$ l substrate (GA12-<sup>14</sup>C). The mixture was incubated for 2 hr at 30<sup>0</sup>C under shaking at 200rpm. Glacial acetic acid (10  $\mu$ l) was added to the mixture and further diluted with 140  $\mu$ l sdH<sub>2</sub>O and centrifuged for 10 min at maximal speed in an Eppendorf centrifuge before HPLC analysis to measure the GA intermediate products catalyzed by this protein (GA20 ox1).

#### 4.3.12 Southern blot analysis

A DNA hybridization probe of 450 bp was synthesized using specific primers for a coding region of GA20 ox and a DIG labelling probe synthesis kit (Roche, Switzerland). Purified genomic DNA (15  $\mu$ g) was digested overnight with the restriction enzymes BamHI, BglII, EcoRI and HindIII. Digested genomic DNA was fractionated on a 0.7% agarose gel and transferred by the alkaline transfer technique (Roche, UK) to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech, UK). Transferred DNA was hybridized with a DIG-labelled probe in an oven at 42<sup>0</sup>C overnight. Membranes with hybridized DNA were incubated in a color substrate solution in the dark without shaking for at least 30 min for detection of hybridized DNA bands.

## 4.4 Results

### 4.4.1 Isolation of GA genes from *E. tef*

Identification and cloning of a *E. tef* ortholog of the rice *SD-1* (*SEMI-DWARF 1*) gene was carried out using a total of 5 degenerate oligonucleotides that were designed based on orthologous sequences of the conserved amino acid regions from rice, sorghum, maize, wheat and barley. Sequences were retrieved from the GenBank database using accession numbers of known gene sequences from rice and wheat using BLAST search (NCBI). A partial sequence closely related to the 2-oxoglutarate dependent oxygenase gene family was first obtained by PCR amplification from *E. tef* DNA, which shares conserved domains of GA biosynthetic pathway genes. However, analysis revealed that this sequence is lacking the important domains that are characteristic of the 2-oxoglutarate dependent dioxygenase (2ODD) genes (*GA20ox*, *GA3ox* and *GA2ox*) involved in the GA biosynthetic pathway (Hedden and Kamiya, 1997). When primers F1, F2, F3 and R1 were used, three PCR fragments (*E. tef1*, *E. tef2*, *E. tef3*) were obtained with sizes between 422-523 (Figs. A.1A and B; Fig. A.2C, D, E and F see Appendix). After cloning of fragments into the plasmid pGEM-T Easy and sequencing the nucleotide sequence of these fragments showed a >60% homology with known *GA20ox* orthologous sequences from closely related cereal species such as sorghum, maize, rice, wheat and barley.

Using an identical PCR approaches GA signaling gene from *E. tef* homologous to the wheat REDUCED HEIGHT (RHT) gene was isolated. Additional gene sequences isolated were the rice Elongated Uppermost Internode (EUI) and the Cytochrome P450 monooxygenase gene involved in brassinosteroid (BR) deactivation both involved in plant height development. Primer pairs RhtF1/ F2, RhtR1/R2 and Rht SPF and RhtSPR for Rht gene; EUIF1 and EUIR1 for Eui gene

and BRF1 and BRR1 for BR gene (Tables A.1 and A.2, see Appendix) allowed PCR amplification of the following fragments: (1) 1395 bp (near full coding region of *Rht* gene) that shares over 85% homology with the above species (Figs. 5.9, 5.10 and A.6 (see Appendix)), (2) 344 bp (*Eui* gene) which shares over 60% homology with *EUI* orthologous sequences from sorghum, wheat, maize, brachypodium and rice (Figs. A.3 and A.4 and A.5, see Appendix); and (3) 749 bp (*BR* gene), another Cytochrome P450 monooxygenase gene but with brassinosteroid deactivation activity was partially cloned using semi-degenerate oligonucleotides (Figs. A.7K and L; A.8 and A.9, see Appendix). The peptide sequence shared 49 - 52% homology with orthologous peptide sequences of genes involved in BRs catabolism.

#### 4.4.2 Putative *E. tef* GA20 ox isolation and cloning

Based on the known sequence information obtained previously for the three putative sequences of *E. tef*1-3 sequence specific primers (SP primers) (Table 4.2) were designed and used for nested PCR amplification. Generation of full-length sequences of coding regions was done using 5'/3' RACE (Random Amplified cDNA ends). Application of RACE for *E. tef* GA20ox resulted in two 5' and two 3' short sequences (contigs) with sizes of 238, 263 and 121 and 305bp. No further sequence extension could be obtained for *E. tef*2 and *E. tef*3 and GA20ox2. The four fragments of *E. tef*1 were aligned using "contig assembly" of the vector NTI program (Vector *NTI Advance*<sup>TM</sup>9.0). The consensus sequence of 1448bp was translated using the ExPASy protein translation tool. The sequence consisted of an open reading frame encoding a putative polypeptide of 482 amino acids. Comparison of the deduced amino acid sequence with other plant species (sorghum, rice, maize, wheat and barley) showed the presence of all characteristic conserved (consensus) amino acids sequences (domains):

LPWKET, (144-149 aa) and NYYPXCXXP (228-236 aa of the putative sequence) and three His residues for binding Fe<sup>2+</sup> (245-247 aa counting from the start codon). BLAST results of the full coding region of putative *E. tef GA2ox1* showed high identity scores with orthologous genes from *S. bicolor* (68.3%), *Z. mays* (68.7%), *O. sativa* (59.3%), *L. perenne* (64.6), *T. aestivum* (66.6%), *H. vulgare* (67.5%) and *Z. japonica* (54.5%) (Figs. 4.1 - 4.6).

Similarity among the *tef GA2ox* homologs and comparison with orthologs of other monocot species was determined based on amino acid sequences using partial sequences trimmed to core areas present in all the three sequences. *E. tef1* showed 89.6% and 59.8% homology to *E. tef3* and *E. tef3* sequences, respectively. *GA2ox* isolated from different species exhibits a conserved domain of amino acids with identity ranging from 50 % - 75% (Hedden and Kamiya, 1997). *E. tef1* and *E. tef3* sequences were found to be closely related to each other and *E. tef GA2ox2* sequence was found to be closely related to *GA2ox2* genes sequences from various other monocot species (Figs. 4.4 – 4.6). The three homologous sequences were tentatively named as *E. tef GA2ox1a*, *E. tef GA2ox1b* and *E. tef GA2ox2*.

The *E. tef GA2ox2* partial coding sequence has high polymorphism at the N-terminus when aligned and compared to orthologs from other closely related monocot cereal species. Generally, alignment and phylogenetic relationship further showed identity with *GA2ox* sequences from other plant species such *S. bicolor* (81%), *O. sativa (sd1)* (68%), *O. rufipogon* (72%), *T. aestivum* (57%), *H. vulgare* (58%), *Z. mays* (56%), *A. thaliana* (57%) and *L. perenne* (58%) when 112-122 aa sequences present in all sequences from those species were aligned (Figs. 4.3 and 4.4). *EtGA2ox2* also has a 120 bp intron between the two conserved functional domains (Fig. 4.3). Further, *EtGA2ox1a*, *EtGA2ox1b* groups closely

with GA20ox1 sequences from sorghum, maize, *Zizania* spp. and rice. A similar result was found for *EtGA20ox2* closely grouping with GA20ox2 orthologs from these plant species.



**Figure 4.1** Nucleotide sequence alignment of three putative *E. tef* *GA20ox* sequences. Putative Fe<sup>2+</sup>- binding consensus regions are indicated with highlighted asterisks (\*), the 2-oxoglutarate-binding motif is indicated over-lined, and the nucleotide sequence for the conserved LPWKET region, considered to be involved in the binding of GA substrate, is shown with double over-line. In *GA20ox2*, the 120 bp intron between LPWKET and NYYPPCPEP functional domains is not included in the alignment. Dashes (-) have been inserted to maximize sequence homology. Identical and similar regions are shown by light and dark shaded areas and dots whereas number indicates the position of the conserved regions within the predicted nucleotide sequence.



**Figure 4.2** Derived amino acid sequence alignment of two putative *E. tef* *GA20ox* (*E. tef1* and *E. tef3*) sequences with orthologous *GA20ox* gene sequences from sorghum (*S. bicolor*; Acc No. XP\_002463483.1), maize (*Z. mays*; Acc No. ACF83905.1), rice (*O. sativa*; Acc No. P93771.2), wheat (*T. aestivum*; Acc No. 004707.1 ), barley (*H. vulgare*; Acc No. AAT49058.1), lolium (*L. perenne*; Acc No. AAG43043.1), Zyocia (*Z. japonica*: Acc No. ABG33927.1) and Dasypyrum (*D. villosum*; Acc No. ACU40946.1). Putative Fe<sup>2+</sup>- binding consensus regions are indicated with asterisks (\*), the conserved motifs, NYYPXCXXP and LPWKET are shown with deep dark shades. Identical and similar regions are shown by light and dark shaded areas and dots whereas number indicates the position of the amino acid within the predicted peptide.

*E. tef* 2

LPWKETLSFGHRD-----VVEYFTSTLGSDFKPLGEVFRDYCQSMKEVSLAIMEVLGASLGVGRRYCRDFTADGC 70  
 TIVEIASFATFFFVTRDRSSESFYNDCTDRAMHGRR

*S. bicolor* GA20 ox2

*O. sativa* sd1

*O. sativa* GA20 ox2

*O. rufipogon* GA20 ox2

*P. sativum* GA20 ox

*A. thaliana* GA20 ox2

*Z. mays* GA20ox

*L. perenne* GA20ox

*T. aestivum* GA20ox1A

*H. vulgare* GA20ox1

.....	RRTSG--SHV	.D	.....	V	YQN	NA	.....	I	V	.....	S	Y	.....	S	78									
.....	FH.RAAAP----	V	AD	.S	.P	.A	M	R	YQK	EE	.L	.T	.L	EL	.....	E	G	Y	E	.....	SS	76		
.....	HANAAGNNSST	AD	.S	..	D	.H	..	YQE	EA	E	.TK	.A	..	E	.....	GG	Y	E	.....	SS	79			
.....	FH.RAAAP----	V	AD	.S	.P	.A	M	R	YQK	..	E	.L	.T	.L	EL	.....	E	G	Y	E	.....	SS	76	
.....	QFS.EKNS---	SNI	KD	LSN	.E	.Q	QF	..	YQE	EA	SKL	.G	..	L	M	.....	KECF	.....	EE	NK	77			
.....	QFSNDNSG---	SRT	QD	.SD	..	QE	EQF	K	YQ	..	EA	SSL	.K	..	L	..	N	D	F	G	.....	EE	ND	77
.....	RYT.DDDGDKSKDV	AS	.V	D	K	EGYRHH	..	YGR	SE	SRL	.EL	..	E	.....	HF	R	.....	Q	G	N	80			
.....	RSCPSE-----	PDL	.D	I	V	A	..	E	HRR	..	YAR	SE	SRL	.E	.....	AHY	R	.....	E	G	N	75		
.....	RSCPSD-----	PAL	.D	I	V	A	..	E	HRR	..	YAR	SE	SRL	.E	.....	AHY	R	.....	E	G	N	75		
.....	RSCPSD-----	PAL	.D	I	V	A	..	E	HRR	..	YAR	SE	SRL	.E	.....	AHY	R	.....	E	G	N	75		

