Isolation, characterization and expression of the rice *sd-1* (*GA20ox*) gene ortholog in *Eragrostis tef*

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Abstract

Eragrostis tef (Zucc.) Trotter, a tetraploid cereal of Ethiopia, has a tall and slender stem susceptible to lodging causing yield loss of ~15-45% each year. Genes conferring lodging resistance through the control of plant height involves GA metabolism genes that have not been identified and characterized in *E. tef*. The aim of this study was to isolate and characterize *E. tef GA20ox,* a key gene in plant height control. Three putative *EtGA20ox* orthologs to the rice *sd-1* gene were identified in *E. tef* with characteristic GA20ox domains. Sequence comparison revealed that *EtGA20ox* sequences share high homology with sorghum, maize and rice sequences. Heterologous expression of the *EtGA20ox1a* protein in *E. coli* catalyzed conversion of $[^{14}C]$-labeled GA_{12} to GA_9. *EtGA20ox1a* and *EtGA20ox1b* had highest transcription in the uppermost internodes. *EtGA20ox1b*, very likely a functional equivalent to the rice *sd-1* gene, is predominantly expressed in the stem and could be a target for height regulation. Sequence information will allow advanced biotechnology applications, such as plant transformation and mutant selection, to improve lodging resistance in *E. tef*.

Introduction

Eragrostis tef (Zucc.) Trotter is an allotetraploid small-seeded full grain nutritious cereal grown mainly in Ethiopia. Lodging in *E. tef* is severe due to its tall and slender stature with yield losses of ~15-45% each year. In Ethiopia alone it constitutes over two-third of the national diet for a 90 million people. However, productivity has been very low (1-2 t ha^{-1}) due to the low yield of unimproved varieties greatly aggravated by lodging (Assefa *et al.* 2010). The large acreage of cultivation, about 2.6 million ha (CSA 2008) despite low productivity, has been due to compelling desirable features. These include tolerance to drought and water logging stress (Tefera and Ketema 2001), high nutritional quality, best quality pancake, feed quality of straw and other uses.

Several studies have shown lodging improvement by modifying plant stature using plant dwarfing genes (Asano *et al.* 2009; Wang and Li 2008; Berry *et al*. 2004). In most studies, GA genes promoted internodal elongation in a grass species (Taiz and Zeiger 2006). However, mutant alleles of the dwarfing genes negatively affect plant performance, often decreasing fertility. Some mutant alleles have been exploited for crop improvement, such as semi-dwarf rice and wheat genes, leading to the Green Revolution in the 1960s. Importance of GA genes was demonstrated by the discovery and subsequent studies on GA-deficient mutants in maize (Phinney 1956), rice (Murakami 1970) and *Arabidopsis* (Koornneef and Vanderveen 1980). These mutants had typically a dwarf, or semi dwarf phenotype, with a reduced bioactive GA amount.

In GA-biosynthesis, *GA20ox* is an important pathway regulator. It catalyses conversion of GA_{12} and GA_{53} to GA_9 and GA_{20} in parallel pathways. They are then converted to bioactive GA_4 and GA_1 by *GA3ox* (Fig. 1). In many species *GA20ox* is encoded by several genes with a