*E. tef* 2

*S. bicolor* GA20 ox2

*O. sativa* sd1

*O. sativa* GA20 ox2

*O. rufipogon* GA20 ox2

*P. sativum* GA20 ox

*A. thaliana* GA20 ox2

*Z. mays* GA20ox

*L. perenne* GA20ox

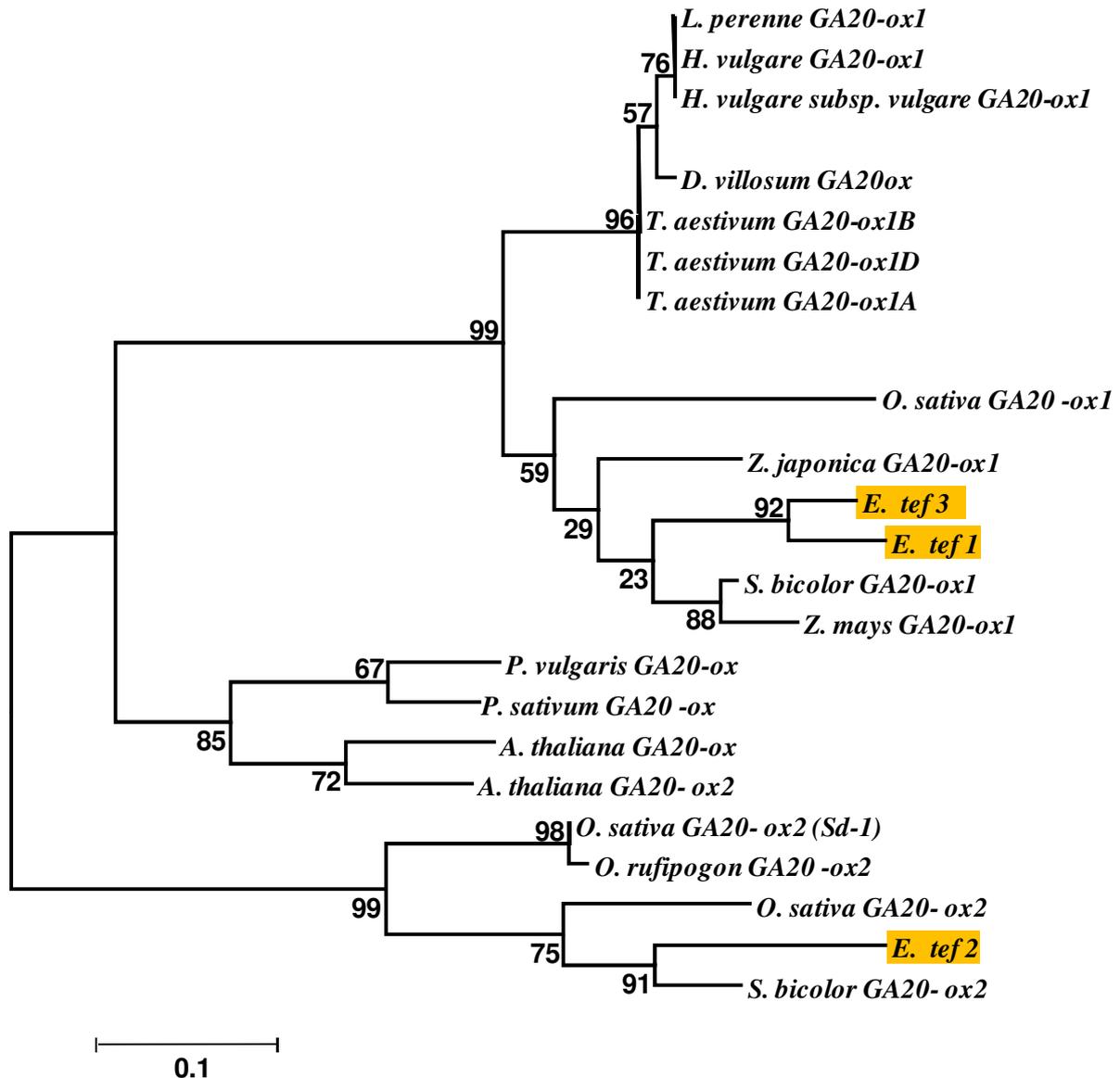
*T. aestivum* GA20ox1A

*H. vulgare* GA20ox1

SIMRCNY	YPPCPEPDR	TLGTGPHCDPA	AHTLL	LLQDDVDGLQ-	112	100															
.....	E	.....	S	L	V	.....	G	.....	117	81											
.....	E	.....	T	L	I	.....	-	G	EV	118	68										
.....	E	.....	S	L	V	.....	G	.....	V	122	72										
.....	E	.....	T	L	I	.....	-	G	EV	118	69										
.....	L	.....	Q	K	..	L	.....	T	S	L	I	H	..	-	Q	G	..	V	119	57	
.....	L	H	.....	Q	T	..	L	.....	S	S	L	I	H	..	-	H	N	..	V	119	57
.....	L	.....	Q	R	YD	.....	T	S	L	I	H	..	-	G	..	V	122	56			
.....	L	.....	Q	R	NE	.....	T	S	L	I	H	..	-	G	..	V	117	58			
.....	L	.....	Q	R	LE	.....	T	S	L	I	H	..	-	N	G	..	V	117	57		
.....	L	.....	Q	R	LE	.....	T	S	L	I	H	..	-	G	..	V	117	58			

Identity (%)

**Figure 4.3** Derived amino acid sequence alignment of putative *E. tef* GA20ox2 sequence (*E. tef2*) with orthologous GA20ox gene sequences from other grass species namely sorghum (*S. bicolor*; Acc No. XP\_002441117.1), maize (*Z. mays*; Acc No. ACN25832.1), rice (*O. sativa* and *O. rufogen*, Acc No. AAT44252.1 and BAK39011.1), wheat (*T. aestivum*; Acc No. 004707.1) barley (*H. vulgare*; Acc No. BAK04752.1), pea (*P. sativum*; Acc No. AAF29605.1) and Arabidopsis (*A. thaliana*; Acc No. NP\_199994.1). The position of the 37 aa intron region is indicated in the first row 23aa downstream LPWKETL motif between amino acids “G” and “E”. Identical and similar regions are shown by light and dark shades respectively and numbers indicate the position of the amino acid within the predicted peptide. Percentage of identity is shown in the parenthesis.

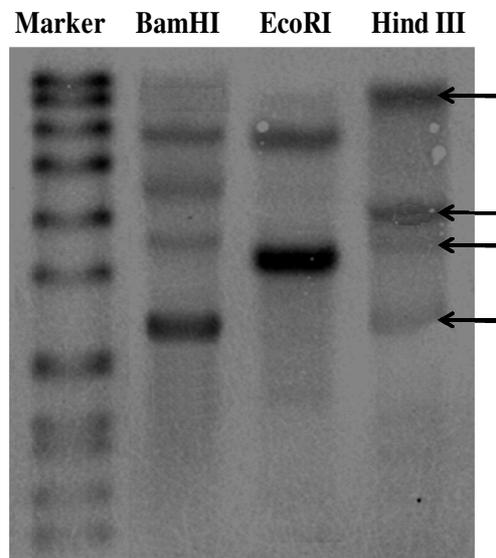


**Figure 4.4** Molecular phylogenetic analysis of the homologous of *E. tef* GA20ox sequences (*E. tef* GA20ox1, GA20ox1b and GA20ox2; underlined). The tree was inferred by using the Maximum Likelihood method based on the JTT matrix-based model. Initial alignment was done by mafft (<http://mafft.cbrc.jp/alignment>) software. The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows: when the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale with branch lengths measured in the

number of substitutions per site. All positions containing gaps and missing data were eliminated. There were a total of 105 positions in the final dataset.

#### 4.4.3 *EtGA20ox* copy number

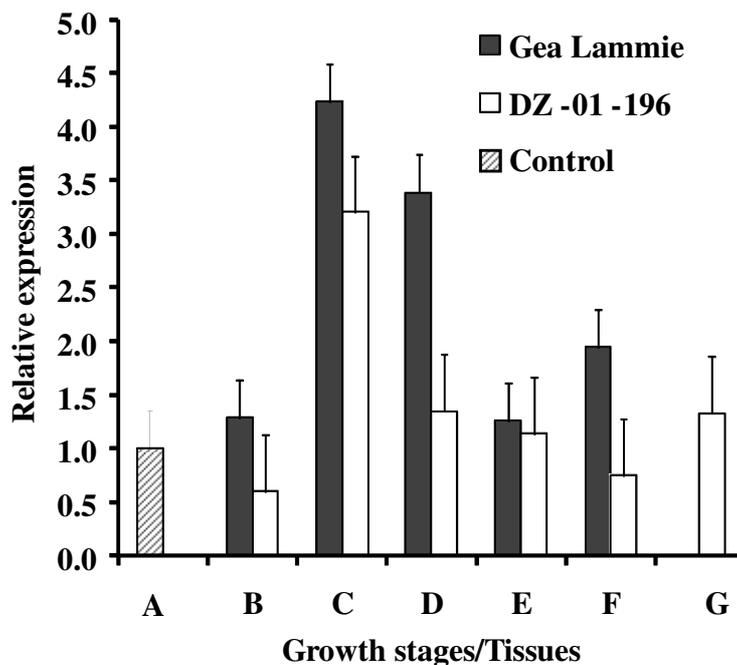
To determine the copy number of *EtGA20ox* gene in *E. tef*, Southern blot analysis of genomic *E. tef* DNA restricted with different enzymes (BamHI, EcoRI and HindIII) and probed with a 450-base-pair coding sequence of *EtGA20ox* was carried out. The probe hybridized with four fragments after the HindIII digest (one is very slightly visible); four fragments after BamHI digest, and two very visible and two hardly detectable fragments after EcoRI digest (Fig. 4.5). Therefore, four copies of *EtGA20ox* are possibly present in the allotetraploid *E. tef* genome.



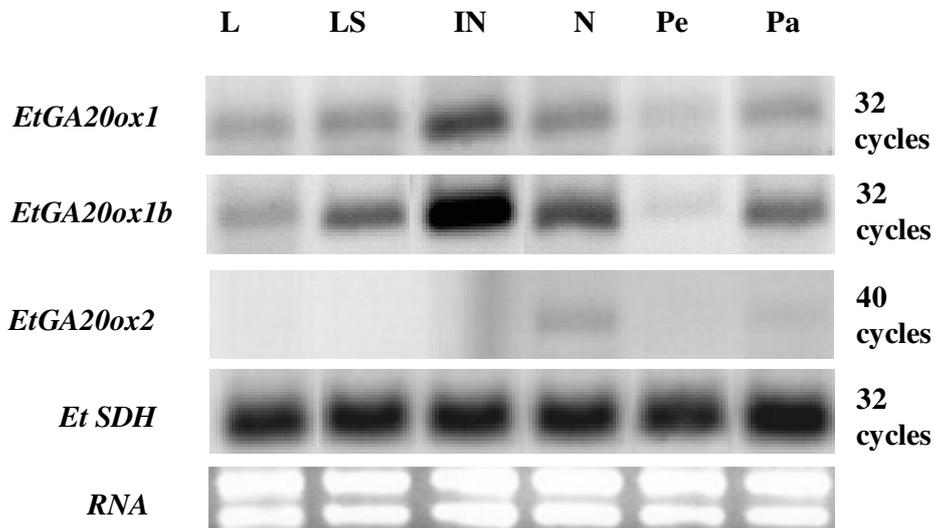
**Figure 4.5** Detection of *EtGA20 ox* gene copies in the *E. tef* genome after restriction enzyme digest using Southern blotting.

#### 4.4.4 *GA20ox* expression in *E. tef*

In both *E. tef* genotypes, Gea Lammie (short phenotype) and DZ-01-196 (long phenotype), *GA20ox1* was expressed in germinating hypocotyls, leaf, stem and inflorescence. Relatively higher expression was found at earlier stages in young stem and leaf (Fig. 4.17) with slight differences between the two genotypes. Further, when semi-quantitative PCR (RT-PCR) was used to study expression of the three *E. tef* *GA20ox* homologous sequences in leaf, leaf sheath, uppermost two internodes, nodes from these internodes, peduncle and inflorescence both *EtGA20ox1a* and *EtGA20ox1b* expression was highest in the uppermost internodes followed by nodes (Fig. 4.7). However, *EtGA20ox1b* expression was greater than *EtGA20ox1a* expression in the internodes, nodes, leaf sheath and panicle. In contrast, *EtGA20ox2* was expressed at relatively lower rates in the nodes and panicle but also in the upper most internodes when compared to the other two genes.



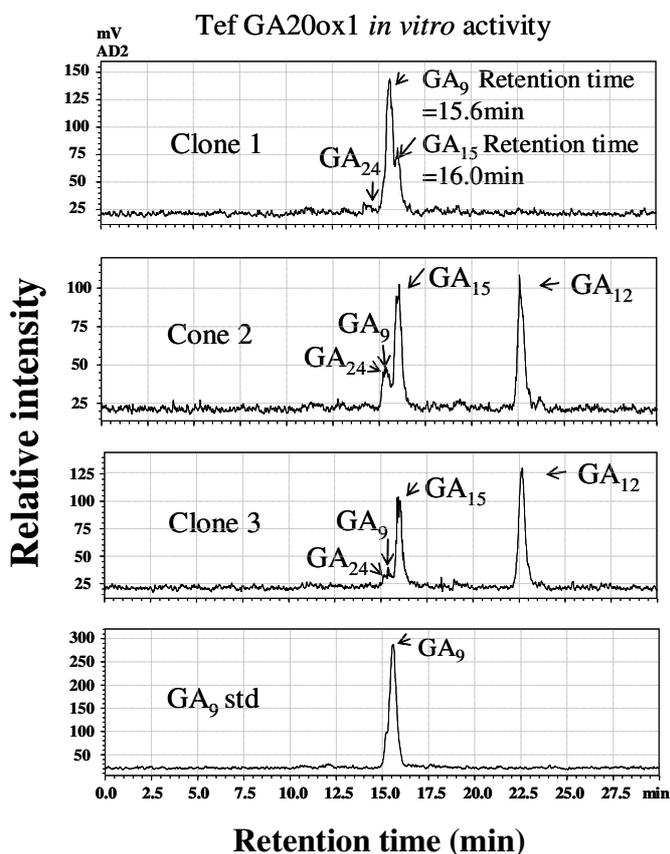
**Figure 4.6** Relative expression of GA20ox1 in two *E. tef* genotypes DZ-01-196 and Gea Lammie in different plant tissues and growing stages. (A) Control; (B) 3<sup>rd</sup> week stem + leaf; (C) 5<sup>th</sup> week stem + leaf; (D) 8<sup>th</sup> week stem; (E) 8<sup>th</sup> week leaf (F) Old (10<sup>th</sup> week) stem and (G) 10 days old inflorescence. The qRT-PCR was repeated three times, and gene expression level was calculated following the expression  $2^{-DCt}$  (Yang *et al.*, 2005).



**Figure 4.7** Semi quantitative RT-PCR Expression analysis of three *EtGA20ox* genes in various plant tissues (L=Leaf; LS=Leaf sheath; IN= Internode; N= Node; Pe= Peduncle; Pa= Panicle) from cultivar DZ-01-196 sampled at the stage of stem elongation and panicle initiation (for panicle). RT-PCR was performed with 32 and 30 cycles for *EtGA20ox* and *EtSDH*, respectively. The experiment was repeated three times with similar result. *EtSDH* was used as an internal PCR amplification control.

#### 4.4.5 *In vitro* enzymatic activity of *GA20ox* in a heterologous system

In *E. coli* expressed *GA20ox1* protein catalyzed the conversion of the [ $^{14}\text{C}$ ]-labelled gibberellin precursor  $\text{GA}_{12}$  to  $\text{GA}_9$  which could be detected by full-scan GC-MS. In some clones only a partial conversion of the substrate was found, possibly due to sub optimal reaction conditions, and intermediates of the reaction pathway,  $\text{GA}_{24}$  and  $\text{GA}_{15}$ , were also detected by HPLC analysis (Fig. 5.8). Clone 1 showed optimum activity by complete conversion of  $\text{GA}_{12}$  into  $\text{GA}_9$ . Clones 2 and 3 showed only partial activity.



**Figure 4.8** Radiochromatograms after HPLC of *E. tef GA20ox1* activity products after incubation with a  $^{14}\text{C}$ -labeled  $\text{GA}_{12}$  as a substrate (standard shown at the bottom).

#### 4.4.6 Isolation of the *Rht* and other genes

For isolation and cloning of *E. tef Rht*, specific primers (SP) (Table A.1) were designed based on a 344 -base-pair known sequence for the *Rht* gene. Primers were used for nested PCR to amplify a putative *Rht* orthologous gene from *E. tef* similar to the procedure used for *GA20ox* to obtain a full-length sequence. PCR amplification resulted in two 5' and two 3' contigs providing a total fragment size of 1395 bp after assembling the contigs. The sequence analysis of the amplified cDNA fragment (1395bp) consisted of an open reading frame encoding a putative polypeptide of 465 amino acids. Comparison of the *E. tef Rht* amino acid sequence with sequences from other plant species, such as sorghum, rice, maize, wheat and barley, showed the characteristic conserved amino acids domains such as the DELLA and GRAS domains for the *Rht* gene (Figs. 5.9 and 5.10). When the aligned sequences were trimmed to core areas present in all sequences (438-465 aa) a high identity to sequences of *Z. mays* (90%), *O. sativa* (86%), *S. bicolor* (85%), *T. aestivum* (84%) and *A. thaliana* (55%) were found. The above gene sequence in *E. tef* was named *EtRht* according to the nomenclature used by Cloes *et al.* (1999).

Partial sequence for brassinosteroid inactivating gene that encodes the Cytochrome P450 monooxygenase family gene, was also cloned using semi-degenerate oligonucleotides. After optimization of PCR conditions degenerate primers F1: 5' - ACA GCC GCA GCG TCT CGT T(GCT) - 3' and primer R1: 5' - A(GA)(CG) (CG)(TC)C (CA)AC GGC GA(CGA) (GC)(CG)(AT) GTT, were used to obtain 749 bp long fragment. Further sequencing and comparison through BLAST-search showed homology range between 49 - 52% with ortholog gene sequences of the Cytochrome P450 monooxygenase family for closely related species including the rice CYP734A1 involved in BRs catabolism (Fig. A.10 and Fig. A.11).



Domain I

Domain II

```
EE-VDELLAALGYKVRSSDMADVAQKLEQLEMAMGMGGVP---AADDGFVSHLATDTVHYNPSDLSSWVESMLSELNAPPPLPPAPAPPAPQL-VSTSSTVT-GGGSG 103
D.....GGAGAT.....GGAGAT.....A.....-T.....R.-A.....S.AAA. 106
-M.M.V.....GGAGAT.....I.....-.....R.-A.....S.AAA. 100
D.....SAPG.....L.....I.....-.....ARH-A.....G....- 104
GNMD.V.E.E.L.TM.SN---VQE-L.E.Y.LDN.P.L.....A.SNGLDP----- 82
.....A.....G-AGA.P.S.AT.....T.....-.....NA.....GS.--- 100
```

```
AGYFDPPPAAVDSSTYALKPIP-SPVAA-PADP---SADSAREPKRMRTGGGSTSSSSSSSSSMGGGGARS SVVEAAPP---ASAAANAPAVPVVVVDTQEAGIRLVH 204
.....L.....PS.....T.....D.RT.....ATQ.....G.....M.P 211
.....L.....V-S..S---T.T.....D.RT.....ATQ.....G..... 205
-F.EL.A.A.....R.S-L.V-T..S---A.....DT.....L.AS.G.....AMQGA..... 208
--VLPS.EICGFPA.D.D.V.GNAIYQF.IDS---SS.NNQN.LKSCSSPD.MVT.T.TGTQI.VIGTT.TTTTT---TTT.AESTRS.I.L.S.N.V..... 184
-...L.S.....I.R.....AG-T.PADL---V.D.....S.....L.....-V.A.NAT.L..... 202
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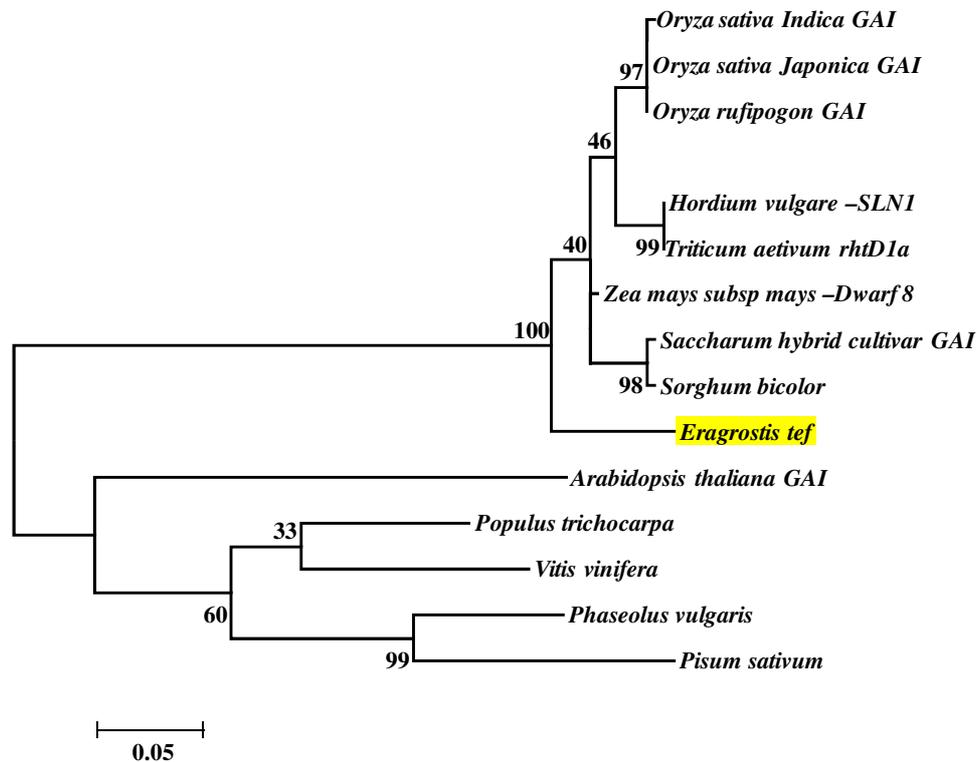
```
ALLACAEAVQOENFSAAEALVKQIPMLASSOGGAMRKVAAYFGEALARRVYRSPPPPTAPSSSTPPSPTSSTPHFYESCPYLKFAHFTANQAFLEAFAGCRRVHVVDVFGI 314
.....FR..DSS-LLDAAFADLLHA.....I..... 320
.....D.....FR.T.DSS-LLDAAVADFLHA.....I..... 314
.....A.....T.A.....FR.-ADST-LLDAAFADLLHA.....I..... 316
M.I.N.LTL.....GC.V.A.....T.A.....I.LS-QNQ--IDHCLSDTLQOM.....T.....I.E.KK.....I.SM 291
.....L.....L.A.....F.FR.Q.DSS-LLDAAFADLLHA.....I..... 311
```