distinct spatial and temporal expression pattern and with some overlapping function regulated by environmental signals and endogenous factors (Hedden and Phillips 2000; Lange 1998). This is caused by multiple alleles and null mutations in some of the genes (Yamaguchi 2008). An example is the semi-dwarf (*sd-1*) mutant in rice where an overlap between expression and mobility of GA products from other genes prohibit severe dwarfing of the stem (Hedden and Phillip 2000). The rice genome carries four GA20-oxidase genes, *GA20ox1-4*, and the rice *Sd-1* (*OsGA20ox2*) gene is highly expressed in leaves and flowers (Yamaguchi 2008). The mutant *sd-1* has a 280 bp deletion in the *GA20ox* coding region which results in rice in a semi-dwarf phenotype. Similar phenol-morphic effects have been also found in plants like *Arabidopsis* carrying three GA20 oxidases (Spielmeyer *et al*. 2002). Rice plants with this mutation had a greater harvest index allowing for increased use of nitrogen fertilizers. Generally, due to their regulatory role, which determines also GA concentration, members of this GA20 oxidase gene family have been targets for genetic manipulation and introduction of agronomically useful traits (Appleford *et al*. 2006; Carrera *et al*. 2000; Sakamoto *et al*. 2003).
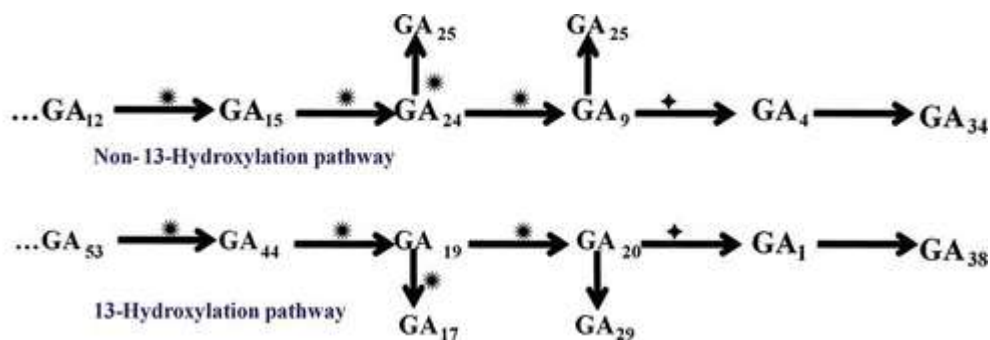
Figure 1. Partial scheme (cytosolic) of the gibberellin (GA) biosynthesis pathways catalyzed by *GA20ox,* a multifunctional gene catalyzing several steps as shown by the symbol "✳" and the final step catalyzed by *GA3ox* (✦).

In wheat, a semi-dominant response mutant, the *reduced height* (*Rht*) gene, has been identified being GA insensitive and encoding a GA repressor in the response pathway (Peng

et al. 1997). This mutant has increased endogenous GA levels due to a negative feed-back regulation (Alvey and Harberd 2005). Introgressed in modern varieties of wheat, the *Rht* gene results in a semi-dwarf plant stature with increased lodging resistance which contributed to the success of the green revolution (Peng *et al*. 1999). *Rht* mutants have also multiple allelic variants providing variation in plant height and productivity (Peng *et al*. 1999).

In *E. tef*, very little genomic research has been carried out and genes for plant height control are unknown. Due to the agronomic significance of inducing dwarfism for the control of lodging, this study was carried out to identify and clone for the first time target *E. tef* GA genes encoding multifunctional GA20-oxidases. Also investigated was the characterization of the sequences in an *in vitro* functional assay and determining expression of *GA20ox* homologues in different plant tissues using internodes, nodes, leaves and panicles aimed to predict the functional equivalent orthologous gene(s) to the rice *sd-1*.

Materials and Methods

Plant material and plant cultivation

Seed material for the *E. tef* varieties DZ-01-196, an improved tall variety grown widely but susceptible to lodging, and Gea Lammie, a short landrace variety, were obtained from the Holetta Agricultural Research Center, Ethiopia. Variety DZ-01-196 was developed for higher yields in a conventional breeding program (Teklu and Tefera 2005). Seeds from both genotypes were germinated on germination mix soil and the seedlings were grown in pots (about 225 plants per m^2) under a 26±2/18°C day/night temperature and a 14 hr day length.

Plants were further supplemented with slow release fertilizer containing 15-11-13 NPK plus micronutrients until all samples were collected.

DNA and RNA isolation and complementary DNA (cDNA) synthesis

All DNA and RNA isolation was done with variety DZ-01-196 and Gea Lammie. Young leaves were homogenized in liquid nitrogen and frozen and stored at $^-80^0$C for further use. The fine-powdered plant material was immediately incubated after homogenization and transferred to a reaction tube containing pre-heated (60^0C) extraction buffer according to the method of Harini *et al.* (2008). Isolated genomic DNA was finally dissolved either in TE buffer (10 mM Tris-HC1, 1 mM EDTA, pH 8.0) or dsH$_2$O and further treated with RNase and precipitated again as outlined above.