```
EOGMOWPALLQALALRPGPPSFRLLTGVGPPQPDETDALQOVGWKLAQFAHTIRVDFQYRGLVAATLADLEPFMLQPEGE-ENDEEPEVIAVNSVFEMHRLLAQPGALEK 423
K.....D-DT.D.....L..... 429
K.L.....H.....D-DK.....L..... 423
K.....ADAN.....L..... 426
N.L.M.E.T.I.A.NS.H.H.E.C.L.EA.H.E.E.F.NS.DAS.ELRPS---DT.AV.....L.K.GR.GI 396
K.....EDPN..... 421
```

Identity (%)

VLTGTVRAVRPKIVTVVEQEANHNSGSFLDRFTQSLHYYSTMF	465	100	<i>E. tef Rht</i>
.....R.....T.....E.....	471	90	<i>Z. mays D8</i>
.....R.....T.....E.....	465	85	<i>S. officinarum GAI</i>
.....H.....R.....E.....	468	86	<i>O. sativa Indica GAI</i>
..V.KQIK.V.F.....S..GPV.....E.....L	438	84	<i>A. thaliana GAI</i>
.....R.....T.....E.....	463	55	<i>T. aestivum rht-D1a</i>

**Figure 4.9** Amino acid sequence alignment of the putative *tef Rht* sequences with orthologous amino acid sequences from maize (*Z. Mays*; Acc No. AAL10325.1), rice (*O. sativa*; Acc No. EAY91579.1), wheat (*T. aestivum*; Acc No. Q9ST59.1) and sugarcane (*S. Officinarum*; Acc No. AAZ08571.1) and Arabidopsis (*A. thaliana*, Acc No. BAC42642.1). The putative sequence shows the characteristic domains (dark shaded) for this gene: a conserved N-terminal: DELLA motif (Domain I), a VHYNP motif (Domain II) and VHVVD. The red boxes in *Z. maize* and *T. aestivum* in domain I and domain II show positions of small internal deletions and introduction of a stop codon (in wheat) in alleles that cause semi-dwarf phenotype (Benetzen and Mulu, 2000). Identical and similar regions are shown by grey and light grey shaded areas and dots whereas numbers indicate the position of the amino acid within the predicted peptide.



**Figure 4.10** Molecular Phylogenetic analysis of *E. tef* putative *Rht* gene by Maximum Likelihood method. The evolutionary history was inferred based on the JTT matrix (Jones *et al.*, 1992). Initial alignment was done by mafft (<http://mafft.cbrc.jp/alignment>) software. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 478 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

#### 4.4 Discussion

In this study full and partial coding sequences of three GA biosynthesis gene homologs for the GA20ox has been isolated, characterized and the expression analyzed. The putative *E. tef* GA20ox genes showed close homology with genes of other cereal species (sorghum, maize, rice, wheat, barley) and a grass species *Zyocia japonica*. The putative GA20ox genes belong to the GA 2-oxoglutarate-dependent dioxygenase (2-ODD) gene family as confirmed by the heterologous expression assay producing final and intermediate products of the GA20ox enzymatic actions. For convenience, gene nomenclature has been kept consistent with the naming of genes used in several recent publications about GA-biosynthesis and signalling genes in plant hormone metabolism (Thomas *et al.*, 1999; Sakamoto *et al.* 2004; Spielmeier *et al.* 2004).

The three *E. tef* GA20 oxidases also have characteristic conserved amino acid residues, namely the 2-oxoglutarate co-factor, LPWKET motif, very likely involved in the binding to the GA substrates (Xu *et al.*, 2002), and the NYYPXCQKP motif for common co-substrate binding (Miao *et al.*, 2010). The conserved H and D residues are involved in Fe<sup>2+</sup> binding at the active site of isopenicillin N synthase (Hedden and Phillip, 2000 and Xu *et al.*, 1995). In *E. tef* GA20ox2 an intron (120 bp) has been further found 23 aa downstream of the LPWKET domain.

The three *E. tef* GA20ox genes (hereafter named EtGA20ox) showed differential transcription in different plant tissues when semi-quantitative RT-PCR was applied. Generally, *EtGA20ox1a* and *EtGA20ox1b* were similarly expressed in tissue with highest transcription in the uppermost

internodes followed by nodes. In contrast, *EtGA20ox2* transcription was relatively lower than transcription of either *EtGA20ox1a* or *EtGA20ox1b*. These two genes might therefore act to promote internode elongation and panicle growth and might further also be involved in reproductive growth of the panicle. Such overlapping transcription has also been found in *Arabidopsis* where *AtGA20ox1* and *AtGA20ox2* act redundantly promoting elongation in the hypocotyls and internode, flowering time, and elongation of anther filaments including seeds number per silique (Rieu *et al.*, 2008). Also, in *Arabidopsis* GA20ox1 greatly contributes to internode elongation (Galun, 2010). Further, in rice *OsGA20ox2* (*SD-1*) is a predominant dwarfing gene with a role in height control (Spielmeyer *et al.*, 2002, Monna *et al.*, 2002). Results in this study indicate, however, that *EtGA20ox1b*, and not *EtGA20ox2*, is the functional gene in *E. tef* for height control equivalent to *OsGA20ox2* (*SD-1*). However, a future study is required to investigate whether *EtGA20ox1b* regulation will affect *E. tef* plant growth as previously found in rice.

*EtGA20ox1a* also showed differential transcription when quantitative RT-PCR was used. This was dependent on growth stages and plant parts in the two genotypes, Gea Lammie and DZ-01-196. *EtGA20ox1a* transcription was found in DZ-01-196 in the immature (emerging) inflorescence, germinating hypocotyls, young stem as well as leaf. However, *EtGA20ox1a* transcript abundance was generally higher in the shorter genotype, Gea Lammie, than in the taller genotype DZ-01-196. It is still unclear if higher transcript abundance in the short genotype depends on a feedback response interfering GA biosynthesis since the level of bioactive GA measured in Gea Lammie in this study was about half the amount in DZ-01-196. Increased transcript levels (reduced sensitivity to exogenous GA application) have been reported for

response pathway mutants in different crops due to impairment in the response genes (Olszewski, *et al*, 2002). Also a low bioactive GA amount despite relatively high transcription of *GA20ox* has to be further investigated.

In a heterologous expression, *EtGA20ox1a* substrates are converted through several successive oxidations at C-20 of GA<sub>12</sub> to the alcohol and aldehyde intermediates GA<sub>15</sub>, GA<sub>24</sub> and GA<sub>9</sub> (Hedden and Kamiya, 1997 and Yamaguchi 2008). Further, it has been shown that GA<sub>9</sub> is converted by 3 $\beta$ -hydroxylation to the bioactive GA<sub>4</sub> (Junttila *et al.*, 1992 and Rood and Hedden, 1994). All these products could be detected when *EtGA20ox1a* was expressed in *E. coli* and reacted also with GA<sub>12</sub> as a *EtGA20ox1a* substrate instead of GA<sub>53</sub> which is the natural substrate in *tef* plants. This sequence was successfully expressed converting substrates to final intermediates product except in few cases where GA<sub>15</sub> was a more abundant product due to incomplete downstream reactions.

In this study, a *Rht* ortholog and a partial sequence of an *Eui* and a BR deactivating gene were also isolated from *E. tef*. However, none of them were further characterized regarding their transcription in *E. tef*. The *E. tef Rht* gene has a conserved N-terminal DELLA motif (Alevy and Harberd, 2005). In general, when the *Rht* gene is expressed it represses downstream genes in the absence of GA inhibiting plant growth (Hedden, 2006). Other conserved *Rht* motifs which were also found in *E. tef Rht* include the TVHYNP domain essential for the perception of an upstream GA signal, VHVVD, and a C-terminal GRAS domain which are the functional domains responsible for transcriptional regulation such as the suppressive function of DELLA proteins against GA action (Peng *et al.*, 1997; Silverstone *et al.*, 2001; Ueguchi-Tanaka *et al.*, 2005). Two

conserved N-terminus DELLA domains (I and II) (Fig. 12; see Appendix) found in *E. tef Rht* are highly conserved regions in angiosperms (Yasumura *et al.*, 2007) presumably necessary for DELLA interaction of the peptide during GA signalling process (Alevy and Harberd, 2005).

The *Eui* gene also isolated from *E. tef* is a single recessive gene responsible for culm length modifications. *Eui* mutants have a longer culm length due to elongation of the uppermost internodes (Zhang *et al.*, 2008). Ectopic expression of the *Eui* coding sequence under the control of the rice *GA3ox2* and *GA20ox2* gene promoters reduced plant height (Zahang *et al.*, 2008). Further, the isolated *E. tef BR* gene has been found to be closely related to the rice CYP724As (Cytochrome P450 monooxygenases) involved in BR catabolism (Sakamoto *et al.*, 2011). Several of closely related sequences also identified through a BLAST search in this study were also related to BR inactivation. Studies have shown that BR mutants develop a dwarf phenotype. In rice, CYP734As control bioactive BRs by direct inactivation of castasterone (CS), a bioactive BR, and by the suppression of CS biosynthesis thus decreasing the levels of BR precursors. In Arabidopsis, CS and brassinolide (BL) are inactivated mainly by two Cytochrome P450 monooxygenases (CYP734A1/BAS1 and CYP734A1/BAS1) that inactivate CS and BL by C-26 hydroxylation (Ohnishi *et al.*, 2009).

In summary, genes were isolated in this part of the study allowing further characterization for their function. Given the redundancy of the GA biosynthetic genes reported in this study for tetraploid *E. tef*, it can be assumed that finding for instance a recessive dwarfing *sd-1* homologue mutant may be challenging since it requires independent mutations in both its A and B genomes. In such a case the semi-dominant *Rht* mutation version (full coding region cloned) is an easier

alternative for TILLING application and easy to express phenotypically due to its semi-dwarf nature. They can now be directly used for genetic engineering approaches using the transformation protocol developed for *E. tef*, which is part of this PhD study. Further, sequences might allow mutagenesis and selection through TILLING or Eco-TILLING and marker assisted breeding in the conventional and modern *E. tef* development process targeting lodging resistance. The genomic information developed through this part of the study therefore provides useful information for further studies to understand and establish the precise roles in plant growth of these isolated genes and for direct use in lodging resistance improvement through modern and conventional techniques.

## **CHAPTER 5**

### **EVALAUTION AND ANALYSIS OF MUTANT TEF (*Eragrostis tef*) LINES FOR DWARFISM FOR LODGING RESISTANCE**

## 5.1 Abstract

To evaluate *E. tef* plants generated via mutagenesis to induce dwarfism, selected mutant lines were evaluated for traits including culm height and diameter, internode number and length, panicle length, shoot biomass, tillering and grain yield under GA sprayed and non-sprayed conditions. Semi-dwarfed phenotypes could be developed in *E. tef* through mutagenesis approach and culm height was significantly reduced (23.1 cm - 41.7 cm) in three mutant lines. These mutants were semi-dwarfed with short culm and peduncle length. Regardless of height, grain yield was considerably reduced in all the mutants showing severe defects in fertility except mutant GA-10 which gave a reasonable yield. Line GA-10 also had a significantly higher diameter at 2<sup>nd</sup> - 5<sup>th</sup> and at 7<sup>th</sup> internodes contributing to the stiffness of the stem and also had the highest panicle dry weight among mutant lines tested. Internode diameter showed consistent increase acropetally with weak tapering. All semi-dwarfed mutants did not respond to GA treatment. Plants of G-10 possibly harbours a mutation in GA signalling. Since biomass production in mutant line GA-10 was not reduced this line might be used for crossings with other parental lines to restore yield without losing its other useful traits.

## 5.2 Introduction

Several studies have shown morphological traits that are related to the lodging in *E. tef* to be related to plant height, stem diameter of lower internodes, panicle length, biomass and seed weight (Chanyalew, 2010; Hundera *et al.* 1999; Ketema, 1983; Mengesha *et al.* 1965). Considerable efforts have been made over the last 50 years to incorporate by conventional breeding desirable agronomic traits into *E. tef*. However, no lodging resistance traits, such as reduced height and stiff straw, have been so far reported using conventional breeding (Assefa *et al.*, 2010).

Mutation breeding has been carried out in cereals to induce semi-dwarfness (Narahari, 1985) and also to solve the lodging problem (Maluszynski and Szarejko, 2003). In general, inducing mutation in target genes using various mutagens provides rapid generation and enhancement of genetic variability (Nichterlein, 2000). Short stature mutants without changing the background character of important traits have been beneficial not only to improve lodging resistance but also to increase productivity. This has been possible because of a more efficient partitioning of the dry matter resulting in high harvest index and increased grain yields in dwarf mutants in cereals due to pleiotropic effects (Hanson *et al.*, 1982; Hu, 1973; Nichterlein, 2000). In *indica* type rice, a spontaneous mutation resulting in semi-dwarfs led to the development of the high-yielding variety 'IR8'. This variety, together with its descendants, was responsible for the Green Revolution in Asia. More than 60 dwarf or semi-dwarf mutant lines have been so far reported for rice. Some of these are allelic to *sd1*, but no mutation, other than in the *sd1* locus, resulted in improvement of agronomic performance. In barley several lines with induced mutation has

resulted in superior breeding material (Maluszynski and Szarejko, 2003) and in wheat, semi-dwarf phenotypes have been obtained through mutation in the *Rht* loci.

Recently a TILLING (Targeting Induced Local Lesion IN Genomes) approach has been carried out for *E. tef* to generate variability in plant height and to develop semi-dwarf phenotype for lodging resistance in *E. tef* (Esfeld and Tadele, 2010). In this part of the overall study, the first objective was to select mutant lines from the TILLING M3 population to evaluate them for morphological (plant stature) and yield traits to possibly identify any desirable mutation for lodging resistance development in *E. tef*. The second objective was to characterize potential candidate mutant lines for changes in the DNA sequence of particular target genes (*GA20ox* homologues and *Rht*).

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### **5.3 Materials and Methods**

#### **5.3.1 Plant material**

Seed material of mutagenized *tef* (*Eragrostis tef*) lines (M3 progenies) of variety DZ-Cr-37 (fairly tall) was obtained from Dr. Zerihun Tadele, University of Bern, Switzerland.

#### **5.3.2 Plant growth and GA treatment**

Plant growth experiments were carried out in an environmentally controlled greenhouse at Rothamsted Research, UK, from May to August 2010. Up to 36 seeds per each mutated line ( 9

per pot) were germinated and thinned down to 3 per pot and grown on a compost mix consisting of peat (75%), sterilized loam (12%), vermiculite (3%) and grit (10%). The mix was supplemented with a slow releasing fertilizer containing 15-11-13 NPK plus micronutrients. Selected seedlings were maintained in the same pot [15 cm diameter (top) x 12.5 cm (height) and 10 cm (bottom)] maintaining 3 seedlings per pot. Since these mutants were from M3 seeds (successively selfed plants), uniformity was kept among the segregating plants by removing seedlings with phenotypes that resembled the wild-type. When the mutant phenotype was very close to the wild-type, selection was not possible and seedlings were selected at random. Six plants per treatment were hand-sprayed with 100  $\mu\text{M}$  GA3 every week on the surface of the leaves. Seedlings were grown for 14 wks until plant maturity in an environmentally controlled greenhouse using a 16 h photo-period provided by natural light supplemented with light from sodium lamps to maintain a minimum PAR of 350  $\mu\text{mol m}^{-2}\text{s}^{-1}$ .

#### 5.3.2.1 Growth measurements

Culm length was measured from main culms from the ground to the base of the spike. Culm diameter was measured at harvest time from the main culm at the basal internode with a digital calliper. Grain yield and shoot biomass were measured for each plant in each replication. Panicle length of main tillers was measured from the node, where the lower base emerges above the peduncle. Tiller height was determined by summing the culm height as well as peduncle and panicle lengths. Internode and tiller number were determined by counting the number of internodes or tillers per plant. Internode diameter was measured about 3 mm above the each node using a standard digital calliper. Dry weight was determined from above-ground plant material

by drying fresh material at 80°C for 2 days in an oven. Grain yield was determined by measuring the weight of seeds from all tillers.

#### 5.3.2.2 Data analysis

Growth and yield data were analyzed using the SAS statistical package (SAS Institute Inc., Cary, NC, USA) for Analysis of Variance (ANOVA) and Pearson Correlation Coefficients. Statistical significance of difference between treatment means was determined using the Tukey's Studentized Range (HSD) test. A *P*-value of  $\leq 0.05$  was considered as significant.

## 5.4 Results

### 5.4.1 Culm height, internode length and diameter

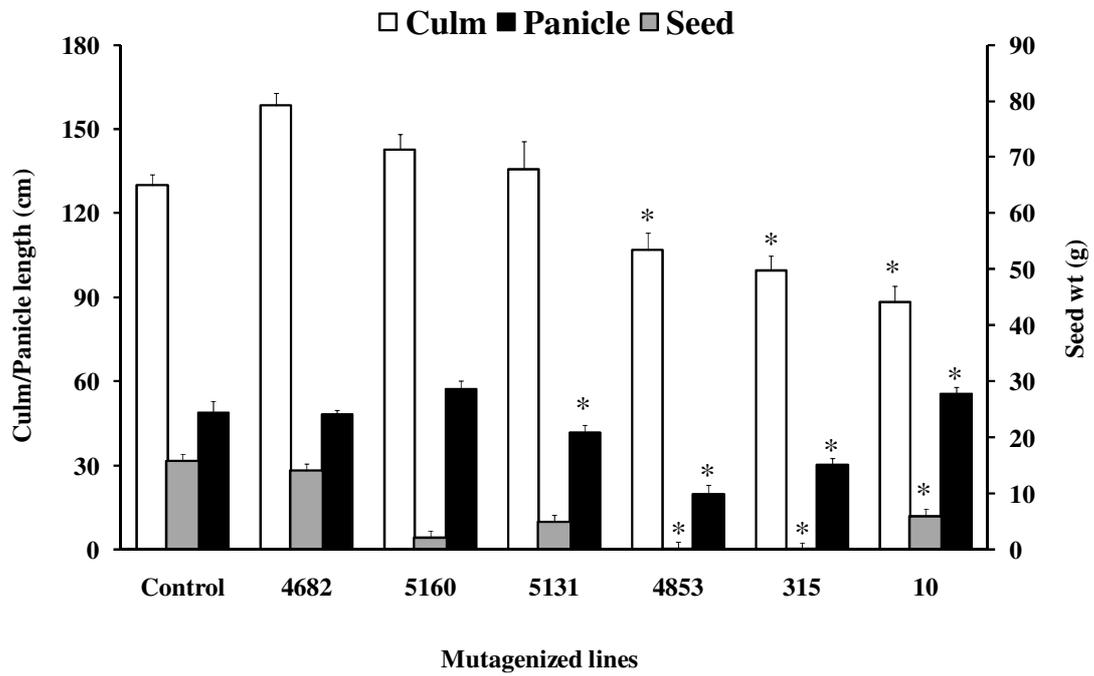
Three lines (3-7-4852-1, 3-7-315-12 and GA-10 ) had significantly shorter ( $p < 0.05$ ) and two lines (3-7-4682 and 3-7-5160) had significantly ( $p < 0.05$ ) taller culm length than the wild-type plants (Fig. 5.1). Most dwarfed lines (3-7-4852-1, 3-7-315-12 and GA-10) were not significantly different ( $p > 0.05$ ) from each other in culm length despite a 18.5cm culm length difference between them. Culm height difference between plants of the tallest and shortest mutagenized lines was about 70cm (Fig. 5.1). Decrease in culm length in plants of the three shorter lines derived from either a decrease in internode number or length or a decrease in peduncle length (Figs. 5.1 and Table 5.3). Lines 3-7-5131-2 and 3-7-4852-1 gave significantly ( $p < 0.05$ ) higher number of internodes (8.3) when compared to wild-type plants (6.9; Table 5.1).

Application of GA did not significantly increase ( $p > 0.05$ ) culm or internode length in plants of the three dwarfed lines (3-7-4852-1, 3-7-315-12 and GA-10) when compared to the wild-type control. GA treatment further did not significantly ( $p > 0.05$ ) affect peduncle and panicle length including the wild-type control. Dwarfed plants of mutant lines did not significantly ( $p > 0.05$ ) change culm, panicle or peduncle length when treated with GA (Figs. 5.2 and 5.3). Also, mutant plants of line GA-10 had a significant ( $p < 0.05$ ) increase in internode diameter when compared to wild-type plants when either treated or not treated with GA<sub>3</sub> (Table 5.4). Further, internode diameter had a slight, but steady, increase upward in plants of all mutant lines (Table 5.4).

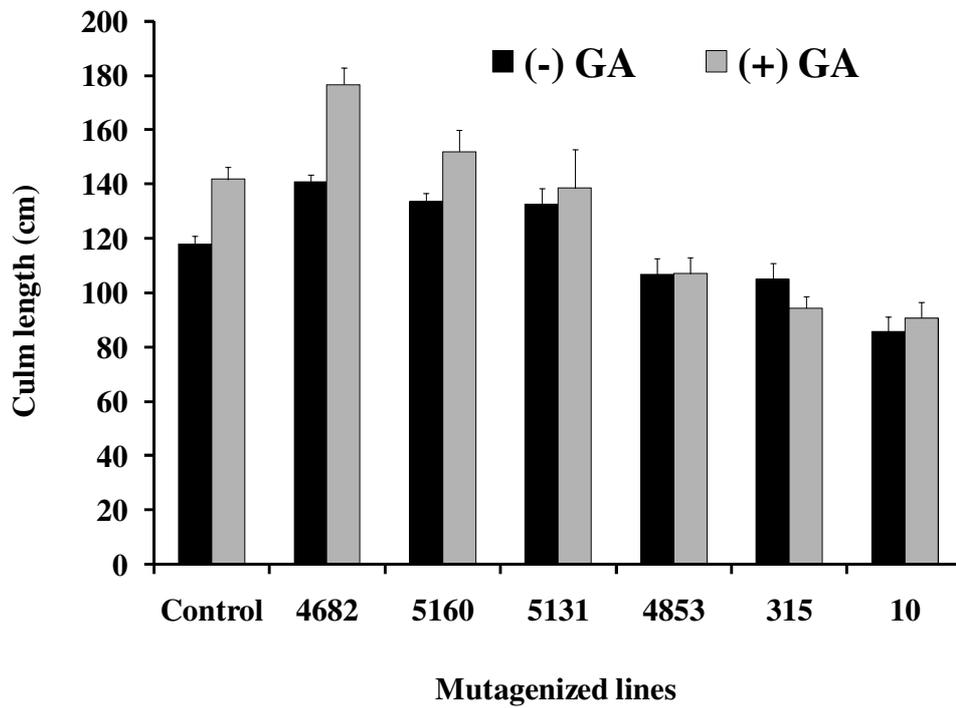
**Table 5.1** Peduncle length, internode number, number of tillers, culm and panicle dry weight of plants of different mutant *E. tef* lines and wild-type control (variety DZ-Cr-37).

<b>Line</b>	<b>Peduncle length (cm)</b>	<b>Internode number</b>	<b>Tiller number</b>	<b>Culm + Leaf DWT (g)</b>	<b>Panicle DWT (g)</b>
<b>DZ-Cr-37</b>					
<b>(wild-type)</b>	29.92a	6.8bc	13.08dc	25.8b	9.60a
<b>3-7-4682-2</b>	24.58ab	7.5ab	13.08dc	26.8b	9.12a
<b>3-7-5160-1</b>	25.72ab	6.9bc	16.23bc	32.5a	3.72c
<b>3-7-5131-2</b>	27.65ab	8.3a	10.23d	25.1b	5.06b
<b>3-7-4852-1</b>	13.08d	8.3a	20.75a	15.1c	0.31d
<b>3-7-315-12</b>	18.04cd	7.4abc	18.73ab	10.3d	0.40d
<b>GA-10</b>	23.67cb	6.4c	14.42c	25.5b	9.49a
<b>Mean</b>	23.24	7.37	15.22	23.1	10.22
<b>P</b>	***	**	***	***	***

DWT= dry weight; Letters within a column denote significance as determined by the Tukey's Studentized Range (HSD) test. Data shown represent mean values  $\pm$  SE of 12 individual plants. Significance level was determined using ANOVA (\*\*\*)  $P < 0.001$ ; \*\*  $P < 0.01$ ). Means followed by the same letter are not significantly different.



**Figure 5.1** Culm and panicle length and seed weight in plants of different mutant lines and the control (DZ-Cr-37). Data represent the mean  $\pm$  SE of 12 individual plants.



**Figure 5.2** Culm length of plants of different mutagenized lines and wild-type plants (DZ-Cr-37) with (+) GA and without (-) GA treatment. Data represent the mean  $\pm$  SE of 6 individual plants.

#### 5.4.2 Panicle length, tillering, biomass and yield

Among the plants of the three dwarf mutant lines with the highest reduction in culm length, plants of lines 3-7-4852-1 and 3-7-315-12 had significantly ( $p < 0.05$ ) lower panicle length when compared to wild-type control plants. However, plants of the most dwarfed mutant line GA-10 had an identical panicle length when compared to the wild-type control (Fig. 5.1) regardless of additional GA treatment (Fig. 5.3). Plants of the two mutant lines 3-7-4853 and 3-7-315 had further the shortest panicle length (Fig. 5.1) and plants of these lines had also severe defects in fertility and produced only little grain.

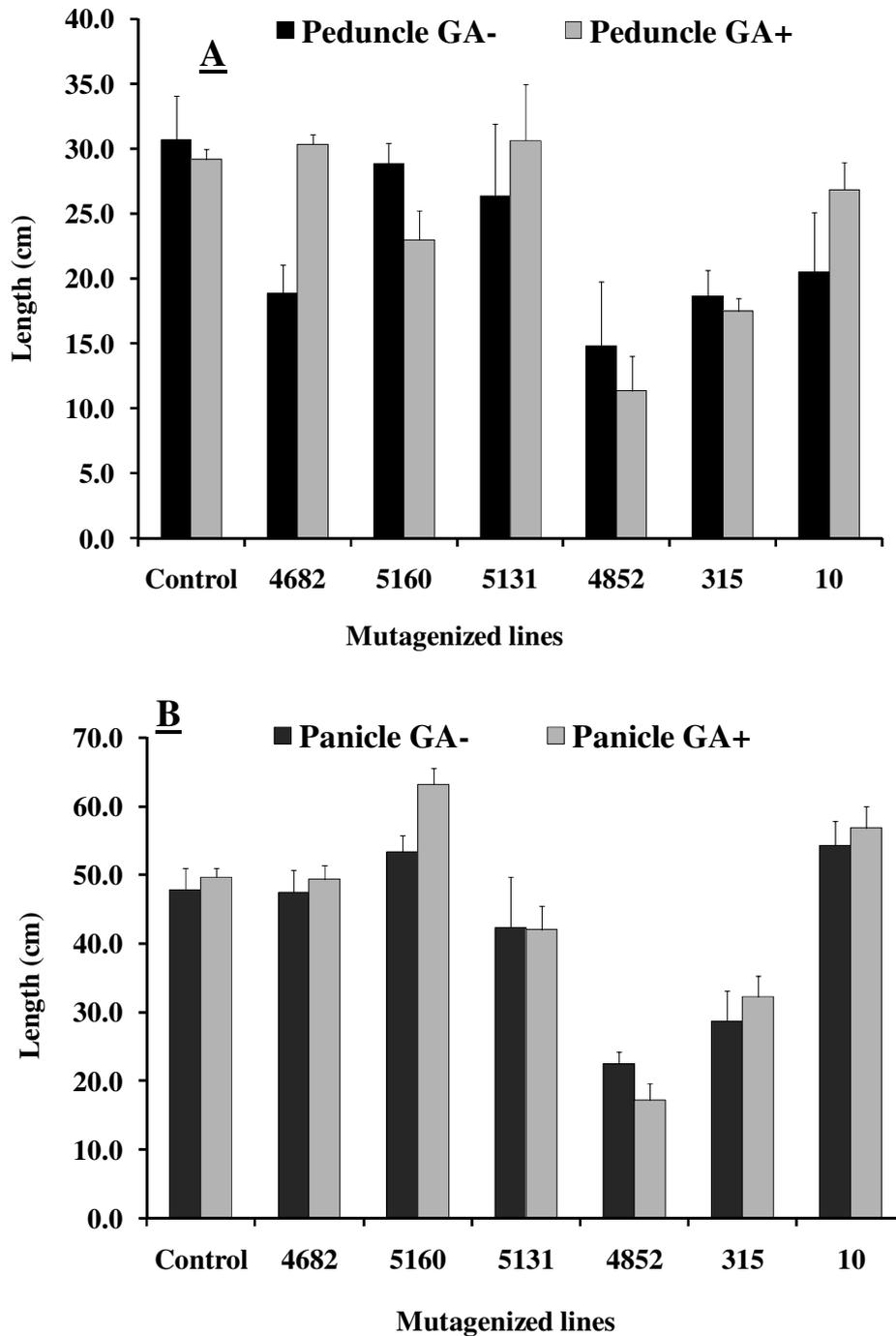
Tillering in plants of the two mutant lines (3-7-4852-1 and 3-7-315-12) was further significantly ( $p < 0.05$ ) higher than in plants of the shortest mutant line (GA-10) and the wild-type control (Table 5.1). Tillering was also not reduced by GA treatment (Table 5.3) and tiller number was also not related to culm length (data not shown).

Plants of the two mutant lines with reduced height (3-7-4852-1 and 3-7-315-12) had significantly ( $p < 0.05$ ) lower panicle dry weight and reduced yield when compared to the control or all other mutant lines (Table 5.3). Plants of mutant line GA-10 produced about half the yield (2.5 g) of the wild-type control (4.8 g). In all plants of dwarf mutant lines yield was not increased by GA treatment (Table 5.3).

**Table 5.2.** Response of mutant lines in terms of internode length to exogenously applied GA<sub>3</sub>

GA	INT1		INT2		INT3		INT4		INT5		INT6		INT7		INT8		INT9	
	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
<b>Wild type</b>	6.7	12.1	14.6	16.5	16.4	22.6	18.3	20.4	20.6	24.1	20.2	27.8	21.2	27.5	-	-	-	-