Frozen tissues from different developmental stages, one week old germinating shoot, stem and/or leaf (5, 8 and 10 weeks old) and young (10 days old) inflorescence from the two *E. tef* varieties DZ-01-196 and Gea Lammie were used for sample preparation. The samples were homogenized in liquid nitrogen followed by total RNA extraction using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instruction. Total RNA was then used for isolation of full-length sequences of coding regions using 5´/3´ RACE employing the RLM-RACE (RNA ligase mediated -Rapid amplification of cDNA ends) kit (FirstChoice® RLM-RACE Kit, Applied Biosystems), This method does not require 2nd strand cDNA synthesis. It further involves an adapter ligation to the de-capped mRNA and application of an inner and outer specific primer 5'/ 3' RACEs for generation and amplification of cDNA. Up to 2 µg of total RNA was used for cDNA synthesis with the First-Strand Synthesis System (Invitrogen, UK) according to the manufacturer's instruction.

Gene identification and isolation

Identification and cloning of GA biosynthesis genes, the rice *sd-1* orthologs (*GA20ox*) in *E. tef* were carried out with specific sense and antisense primers (Table 1). Primers were designed using the primer3 program (Rozen and Skaletsky 2000) based on conserved domains of rice, wheat, sorghum and maize *GA20ox* gene sequences accessed from GenBank (Spielmeyer *et al*. 2002) . The PCR conditions were optimized using these primers for DNA or cDNA amplification.

Table 1. Primers used for extending *GA20ox* sequences in *E. tef* using RACE-PCR amplification

Target gene	Primers	Primer sequence (5' – 3')
EtGA20ox1	GA20 ox1SPR3	GCGGCAGCGTGAAGAAGGCGTCCAT
	GA20 ox1SPR2	CACATCATCATCATCATCATCGGT
	GA20 ox1SPR1	CTCGGCGGGTAGTAGTTGAGGCGCAT
	GA20 ox1SPF1	CCTTCGTCGTCAACATCGGCG
	GA20 ox1SPF2	CGGAGACAACCAAAGGAGGCG
EtGA20ox2	GA20 ox3SPR3	GTCTCCTTCCACGGCAGCAATCG
	GA20 ox3SPR2	CGATTGGCAGTAGTCTCGGAACA
	GA20 ox3SPR1	CGGGCACGGCGGGTAGTAGTTG
	GA20 ox3SPF1	GCAGGGACTTCTTCGCCGACG
	GA20 ox3SPF2	CGGGAGCCATCGTCGTCAACATC

Primers for sense were F1: GCT GCC GTG GAA GGA GAC; F2: CAC CGA TGA TGA TGA TGA TGA TG; F3: CTA CGC GAG CAG CTT CAC G and for antisense R1: CGC CGA TGT TGA CGA CGA. Primers for *GA20ox* were targeted to the region encoding the amino acid sequence KLPWKET (sense) and NYYPXCQKP (antisense). PCR amplification was done with a 50 µl reaction mixture consisting of 5x PCR buffer (Green GoTaq Flexi

buffer containing 10 μl MgCl$_2$ (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 GoTaq Taq DNA-polymerase (5 U/μl); 33.75 μl H$_2$O. PCR reactions were run at 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) and final extension at 72°C for 7 min and then holding the reaction at 4°C.

Amplification with a high-fidelity polymerase was done to minimize PCR reading errors in quantitative PCR to monitor differences in transcript abundance. In such cases the "Phusion" polymerase proof reading enzyme (Finnzymes) was used. The PCR reaction contained the following: 10 μl 5x Phusion "GC" buffer; 1 μl dNTPs (10 mM each); 1 μl each for forward and reverse primers (10 μM each); 1μl DNA (100 ng/μl); 1 μl DMSO; 0.5 μl Phusion polymerase (2 U/μl); 34 μl H$_2$O. PCR cycling conditions were: 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. Genomic DNA amplifications were done with GC-rich buffer and addition of DMSO (molecular grade). PCR products were excised from the gel and purified for cloning or nested PCR applying the conditions described above.