<b>3-7-4682-2</b>	10.2	11.5	18.7	16.3	17.8	18.0	18.8	24.8	22.3	24.1	24.1	23.3	28.6	27.3	-	23.7	-	23.0
<b>3-7-5160-1</b>	9.1	13.0	15.8	18.9	19.6	22.1	18.1	21.8	24.1	27.9	26.3	27.9	29.4	26.1	-	-	-	-
<b>3-7-5131-2</b>	7.7	7.1	15.4	9.46	15.8	15.8	16.3	17.0	19.9	17.3	24.0	20.9	25.6	15.6	25.0	11.4	-	16.5
<b>3-7-315-12</b>	5.3	6.5	10.5	8.8	13.0	10.6	15.5	14.9	18.5	16.1	17.8	16.2	16.6	13.1	17.1	15.3	-	-
<b>3-7-4852-1</b>	3.6	8.1	6.8	10.8	8.8	12.1	9.5	14.0	10.1	14.0	13.1	19.7	15.6	16.8	17.1	12.5	-	-
<b>GA-10</b>	5.1	4.1	9.58	9.4	12.4	12.5	13.8	13.7	16.1	18.3	18.1	19.7	20.6	23.7	-	-	-	-
<b>GA Mean</b>	<b>7.0b</b>	<b>8.8a</b>	<b>13.3a</b>	<b>13.2a</b>	<b>15.4a</b>	<b>16.4a</b>	<b>16.5b</b>	<b>18.6a</b>	<b>19.5a</b>	<b>20.8a</b>	<b>21.2a</b>	<b>22.2</b>	<b>23.1a</b>	<b>21.8a</b>	<b>19.7a</b>	<b>17.9b</b>	<b>15.8a</b>	<b>19.7a</b>
<b>GA</b>	*		NS		NS		**		NS		NS		NS		***		NS	
<b>LinexGA</b>	NS		*		*		NS		NS		**		**		NS		NS	

Significance level was determined using SAS GLM for ANOVA (\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ); L!= Line; Letters for mean denote significance as determined using the Tukey's Studentized Range (HSD) Test. Data shown represent mean values  $\pm$ SE of 6 individual plants. Means followed by the same letter are not significantly different. NS = Non-significant; (-) = control or unsprayed; (+) = Sprayed with GA<sub>3</sub> (100uM).



**Figure 5.3** Effect of GA application on peduncle (A) and panicle (B) length of mutant lines and the control (DZ-Cr-37). Data represent the mean  $\pm$  SE of 6 individual plants.

**Table 5.3** Peduncle length, internode number, number of tillers, culm and panicle dry weight of plants of different mutagenized *E. tef* lines and wild-type control plants (cv. DZ-Cr-37).

Line	Internode number		Tiller number		Culm DWT (g)		Panicle DWT (g)		Seed WT (g)	
	GA-	+	-	+	-	+	-	+	-	+
<b>Wild-type</b>	7.0	6.5	13.5	12.7	26.1	25.4	10.2	8.9	4.9	4.6
<b>3-7-4682-2</b>	7.0	8.0	12.7	13.5	26.4	27.2	9.1	9.1	4.5	4.9
<b>3-7-5160-1</b>	6.7	7.2	15.8	16.4	33.8	31.2	3.4	4.1	0.4	1.1
<b>3-7-5131-2</b>	7.3	9.2	8.7	11.8	21.6	29.3	4.3	5.9	1.3	2.4
<b>3-7-4852-1</b>	9.2	7.5	22.7	18.8	14.2	15.9	0.4	0.3	0.07	0.08
<b>3-7-315-12</b>	7.3	7.4	21.7	14.6	11.4	9.1	0.3	0.5	0.00	0.01
<b>GA-10</b>	6.5	6.3	13.7	15.2	24.7	26.2	8.8	10.2	2.2	2.8
<b>Mean</b>	<b>7.3a</b>	<b>7.3a</b>	<b>15.2a</b>	<b>14.6a</b>	<b>22.9a</b>	<b>23.3a</b>	<b>5.60a</b>	<b>6.10a</b>	<b>2.25a</b>	<b>2.70a</b>
<b>GA</b>	NS		NS		NS		NS		NS	
<b>Line x GA</b>	***		NS		***		***		***	

DWT= dry weight; Significance level was determined using SAS GLM or ANOVA (\*\*\*  $P < 0.001$ ). Letters for mean denote significance as determined using the Tukey's Studentized Range (HSD) test. Data shown represent mean values $\pm$ SE of 6 individual plants. Means followed by the same letter are not significantly different. NS = Non-significant; GA (-) = control or unsprayed; GA (+) = sprayed with gibberellic acid (GA<sub>3</sub> at 100  $\mu$ M).

**Table 5.4** Internode diameter of different mutant lines and wild-type control (cv. DZ-Cr-37) plants in response to GA<sub>3</sub> treatment.

Line	INTØ1*		INTØ2		INTØ3		INTØ4		INTØ5		INTØ6		INTØ7		INTØ8		INTØ9		
	GA-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	
<b>Wild type</b>	1.84	1.55	2.30	1.92	2.50	2.44	2.74	2.51	2.89	2.66	3.06	3.02	3.29	3.16	-	-	-	-	
<b>3-7-4682-2</b>	1.97	1.61	2.67	1.43	2.95	2.24	3.04	2.73	3.48	2.74	3.42	2.80	3.65	2.83	-	2.52	-	1.98	
<b>3-7-5160-1</b>	2.08	1.59	2.61	1.80	2.80	2.46	3.15	2.68	3.11	2.78	3.44	3.06	3.86	3.13	-	-	-	-	
<b>3-7-5131-2</b>	1.93	1.52	2.25	1.86	2.89	2.34	3.23	2.57	3.28	2.69	3.84	2.88	3.92	2.84	3.63	3.02	-	1.92	
<b>3-7-4852-1</b>	1.41	1.02	1.44	1.13	1.42	1.26	1.61	1.51	1.61	1.84	1.71	1.83	1.61	1.55	1.55	1.30	-	-	
<b>3-7-315-12</b>	1.50	0.77	1.87	1.09	2.03	1.40	2.03	1.41	2.33	2.02	2.32	1.83	2.54	1.54	2.26	1.28	-	-	
<b>GA-10</b>	1.90	1.85	2.74	2.45	2.86	2.80	3.24	2.95	3.55	3.11	3.24	3.34	3.71	2.90	-	-	-	-	
<b>Mean</b>	<b>1.82a</b>	<b>1.43b</b>	<b>2.28a</b>	<b>1.69b</b>	<b>2.51a</b>	<b>2.14b</b>	<b>2.74a</b>	<b>2.34b</b>	<b>2.92a</b>	<b>2.54b</b>	<b>3.01a</b>	<b>2.69b</b>	<b>3.19a</b>	<b>2.58b</b>	<b>2.48a</b>	<b>2.11a</b>	<b>1.44a</b>	<b>1.74a</b>	
<b>GA</b>	***		***		***		***		***		***		***		***		**		NS
<b>Line x GA</b>	NS		*		NS		NS												

\*IntØ1= internode No. 1 diameter from the base of the stem; Significance level was determined using SAS GLM for ANOVA (\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ). Letters for mean denote significance as determined using the Tukey's Studentized Range (HSD) test. Data shown represent mean values  $\pm$ SE of 6 individual plants. Means followed by the same letter are not significantly different. NS = Non-significant; GA (-) = control or unsprayed; GA (+) = sprayed with gibberellic acid (GA<sub>3</sub> at 100  $\mu$ M).

## 5.5 Discussion

This study has shown that semi-dwarf phenotypes could be developed in *E. tef* through mutagenesis. However, regardless of plant height grain yield was considerably reduced in all the mutant lines, possibly because of other undesirable effects induced in the plants. Some of the mutants showing considerable reduction in height were also found near infertile and produced only a very little amount of grain except for plants of the most dwarfed mutant line GA-10, which gave about half of the untreated wild type control.

Internodal diameter was not increased due to induced semi-dwarf phenotype with the exception of semi-dwarf line GA-10 which had consistently higher internodal diameter from 2<sup>nd</sup> to the 5<sup>th</sup> internode in the GA untreated plants compared to GA untreated wild type control. Such increase in internode diameter contributes to the stiffness of straw (stem) by increasing the ratio for stem basal-diameter to height which would be of benefit for obtaining lodging resistance. On the other hand, the weak tapering of control plants could not be improved in any of the dwarf mutant plants since acropetally steadily increasing internodal diameter was found in all of the mutant plants. Based also on our previous results from GA inhibition studies (Chapter 2) that plant height reduction did not increase stem basal diameter, results of this study also confirmed absence of meaningful relationship between plant height and stem diameter. However, this is under the assumption that the induced semi-dwarf phenotype in the mutant plants in this study also involves mutation in the height controlling genes. This, therefore, is subject to further verification. Therefore, it is possible to speculate that factors, other than GA genes mediated controlling of culm height might also be involved in controlling internode diameter. This speculation is in line with the recent report by Ookawa *et al.* (2010) about enhanced stem strength obtained in japonica rice plants carrying

the *STRONG CULM2* (*SCM2*) gene due to its effect on increasing culm diameter. Therefore, mutagenesis and TILLING application can also be directed to induce change in such genes involved in culm diameter control for lodging resistance improvement. It, however, is not yet clear from the present study if mutant line GA-10 may harbor induced changes in such genes involved in stem diameter control.

None of the semi-dwarf mutant including GA-10 plants showed a response to GA treatment and did not recover height. This means the induced changes are not in the GA biosynthesis since such mutants can easily recover height by exogenous GA application. The alternative is change in GA signalling in which case studies have already shown that GA signalling mutants are insensitive to GA treatment (Milach *et al.*, 2002; Hedden *et al.*, 1998). It might, therefore, be possible that mutant plants not responding to exogenously applied GA harbour a mutation in GA signalling components such as the *Rht* in GA metabolism.

Plants of semi-dwarf lines also had normal tillering but did not have identical biomass amount as the wild-type control except mutant line GA-10. Hence, most tillers were rather weak with slender stem having little biomass to support further growth and sustain grain filling as evident from the very low yield. However, biomass in the semi-dwarf mutant GA-10 was identical to the biomass in the control plants which could be of significant advantage if this line is used for crossings with other parental lines to restore yield without losing its other useful traits such as the semi-dwarf and higher internodal diameter. Plants of GA-10 were non-responsive to GA application implying that it may harbor a mutation possibly in GA-response genes. It also have shown a higher GA content (data not shown: personal communication Dr. Tadelle) which also suggests an impaired GA signalling component such as changes in the *Rht* gene. GA signalling mutants have been shown to have elevated levels

of bioactive GA presumably because of a feedback regulation as a result of reduction in GA-response (Alvey and Harberd, 2005). However, it is necessary to verify the assumptions in future experiments to determine the agronomic importance of mutant line GA-10. Crossing GA-10 with known tall varieties would also be necessary to determine the line's potential as a source of a semi-dwarf trait. Evaluation can be further supplemented with characterization of the DNA sequences for target GA-signalling genes such as *Rht*.

Overall, this study has shown that a TILLING approach can be applied to obtain semi-dwarf *E. tef* plants. Application so far has resulted in different semi-dwarf lines and one of these lines, GA-10, has potential for further characterization including the cause for dwarfism.



# **CHAPTER SIX**

## **GENERAL DISCUSSION AND FURTHER PERSPECTIVE**

The study aimed to be investigate the role of GA in plant height control in *E. tef* and associated changes *in* morpho-physiological parameters of the plant including yield . Results showed the significance of GA genes as prime targets for plant height reduction in *E. tef* to ultimately improve lodging resistance by reduction of culm length.

A first new aspect of this study was the high responsiveness of *E. tef* in terms of plant height reduction following GA inhibition by anti-gibberellins chemical treatment (CCC and PBZ). A further new finding was that growth of the *E. tef* panicle, which constitutes one third of the total plant height, was not very sensitive to GA inhibition. At higher concentration of a potent inhibitor, such as PBZ, stem elongation was severely reduced. Generally, the control of GA inhibitors on stem height and panicle elongation provided strong evidence that targeting the GA biosynthesis pathway is a realistic strategy for the control of plant height in *E. tef*. Further, evidence was provided that decoupling plant height and yield could be achieved in *E. tef*. This would allow developing dwarf lodging-resistant plants in high yielding cultivars.