Isolation of complete *E. tef GA20ox* coding regions was done by extension of *GA20ox* homologs (*GA20ox1-3*) to their unknown 5' and 3'ends with 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) (Table 1). Complementary DNA synthesis was done with manually designed specific primers (Table 2) and cloned PCR products were further sequenced to obtain sequence information.

Table 2. Primers used in quantitative and semi-quantitative PCR amplification of putative *EtGA20ox* sequences in *E. tef.*

Target *E. tef* gene	Primer	Primer sequence (5'-3')
EtGA20oxa	GA20 ox1 02F	ATGTGGTGGGCTACTACGTCAGCAAG
	GA20 ox1 01R	TCATCTCCGAGCAGTAGCGCCCGT
EtGA20ox2	GA20 ox2 02F	CGGCCCACACCCTCTTGCTCCA
	GA20 ox2 02R	TTGACGACGATGGCTCCCGGCTT
EtGA20ox1b	GA20 ox3 02F	GGACTACCTGGTGGGCCGC
	GA20 ox3 01R	TAGTAGTTGAGCCGCATGATGGAGTCG
EtSDH	SDH 01F	CACAGCTGAGCGCTACGTTCTC
	SDH 01R	CCCAATGCAACACCGAAAATACG

DNA sequence analysis and phylogenetic relationship

DNA sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov /blast/) and compared with sequences in the GenBank database. Candidate nucleotide sequences were translated into protein with 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 carried out with amino acid sequences of related genes from the different species applying MAFFT alignment (http://mafft.cbrc.jp/alignment) software followed by CLC Bio Main bench (Version 5.5).

Analysis of GA20ox transcripts

The expression of *E. tef* GA20-oxidases in different tissues and developmental stages was determined by a quantitative real-time PCR (qRT-PCR) with purified total RNA from

duplicate tissues samples of varieties DZ-01-196 and Gea Lammie. The qRT-PCR analysis was done with the LightCycler technique for quantitative reverse transcription of mRNA levels using SYBR Green too produce fluorescence for detection. The *E. tef SDH* gene was taken as an internal control (Table 2) based on a stability test for constitutive expression to optimize qRT-PCR measurements (data not shown). Transcript quantification was further done with purified total RNA from various tissues of variety DZ-01-196 prepared in three sample replicates for the three homologous sequences by RT-PCR analysis. PCR DNA amplification was carried out at 94°C for 1 min followed by a 32 cycles amplification 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. For both quantitative PCRs, specific primers, shown in Table 1, were designed on the basis of polymorphic regions of the three cloned homologous sequences. The length of the expected amplification product ranged between 80-190 bp.

Expression of *EtGA20ox1a* in *E. coli*

The activity of *EtGA20ox1* encoded protein was monitored using a gel-purified PCR product (1246 bp) that was amplified sense primers: 5'-AG<u>G GAT CC</u>A GCC AGC TGC CCG TGA TG-3' and in antisense: 5'-TG<u>A AGC TT</u>**A** ACA GAA CAG GCG GTC ATG GAT GAC-3' from a cDNA template. The PCR amplification was done with "Phusion" Taq polymerase and the blunt-end PCR product was gel-purified and directionally ligated into the restricted pET32a vector (Novagen, UK) applying the restriction enzymes BamHI and HindIII (underlined). Competent *E. coli* DH5-α cells were transformed with the *E. tef GA20ox1* 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 µl) was then added to 50ml 2YT, containing 0.1 mg/ml carbenicillin, and incubated at $37^{o}C$ for 2 hr and then 50 µl of 1M IPTG was added to the cell suspension. Cells were cultivated at $25^{o}C$ for 6 hr under shaking (200 rpm). The cell suspension was finally centrifuged for 5 min and the cell pellet was frozen overnight at $-20^{o}C$.

HPLC analysis

Defrosted cells were re-suspended in 1.5 ml lysis buffer containing 1 ml 1 M Tris-HCl, pH 7.5, 50 µl 1M DTT, 200 µl 50 mg/µl lysozyme, and 8.75 ml sdH$_2$O. The cell suspension 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 $4^{o}C$. The cell lysate was kept at $^{-}80^{0}C$. The lysate (90 µl) was then thawed and incubated with co-factor mix and substrate (GA12-^{14}C) for 2 hr at $30^{0}C$ under shaking at 200 rpm. Glacial acetic acid (10 µl) was added to the mixture, further diluted and then centrifuged before HPLC analysis to measure the GA intermediate products catalyzed by the putative EtGA 20-oxidase1a protein.

Southern blot analysis

A DNA hybridization probe of 450 bp was synthesized using specific primers for amplification of a coding region of *GA20ox* which was labeled with a DIG labeling probe synthesis kit (Roche). The probe was synthesized with an initial denaturation cycle for 2 min at 95°C followed by 30 cycles of denaturation at 95°C (10 sec), annealing 67°C (30 sec) and elongation at 72°C (40 sec). Final elongation was for 7 min at 72°C and cooling at 4°C. Purified gDNA (25 µg) was digested overnight with the restriction enzymes BamHI, EcoRI

and HindIII. Digested genomic DNA was fractionated on a 0.7% agarose gel and transferred to a nylon membrane. Transferred DNA was hybridized with the DIG-labeled probe in an oven at 42°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.

Analysis of endogenous GA content

The upper most two internodes including the nodes at shoot elongation stages and just before panicle initiation were harvested and stored at ⁻80^{0}C until analysis. Endogenous GA levels were monitored by using ground internodal tissue applying a ball mill extraction. Powdered replicate samples of 0.4-0.5g were re-suspended in 80% aqueous MeOH with addition of GA internal standards (2H- and 3H-labeled). The pH of the aqueous extract with ethanol removed was adjusted to 3.0 with 1 mol/l HCl before further partitioning three-times with water-saturated ethyl acetate. Full methylated samples dissolved in methanol were injected onto an analytical C18 reversed phase HPLC column for fractionation. Recovery of fractions was monitored with (3H) internal standards and GAs were quantified with a gas chromatography-mass spectrometry (GC-MS) system using selective ion monitoring.

Results

Isolation of GA genes from *E. tef*

Identification and cloning of *E. tef* orthologs of the rice *SD-1* (*SEMI-DWARF* 1) genes with primer sequences of the conserved amino acid regions from rice, sorghum, maize, wheat and barley yielded three PCR fragments tentatively named as *tef* 1, *tef* 2 and *tef* 3 with sizes

ranging between 422-523 bp (Fig. 3). After cloning and sequencing of fragments, the resulted protein sequence had a >60% identity with known *GA20ox* orthologs from different species.

Putative *E. tef GA20ox* isolation and cloning

Based on the known sequence information, *tef* 1-3, sequence specific (SP) primers (Table 1) were designed for nested PCR amplification. Generation of full-length sequences of coding regions was done using 5´/3´ RACE which resulted in two contigs each for 5´ and 3´ amplifications with sizes of 238, 263 and 121 and 305bp. No further sequence extension could be obtained for *tef* 2 and *tef* 3. The four fragments of *tef* 1 were aligned with the "contig assembly" program (Vector *NTI Advance™9.0*). The coding region of the consensus sequence (1448bp) was translated into a protein (Fig. 2) with the ExPasy protein translation tool. The sequence consisted of an open reading frame encoding a putative polypeptide of 365 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) for *GA20ox*: LPWKET, (143-149 aa) and NYYPXCQKP (227-236 aa of the putative sequence) and three His residues for binding Fe^{2+} (245-247 aa).