Although PBZ had a much stronger GA inhibition on internode elongation in *E. tef* than CCC and also acting at a much lower concentration than CCC, PBZ cost and persistence in the soil would restrict its wider application. In addition, panicle bearing tillers was not increased by PBZ or CCC treatment although PBZ increased tillering many-fold. Unfortunately, CCC treatment did not increase stem diameter, but the diameter to height ratio was increased improving plant standing. Therefore, CCC appears to be a suitable inhibitor and a candidate for further plant height control for reducing the lodging problem under field conditions. Fine-tuning of CCC application and observing response of plants (with reduced height) under field conditions are, however, required to prepare a practical guideline for wider application of the PGR in *E. tef*. This will help to identify a good balance between vegetative (stem and tiller)

and reproductive (panicle and seed setting) growths for effectively reducing height (therefore lodging) without compromising seed yield. The weak tapering in *E. tef* observed in this study together with absence of any promising effect on stem diameter is still a concern suggesting a weak transition between the shoot base, 1<sup>st</sup> lowermost internode and the root collar, in *E. tef* plants. Emphasis should therefore also be given to understand the mechanism, other than those regulated by GA genes, that might involve improving *E. tef* stem-base.

A further new aspect of this study was the optimization of *E. tef* regeneration to produce putative transformed plants from immature somatic embryos via *Agrobacterium*-mediated transformation for the induction of dwarfism over-expressing GA inactivating gene (*GA2ox*) from *Phaseolus coccineus*. In this study, 8 putative transformed plants carrying the insert (*PcGA20 ox* or *nptII* gene sequence) at the T<sub>0</sub> generation were obtained. Regenerated plants were successfully grown into mature fertile plants producing seeds. In the transformation procedure, a combination of different previously reported media for various crops have been successfully applied for embryogenic callus induction, *Agrobacterium* inoculation and co-cultivation and plant regeneration. The success in embryogenic callus induction using less than 1-week old zygotic immature embryos explants for regeneration into shoots was dependent on the use of intermediate size embryos. It was further found that the antibiotic geneticin (G418) fully controlled shoot growth from mature *E. tef* embryos which also requires further optimization for *E. tef* callus.

Molecular assessment of the transgene *PcGA2ox* has been based on results of previous studies where reduction in plant height in other cereals to be a key agronomic feature to limit lodging (Rajala, 2003; Rademacher, 2000). Results in this study showed only a putative T<sub>0</sub> transformants having a positive PCR results for the transgene. Selected putatively transformed T<sub>0</sub> plants were characterized further growing seeds (T<sub>1</sub> progeny) for genotype

and phenotype and inconsistent results were obtained during PCR detection of the presence of the transgene. Selected semi-dwarf T<sub>1</sub> generation plants showed that the reduction in plant height significantly varied even among the semi-dwarf plants. The reduction in height was also associated with amounts of bioactive GA<sub>1</sub>. On the other hand, the accumulation of GA<sub>8</sub> in the semi-dwarf plants was not proportional to the relative height differences or supposedly deactivation of GA<sub>1</sub>. Such phenomenon was also observed in transformed *Solanum nigrum* over-expressing same GA inactivating gene, *PcGA2ox* transgene (Dijkstra *et al.*, 2008). Deficiency through GA deactivation decreases height in rice (Lo *et al.*, 2008) with increasing yield (Ookawa *et al.*, 2010). However, due to the ambiguity of the PCR result, therefore, these plants have to be further characterized to show if *GA2ox* transgene expression such as *GA2ox* transcript abundance in the stem tissue is always associated with reduction in plant height to exclude the possibility of somaclonal variation induced in the tissue culture process using the auxin 2, 4-D (Banerjee *et al.*, 1985) and not due to stable transgene integration.

The fourth new aspect of this study was the successful isolation three *GA20ox* homologous genes from *E. tef*, involved in GA biosynthesis. Additionally, the *Rht* (*Reduced height*) and *Eui* (*Elongation Uppermost Internode*) genes involved in GA biosynthesis and a Cytochrome P450 monooxygenase gene in brassinosteroid deactivation were either fully or partially isolated and cloned. All these genes are involved in plant height control. Genomic analysis identified four copies of *GA20ox* to exist in *E. tef*. The *EtGA20ox1* sequence was successfully expressed in a heterologous bacterial system and allowed converting the substrate GA<sub>12</sub> to products GA<sub>9</sub>, GA<sub>15</sub> and GA<sub>24</sub>. Further, in *E. tef* *EtGA20ox1b* was the functional equivalent to the rice *sd-1* gene. Alignment and phylogenetic relationship of the full coding region of putative *E. tef* *GA2ox1* and partial putative *GA2ox1b* and *EtGA20ox2* sequences showed high identity scores with orthologous genes from *S. bicolor*, *Z. mays*, *O. sativa* and *L. perenne*.

This gene is expressed with highest transcriptional abundance in the uppermost internodes followed by node tissues and any mutation in this *E. tef* gene might specifically control plant height. However, a further study is required to confirm any functional similarity between *EtGA20ox1b* and *EtGA20ox1* in different plant tissues.

A fifth new aspect in this study was the potential usefulness of *E. tef* seed mutagenesis in producing a semi-dwarfed phenotype using variety DZ-Cr-37, which is a fairly tall modern cultivar. However, mutants combining reduced culm length and still good yield were not identified among the mutants screened. Except for semi-dwarf mutant line GA-10, which had a reasonable yield, low yield in tested dwarfed mutant lines was always associated with weak and infertile panicle development possibly due to undesirable random genetic changes not associated with height control. GA-10 also had a significantly higher diameter in most internodes which could directly contribute to the stiffness of stem and lodging resistance. This line also showed normal tillering and culm and panicle biomass and should therefore be tested further. Since all mutants were insensitive to GA application, the mutation in GA-10 might possibly be due to a change in GA signalling genes. Further analysis is therefore required if plants of this line harbour a GA signalling mutation. Also, crossing experiments with tall varieties are also required to verify and predict agronomic significance of line G-10 for lodging resistance.

Overall, support of the original hypothesis that regulation of the GA amount in *E. tef* will change pheno-morphic and also agronomic characteristics that would affect lodging and further decoupling plant height from yield was found in this study. GA inhibition or deactivation in *E. tef* produced a semi-dwarf phenotype without changing panicle development and grain yield except under conditions of severe GA inhibition where panicle

growth was affected and grain yield was reduced. Other pheno-morphic features or plant stand structures, such as diameter of basal internodes, the weak tapering (acropetally increasing stem diameter) and panicle bearing tillers in *E. tef* plant were not improved by reducing the endogenous GA amount.

Based on the findings of this study, any future following-up study might investigate stem tissue specific down-regulation of height controlling genes such as down-regulation of the already cloned *EtGA201a* in *E. tef* through a non-transgenic approach such as mutagenesis and TILLING or through RNAi technology. Panicle elongation was unaffected by GA inhibition, a future lodging-resistant improvement in *E. tef* also has to focus on obtaining compact type panicles in a dwarf or semi-dwarf phenotype background. Moreover, it is essential to further consider other potential threats in the future lodging resistant ideotype development in *E. tef* such as the narrow stem base problem (weak stem -basal to root-collar transition) that render *E. tef* plants weak in anchorage strength. Therefore, further study is required to understand mechanisms involved in stem-base diameter regulation (with emphasis on basal internodes), since the presumed weak transition from stem-base to the root-collar (supposedly low for *E. tef*) appears not to be regulated by GA. Generally based on the analyzed and implicated *E.tef* pheno-morphic/architectural traits in relation to lodging, the need for a more comprehensive intervention through gene regulation is clear and need to combine traits for a short stature, thicker basal-diameter and shorter panicle form in the lodging resistance *E. tef* ideotype development. The prime target genes related to stem height control that have already been cloned in this study can now be used to employ a non-transgenic approach using mutagenesis and TILLING techniques to induce mutation and select semi-dwarf genotypes in *E. tef* as an immediate future intervention strategy.



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# Appendix



**Figure A.1** (A) *E. tef* *GA20ox1* full coding region (nucleotide sequence) and (B) deduced amino acid residues of *E. tef* *GA20ox1* sequence with 482 aa with conserved domains

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-TCGATTGACTTCGTGGTGGAGGACTGCAAGAACATCTACTTCGCGGGGTACGAGACCACC GCGGTGACCGCGGCCTGGT 79
GGT GAG . G . . . . C . . . . CA . . . . . G . . . . . C . . . . . G . . . . . C . . . . . 80
CA CG GA GA A A . . . . . G . . . . . CGT . T . . . . . CA GC . . . . . GT AACCT CT A A . . . . . A 80
GC . GAC . . . . . CA . . . . . C . . . . . G G . . . . . C . . . . . CA . . . . . 80
GC . GAC . . . . . CA . . . . . C . . . . . G G . . . . . C . . . . . A G . . . . . 80
GC . GAC . . . . . CA . . . . . C . . . . . G . . . . . C . . . . . CA . . . . . 80

GCCTGATGCTCCTGGCGCCGCACCCGGAGTGGCAGGACCAGGTGCGCGACGAGGCCTGCCAGGCGTCCG----- 148
AC . . . . . T . . . . . G . . . . . C G . . . . . G . . . . . ----- 149
C ACAG C G C CAT . . . . . G TC CC TC G . . . . . T CT . . . . . A TC . . . . . G ----- 150
. . . . . G . . . . . CG . . . . . C . . . . . A . . . . . C . . . . . TCCT G C TCCT GCACCGGCACC 160
. . . . . G . . . . . CG . . . . . GC CC . . . . . C . . . . . T CT G . . . . . TC . . . . . GCGGCACGGC 160
. . . . . G . . . . . CG C T . . . . . GC C . . . . . C . . . . . T CT G C TC . . . . . GCGGC----- 155

-CAGGTGGCCCG-----CGCGCCGACTTCACCTCTCTCCAGAGGATGAAGA-GCTGACGATGGTGATCCAGGA 217
G CC . . . . . G . . . . . G A . . . . . G . . . . . A . . . . . ----- 216
G CGC T A A . . . . . TTC CG GAGCAG . . . . . ACC A C C . . . . . CA CGG . . . . . A CC GA C . . . . . 216
GGACC . . . . . GGGATCCCCT . . . . . G . . . . . GAGG . . . . . GTC . . . . . C . . . . . GGC . . . . . G G . . . . . 240
G C CC C C . . . . . C . . . . . GA ATGG GTCCC . . . . . CG . . . . . G G . . . . . G G . . . . . 231
C CC . . . . . A AC G . . . . . GA GTGA . . . . . TCC . . . . . A C G . . . . . GGT . . . . . TG G . . . . . 225