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atggtggtggtagcgcaggcgcaggcgcaggcgcagatagaagtagaagcagatgagccgccgcagcagcttgag   75
 M  V  V  V  A  Q  A  Q  A  Q  A  Q  I  E  V  E  A  D  E  P  P  Q  Q  L  E     25
gtggtggtgttcgacgcggcccgtctgagcgggctgagtgacatcccggcccagttttttgtggccggaggaggag  150
 V  V  V  F  D  A  A  R  L  S  G  L  S  D  I  P  A  Q  F  L  W  P  E  E  E     50
agcccgacgcctgacgcggcggaggaggagctggacgttcctctgatcgacctctccggcgacgcgtcggaggtg  225
 S  P  T  P  D  A  A  E  E  E  L  D  V  P  L  I  D  L  S  G  D  A  S  E  V     75
gtccgtcaggtgcgtgaggcctgcgaggcgcacggcttcttccaggtggtgaaccacggcatcgacgccggcctc  300
 V  R  Q  V  R  E  A  C  E  A  H  G  F  F  Q  V  V  N  H  G  I  D  A  G  L    100
gtggcggaggcgcaccgctgcatggacgccttcttcacgctgccgctgccggagaagcagcgcgcccagcgccag  375
 V  A  E  A  H  R  C  M  D  A  F  F  T  L  P  L  P  E  K  Q  R  A  Q  R  Q    125
cccggcgactgctgcggctacgccagcagcttcacgggccgcttcgccagcaagctgccatggaaggagacgctc  425
 P  G  D  C  C  G  Y  A  S  S  F  T  G  R  F  A  S  K  **L  P  W  K  E  T**  L    150
tccttccgggccaaggccaaggccaccgatgatgatgatgatgtggtgggctactacgtcagcaagctgggc  500
 S  F  R  A  K  A  K  A  T  D  D  D  D  D  V  V  G  Y  Y  V  S  K  L  G    175
gaggcctacaggcggcacggcgaggtgtacgggcgctactgctcggagatgagccggctgtcgctggagatcatg  575
 E  A  Y  R  R  H  G  E  V  Y  G  R  Y  C  S  E  M  S  R  L  S  L  E  I  M    200
gaggtgctgggcgagagcctgggcgtggggccgccgctgcttccgcgacttcttccaggacaacgactccatcatg  650
 E  V  L  G  E  S  L  G  V  G  R  R  C  F  R  D  F  F  Q  D  N  D  S  I  M    225
cgcctcaactactacccgccgtgccagcggcccatggagacgctgggcacgggcccgcattgcgaccccacctcc  725
 R  L  **N  Y  Y  P  P  C  Q  R  P**  M  E  T  L  G  T  G  P  **H  C  D**  P  T  S    250
ctcaccatcctgcaccaggaccacgtcgccggcctccaggtcttcgccgccggccggtggctctccatccgcccg  800
 L  T  I  L  H  Q  D  H  V  A  G  L  Q  V  F  A  A  G  R  W  L  S  I  R  P    275
cacgcccaggccttcgtcgtcaacatcggcgacaccttcatggcgctctccaacggccggtacaaaagttgcctg  875
 H  A  Q  A  F  V  V  N  I  G  D  T  F  M  A  L  S  N  G  R  Y  K  S  C  L    300
cacagggcggtggtcaacagcagcgtcccgcggaggtccctggccttcttcctctgcccggagatggacaagctc  925
 H  R  A  V  V  N  S  S  V  P  R  R  S  L  A  F  F  L  C  P  E  M  D  K  L    325
gtcacgctcccgccgcagcttcttcctgatctgcccggagacaaccaaaggaggcgcccctacccggacttcacg 1000
 V  T  L  P  P  Q  L  L  P  D  L  P  G  D  N  Q  R  R  R  P  Y  P  D  F  T    350
tggcgcaccctgctcgaattcacgcagaagcacacagggccgacatga                            1048
 W  R  T  L  L  E  F  T  Q  K  H  T  G  P  T  *                              366
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Figure 2. Putative *E. tef GA20ox1* (Acc. No. JX894241) full coding region with deduced amino acid residues of *GA20ox1* sequence (366 aa) and with 5' and 3' flanking regions (un-translated). The conserved domains are indicated bold and underlined. Conserved domains include the consensus sequences **NYYPXCQKP** of 2-oxogltarate dependent dioxygenase (2ODDs) for binding the common co-substrate and sequence **LPWKET**, which binds to GA substrates, and three histidine residues **HCD** for binding Fe^{2+}. Asterisk (*) shows position of the stop codon. Numbers at the right indicate nucleotide or protein (bold) numbers.