```

		Identity (%)		
<b>AACGCTGCGGCTGTAC</b>	233	66		<i>E. tef Eui</i>
<b>G . . . . .</b>	232	60		<i>S. bicolor</i>
<b>G . . . . .</b>	232	49		<i>Z. mays</i>
<b>G . . . . . T</b>	256	39		<i>T. sylvaticum Eui</i>
<b>. . . . . T</b>	247	40		<i>O. sativa EUI</i>
<b>G . . . . . T</b>	241	30		<i>T. aestivum</i>

indicated (bold and underlined). Conserved domains include (i) the consensus sequence **NYYPXCXXP** of 2-oxoglutarate dependent dioxygenase (2ODDs) for binding the common co-substrate; ii) sequence **LPWKET**, which binds to the GA substrates, and iii) three histidine residues **HCD** for binding Fe<sup>2+</sup>.

## C

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GCTGCCGTGGAAGGAGACGCTCTCCTTCGGCCACCGCGACGTCGTGGAGTACTTCACATCCA
CCCTCGGCAGCGACTTCAAACCCCTAGGGTAACATAAACTGTTGAAATAGCTAGCTTCGCT
ACGTTTTTTTTTGTACGTACAAGAGATCGGTCATCGGAGAGCTTTTACAATGACTAATGCAC
CGATCGAGCCATGCATGGACGCAGGGAGGTGTTCCGAGACTACTGCCAATCGATGAAGGAGG
TGTCGCTGGCGATCATGGAGGTGCTGGGCGCGAGCCTGGGCGTGGGGAGGCGCTACTGCAGG
GACTTCTTCGCCGACGGCTGCTCCATCATGAGGTGCAACTACTACCCGCCGTGCCCGGAGCC
GGACCGGACGCTGGGCACGGGGCCCCACTGCGACCCGGCGGCCACACCCCTTTGCTCCAGG
ACGACGACGTGGACGGGCTCCAGGACGACGACGTTGGACGGGCTCCAGGTGCTCGTGCACGGC
GAGTGGCGGCCCGTGGCGCCCAAGCCGGGAGCCATCGTCTCAACATCGGCG

```

## D

LPWKETLSFGHRDVVEYFTSTLGSDFKPLG\*T\*TVEIASFATFFFVTRDRSSESFYND\*CT  
DRAMHGRREVFRDYCQSMKEVSLAIMEVLGASLGVGRRYCRDFFADGCSIMRCNYYP CPEP  
DRTLGTGPHCDPAAHTLLLQDDVDGLQVLVDGEWRPVRPKPGAI VVNIG

## E

CGATTGCTGCCGTGGAAGGAGACGCTCTCCTTCCGGGCCAGCCCAACGTCGCCGGCCTTGGTGGAGGA  
CTACCTGGTGGGCCGCCTTGGCGACGAGTACAGGCGGCACGGCGAGGTGTACGGGCGCTACTGCTCGG  
AGATGAGCCGGCTGTCGCTGGAGATCATGGAGGTGCTGGGCGAGAGCCTGGGCGTGGGCCGGCGCTGC  
TTCCGCGACTTCTTCCAGGACAACGACTCCATCATGCGGCTCAACTACTACCCGCCGTGCCAGCGGCC  
CAYGGAGACGCTGGGCACGGGCCCGCATTGCGACCCACCTCCCTCACCATCCTGCACCAGGACCACG  
TCGCCGGCCTCCAGGTCTTCGCCGGCGGCCGGTGGCTCTCCATYCGCCCGCACGCCGCCGCCTTCGTC  
GTCAACATCGGCGA

## F

RLLPWKETLSFRASPTSPALVEDYLVGRLGDEYRRHGEVYGRYCSEMSRLSLEIMEVLGESLGVGRRC  
FRDFFQDNDSIMRLNYYPCQRPETLGTGPHCDPTSLTILHQDHVAGLQVFAGGRWLS?RPHAAAFVV  
NIG

**Figure A.2** Homologous sequences of *E. tef* *GA20ox1a* and *GA20ox2*. (C) represents a *E. tef* *GA20ox2* partial coding nucleotide region, (D) deduced amino acid residues of *E. tef* *GA20ox2* sequence with 152 aa (excluding the sequence in grey shade) with conserved domains indicated (bold and underlined) and stop codons (\*); (E) *E. tef* *GA20 ox1b* partial coding nucleotide region and (F) deduced amino acid residues of *E. tef* *GA20ox1b* sequence with 140 aa with conserved domains indicated (bold and underlined). Sequence in box is in “C” and “D” is an intron between the concerned region in *tef GA20ox2* sequence.

**Table A.1** Primers used for PCR amplification of gene fragments of Rht, Eui and BR using *E. tef* genomic DNA

Target gene	Primers	Degenerate/specific primers
Rht	F1	GTGG(TCA)GGACACGCAGGAGGC
	F2	TTCTACGAGTCCTGCCCCTACCT
	R1	T(TCG)GCGGTGAAGTGGGCGAAC
	R2	TCGGGCTCGTCATCCGTGTCAT
Eui	F1	A(GA)(CG)(CG)(TC)C(CA)ACGGCGA(CG)(AG)AT
	R1	ACAGCCGCAGCGTCTCGTT(GCT)
BR	F1	ACAGCCGCAGCGTCTCGTT(GCT)
	R1	A(GA)(CG)(CG)(TC)C(CA)ACGGCGA(CGA)(GC)(CG)(AT)GTT

**Table A.2** *Rht* primers used in RACE- PCR

Target gene	Primers	Degenerate/specific primers
Rht	Rht SPR3	ATCCCGGCCTCCTGCGTGTCCA
	Rht SPR2	CGGTGGCGGGCGGAAGCGATACA
	Rht SPR1	GGGCGAACTTGAGGTAGGGGCAG
	Rht SPF1	TGTATCGCTTCCGCCCGCCACCG
	Rht SPF2	CTGCCCTACCTCAAGTTCGCCC

## G

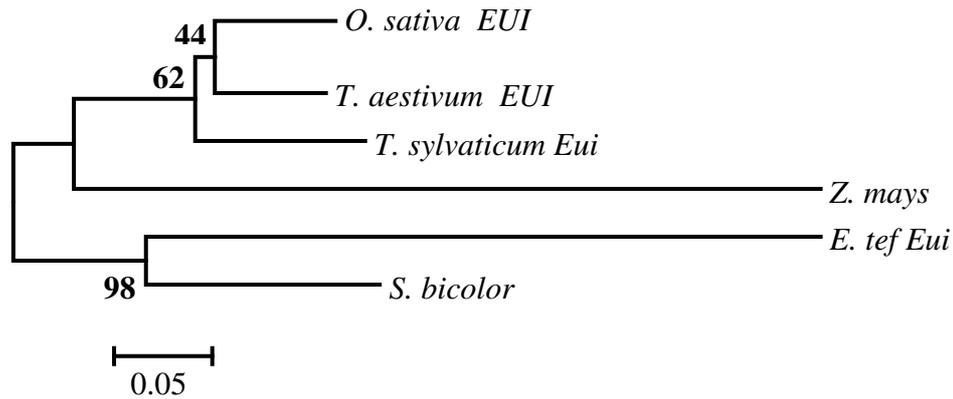
TCGATTGACTTCGTGGTGGAGGACTGCAAGAACATCTACTTCGCGGGGTACGAGACCACCGC  
GGTGACCGCGGCCTGGTGCCTGATGCTCCTGGCGCCGCACCCGGAGTGGCAGGACCAGGTGC  
GCGACGAGGCGTGCCAGGCGTGCGCAGGTGGCGCCGCGGCCAGACTTCACCTCTCTCCAG  
AGGATGAAGAAGGTACTGCAGGTCAAATGAATCACACAGTTCATGCATGCACGTTTCAGTAC  
ACACGCGCGCGCTGATCTGACCAACCTTGTGCATCCAAATATTTGTTTTTTTCGGCAGCTG  
ACGATGGTGATCCAGGAAACGCTGCGGCTGTACA

## H

SIDFVVEDCKNIYFAGYETTAVTAAWCLMLLAPHEWQDQVRDEACQACAGGAAAPDFTSLQ  
RMKKVLQVK\*ITQFHACTFSTHARALI\*PTLCIQIFVFSAADDGDPGNAAAV

**Figure A.3** Partial coding region of the Elongated Uppermost Intenode (EUI) gene in *tef*. (G) represents a *E. tef* EUI partial coding nucleotide region (344bp), (H) deduced amino acid residues of *E. tef* EUI sequence with 115 aa. The underlined region (109 bp) is a predicted intron.

**Figure A.4.** Derived nucleotide sequence alignment of the putative tef *Uppermost Elongated Internode (EUI)* to orthologous monocot sequences from sorghum (*S. bicolor*; Acc No. XM\_002439928.1), maize (*Z. Mays*; Acc No. BT043273.1), rice (*O. sativa* Acc No. AY987040.1), wheat (*T. aestivum* Acc No. AL816398) and brachypodium (*T. sylvaticum*). Identical regions (>80%) are shown by dark shaded areas and dots whereas number indicates the position of the nucleotide within the sequence.



**Figure A.5** Molecular phylogenetic analysis of the rice *EUI* ortholog gene in *tef*. The tree was inferred by Maximum Likelihood method using partial gene sequence. Initial alignment was done by mafft (<http://mafft.cbrc.jp/alignment>) software. The phylogeny was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (1993). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 368 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

# I

GAGGAGGTGGACGAGCTGCTGGCCGCGCTCGGGTACAAGGTGCGCTCGTCGGACATGGCGGA  
CGTGGCGCAGAAGCTGGAGCAGCTCGAGATGGCCATGGGGATGGGCGGCGTCCCCGCCGCGG  
ACGACGGGTTCGTGTGCGACCTGGCCACGGACACCGTGCACACTACAACCCCTCCGACCTGTGCG  
TCCTGGGTGGAGAGCATGCTGTCCGAGCTCAACGCCCGCCGCGCCGCTCCCGCCCGCGCC  
CGCGCCGCGGCCCGCCGAGCTGGTTTCCACCTCGTCCACCGTACAGGGCGGCGGCTCCGGCG  
CCGGGTACTTCGATCCCCCGCCGCGTCTGACTCCTCCAGCAGCACGTACGCGCTGAAGCCG  
ATCCCTCGCCGGTGGCGGCGCCGGCCGACCCGTCCGCGGACTCCGCGGGGAGCCGAAGAG  
GATGCGCACTGGCGGCGGCAGCACGTCGTCTTCCCTCGTCTTCGTCTTCGTCCATGGGCGGCG  
GCGGCGCCAGGAGCTCCGTGGTTGAGGCTGCCCGCCCGCATCCGCGGCGGCGAACGCGCCC  
GCGGTGCCTGTGGTGGTGGTGGACACGCAGGAGGCCGGGATCCGGCTCGTGCACGCGCTGCT  
GGCGTGC GCGGAGGCCGTGCAGCAGGAGA ACTTCTCCGCCGCGGAGGCGCTGGTGAAGCAGA  
TCCCCATGCTGGCCTCGTCGCAGGGCGGCCATGCGCAAGGTGGCCGCCTACTTCGGCGAG  
GCTCTCGCTCGCCGCGTGTATCGTCCCCCCCCCGCCCCGACAGTCCCTCCTCGACGCC  
GCCTTCGCCGACCTCCTCCACGCCCACTTCTACGAGTCTGCCCCTACCTCAAGTTCGCC  
ACTTCACCGCGAACCCAGGCCTTCCCTCGAGGGGTTTCGCCGGCTGCCGTGCGGTCCACGTCGTC  
GACTTCGGCATCGAGCAGGGGATGCAGTGGCCGGCGCTCCTCCAGGCCCTCGCCCTCCGCC  
CGGCGGCCCCCGTCTTCCGCCTCACCGGCGTCGGCCCACCGCAGCCTGACGAGACCGACG  
CCTTGACAGAGGTGGGTGGAGCTCGCCCAGTTCGCTCACACCATCCGCGTCTGACTTCCAG  
TACCGCGCCTCGTGC GCGCCACGCTCGCAGACCTGGAGCCGTTTCATGCTGCAACCGGAGGG  
CGAGGAGAATGACGAGGAGCCCGAGGTGATCGCCGTCAACTCGGTGTTGAGATGCACCGGC  
TGCTGGCGCAGCCCGGCGCCCTGGAGAAGGTCCTGGGCACGGTGC GCGCGGTGCGGCCCAAG  
ATCGTGACCGTGGTGGAGCAGGAGGCCAACACA ACTCCGGCTCGTTCCTGGACCGCTTCAC  
GCAGTCTCTGCACTACTACTCCACCATGTTCGA

# J

EEV**DELLA**ALGYKVRSSDMADVAQKLEQLEMAMGMGGVPAADDGFVSHLATDT**VHYNP**SDLS  
SWVESMLSELNAPPPPLPPAPAPPAPQLVSTSSSTVTGGGSGAGYFDPPPAVDSSSSTYALKP  
IPSPVAAPADPSADSAREPKRMRTGGGSTSSSSSSSSSMGGGARSSVVEAAPPASAAANAP  
AVPVVVVDTQEAGIRLVHALLACAEAVQQENFSAAEALVKQIPMLASSQGGAMRKVAAYFGE  
ALARRVYRSPPPPPTAPSSTPPSPTSSTPHFYESCPLYLKFAHFTANQAFLEAFAGCRR**VHV**  
**D**FGIEQGMQWPALLQALALRPGGPPSFRLTGVGPPQPDETDALQQVGWKLQFAHTIRVDFQ  
YRGLVAATLADLEPFMLQPEGEENDEEPEVI AVNSVFEMHRLLAQPGALEKVLGTVRAVRPK  
IVTVVEQEANHNSGSFLDRFTQSLHYSTMF

**Figure A.6** Full coding region of the *E. tef* RHT gene. (I) represents a *E. tef* Rht near full coding nucleotide sequence (1397 bp), (J) deduced amino acid residues of *E. tef* Rht sequence (465 aa) with characteristic domains (bold and underlined): the DELLA motif, VHYNP and VHVVD of the GRAS domain.

## K

TAAGGCCACGGCGAGGAGTGGGCGCGCCGCCGAAGATCCTCACCCCGCCTTCCACACCGAGAACC  
TCAAGCTGCTGGTGCCGTTTCGTCGGCGAGACGGTGCAGCGGATGCTGGAGGAGCGCGTGCTCTCGCCG  
TCGGCGTCGGCGGCGAACGGCGGCGAGGTGGAGGTGGACGTCGCGGAGTGGTACCCGCGGCTGCCGCA  
GGAGGCCATCACGCTCGCCACGTTTCGGCCGGAACACTACGCCGAGGGCAGCGTCGTGTTCCGGCTGCAGG  
GCGAGCACGCCAGCCACGCCACGGTGGCGCACAGCAAGGTCTTCATCCCGGGGTACAGGTTTCATCCCG  
ACAAGGCGGAACCGGCGCGTGTGGCAGCTGGACAGGGAGATCAAGAGCACCCCTGGCCAAGTTCGTCTGT  
CGCCCTGCAGAGCCGCGGGCGGCGGGTGACCACCACCACCGCCGGGACGAGGGGCGAGCGGACGACG  
GCTTGAGGGACTTCATGAGCTTCATGGCGCCGGCCATGACGGCGGACGAGATCATAGAGGAGTGCAAG  
AACTTCTTCTTCGCCGGAAGGAGACCCTGACCAGCCTCCTCACCTGGGCCACCGTTCGCGCTCGCCAT  
GCACCCGGAGTGGCAGGACCGCGCGCGCCGGGAGGTCTGCTCCGTCTGCGGCCACCGCGGCCTCCCGA  
CGAGAGACCACCTTCCCAAGCTCAAGACCCTGGGGATGATCGTGAACGAGACGCTGCGGCTGTAATCG  
A

## L

KAHGEWARRRKILTPAFHTENLKLVPFVGETVQRMLEERVLSPSASAANGGEVEVDVAEWYPRLPQ  
EAITLATFGRNYAEGSVVFRLOGEHASHATVAHSHKVFIPGYRFIPTRRNRRVWQLDREIKSTLAKFVV  
ALQSRGGGDHHRDEGRADDGLRDFMSFMAPAMTADEIEECKNFFFAGKETLTSLLTWATVALAM  
HPEWQDRARREVSVCGHRGLPTRDHLPLKLTGMIVNETLRL

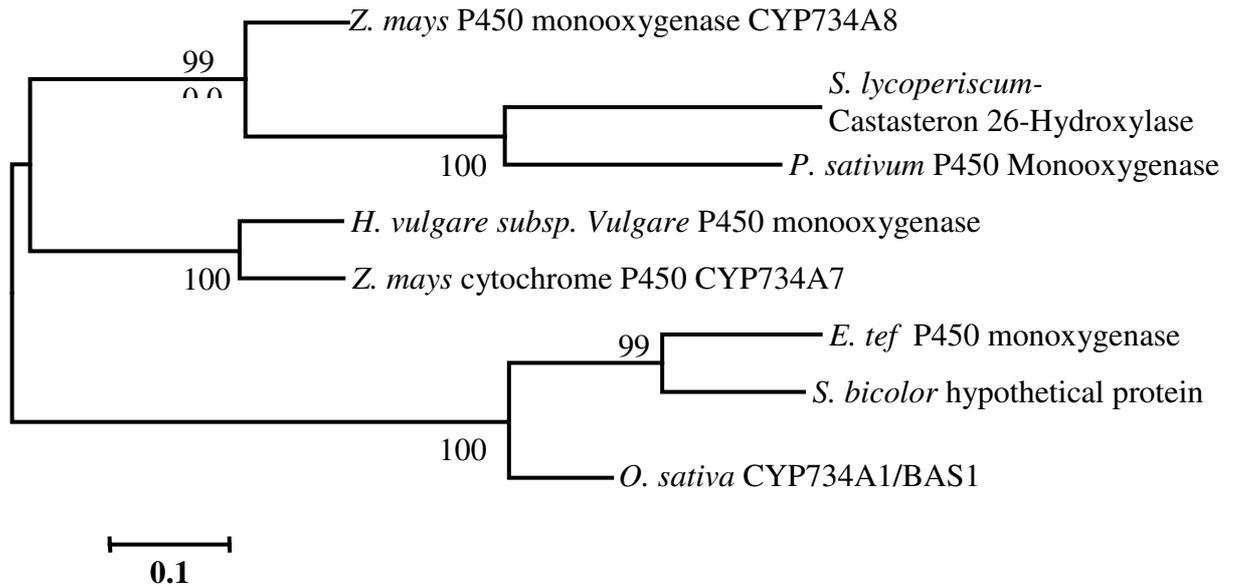
**Figure A.7** Putative *E. tef* brassinosteroid deactivation related Cytochrome P450 monooxygenase gene partial sequence. (K) represents brassinosteroid deactivation gene partial coding region (749 bp), (L) deduced amino acid residues of the sequence (247 aa).

KAHGEWARRRKILTPAFHTENLKLKLVFVGETVQRMLEERVLSPSASAANG-GEVEVDVAEWYPRLPQEAITLATFGR-NYAEGSVVFRLOGEHASHAT 98  
 NL..D.....NA.....A..AD.....L..S...G.S.....V...Q...K...V.....-SD...A.....A...Y.. 99  
 NL..R.....RV.....HRMIA..AG..T...D.--LAERAR.G.A.A.....FQ.V.....F.A...R..DD.AA.....D.L.GY.. 97  
 NL..R.....RV.....FQ.V.....F.A...R..DD.AA.....D.L.GY.. 65  
 SL..DK..LH.RV.V...YPD..NR..H..RS.AALA.R---WR.M.CASG.....FQAVAE...R...-S..S.R...M..RLMAF.S 95  
 SL.DDK..LH.RV...YPD..NR.A.H.ARS.VALA.R---WR.M.SAAG.....QAVAE...R...S-S.DS.R...M..ARLMAF.S 95  
 SLR.DK..H..V...M...L...R..VDVVDK---WHDM..AAS...I..S..FQVVTED...RTA...-S.ED.KA..K..TQLMAF.S 95  
 SLR.DK..H..V...M...L...R..VDVVDK---WHDM..AAS...I..S..FQVVTED...RTA...-S.ED.KA..K..TQLMAF.S 95  
 SLK..K..HH...I..T.YI..R.MI.MM.KSMKE..DK---W.KMSN-AS.K..IE.S.MFST.AEDV...RIV..N-S.ED.KAI.E..AQQMIY.. 94  
 SLK..K..HH.R.IS.T.M.....I.VMATS.VE...N---W.EMS--HK...IE.S.CFQT.TEDV...KTA...S-S.QD.KAI...AQQMVL.A 93

VAHSKVFIPGYRFIPTRRNRRVWQLDREIKSTLAKFVVALQSRGGGGDHHRRDEG-RADDGLRDFMSFMA-----PAMTADIEIEECKNFF 185  
 E.....L.....RRL..L.AG...--D..R..GRDP..G..M.N.....S... 182  
 E...Y.....L...K.....R.H.....TG...--CSSS.GDDA.D.GDGGG.M.E.....G.....S... 183  
 E...Y.....L...K.....R.H.....TG...--CSSS.GDDA.D.GDGGG.M.E.....G.....S... 151  
 E.FR..LV.....L..KK..MS.G...RRG.VQLIGRRSD---AAEEREAEIKDKG---F..LLGL.INARDKK----SQP.PVE.MV...T... 184  
 E.FR..V.....L..KK..LQ.S...RRG.VTLIGHRND--AAQDDDSEPNDKGSSN.F..LLGL.INASDKKKQEEAR..PVEDML...T... 193  
 E.FR.....L..KK.TTS.K..K..RKN..TLIGRR.EA--AD.EKLSG-----CAK.LLGLL.INAGSNG---GKVSP.I.VND.V...T... 182  
 E.FR.....L..KK.TTS.K..K..RKN..TLIGRR.EA--AD.EKLSG-----CAK.LLGLL.INAGSNG---GKVSP.I.VND.V...T... 182  
 E.YQ.....L.SKK..IC.R..KQVRKS.M.LIEERRKK--EE-VLSEE-----CPN.LLEV.IKAGSDD---EYRNTI.VND.V...TI... 180  
 D.FQ.....F...IKS..K..KQ..KS.V.LIERREN--SN-ERIEK-----PK.LLGL.IQASN-----KTNV.V.D.VG...S... 175

	Identity (%)	
AGKETLTSLLTWATVALAMHPEWQDRARREVVSVCGRHGLPTRDHLPKLKTLLGMIVNETLRL 247	100	<i>E. tef</i> P450 monooxygenase
..L..N.....L.....D...R..V..K...R.R...V..... 244	50	<i>S. bicolor</i>
...SN...T...E...A...RGD..K...L... 245	52	<i>O. sativa</i> CYP734A1/BAS1
...SN...T...E...A...RGD..K...L... 213	50	<i>O. sativa</i> P450 monooxygenase
..Q.T.N...L...D.A..Q..LA...PGE..KE..H...L... 246	52	<i>H. vulgare</i>
..Q.T.N...L...D.E..Q..LA...ADE..SKE...L... 255	50	<i>Z. mays</i> P450 monooxygenase CYP734A7
..Q.TSN...T..L...EL..Q..LQ...A.DI..S.EQ.T...L... 241	51	<i>Z. mays</i>
..Q.TSN...T..L...EL..Q..LQ...A.DI..S.EQ.T...L... 244	42	<i>Z. mays</i> P450 monooxygenase CYP734A8
..H.TSN...T..IL...K..EL..D..LT...A.DP.SKQQIS...I..SV-- 240	42	<i>S. lycopersicum</i> P450 monooxygenase
..Q.TSN...T..IL...Q...VQ...D...LKM...S.DV..K..VV...N...S--- 234	48	<i>P. sativum</i> P450 monooxygenase

**Figure A.8** Amino acid sequence alignment of the putative brassinosteroid deactivating gene sequences from *E. tef* with amino acid sequences from other species. sorghum rice (*O. Sativa*; Acc No. EAY84935.1), barley (*H. vulgare*; Acc. No. BAK00002.1), sorghum (*S. bicolor*; Acc No. XP\_002453514.1), maize (*Z. mays*; Acc No. ACG29333.1), pea (*P. sativum*; Acc No. BAF56240.1), and tomato (*S. Lycopersicum*; Acc. No. BAF02550.1). Identical and similar regions are shown by dark (100%) and light shaded areas and dots whereas number indicates the position of the amino acid within the predicted peptide.



**Figure A.9** Molecular Phylogenetic analysis of putative *E. tef* brassinosteroid deactivation gene sequence. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based (Jones *et al.*, 1992). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 269 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).