The three *E. tef GA20ox* sequences were named *EtGA20ox* and were compared for similarity. Amino acid sequences comparison (trimmed to core areas present in all the three sequences) showed that *EtGA20ox1* was 89.6% and 59.8% identical to *EtGA20ox3* and *EtGA20ox2*,
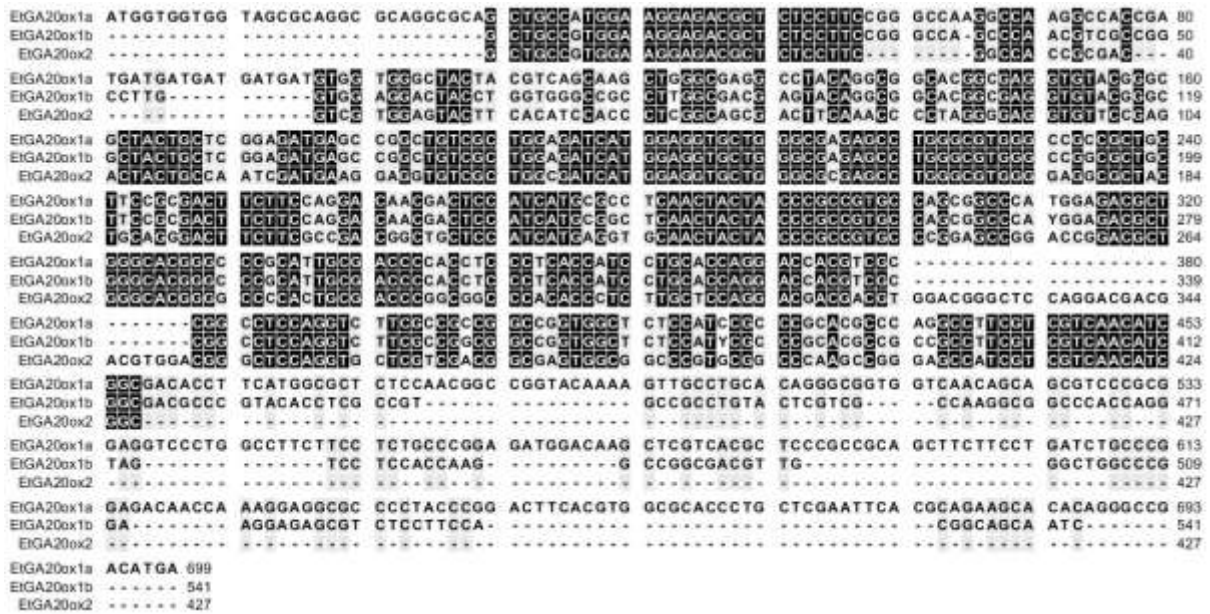
Figure 3. Nucleotide coding sequence alignment of three *E. tef GA20ox* putative sequences. Putative nucleotide sequence for the conserved regions are shown with dots, an over-line and highlighted with asterisk (*). Dashes (-) have been inserted to maximize sequence homology. Identical and similar regions are shown by dark (100%) and light (>66%) shads respectively whereas number indicates the position of the nucleotide within the predicted nucleotide coding sequence where *EtGA20ox1a* has 428+699=1127bp.

respectively. Because of close similarity between *EtGA20ox1* and *EtGA20ox3*, they were renamed as *EtGA20ox1*a and *EtGA20ox1*b. *GA20ox* isolated from different species exhibits a conserved domain of amino acids with identity ranging from 50% - 75% (Hedden and Kamiya 1997). BLAST results of the full coding region of putative *EtGA20ox1a* (Fig. 3) showed high identity scores with GA20 ox orthologs from *Sorghum bicolor* (68.3%), *Zea mays* (68.7%), *Oryiza sativa* (59.3%), *Lolium perenne* (64.6%), *Triticum aestivum* (66.6%), *Hordeum vulgare* (67.5%), *Dasypyrum villosum* (67%) and *Zyocia japonica* (54.5%).
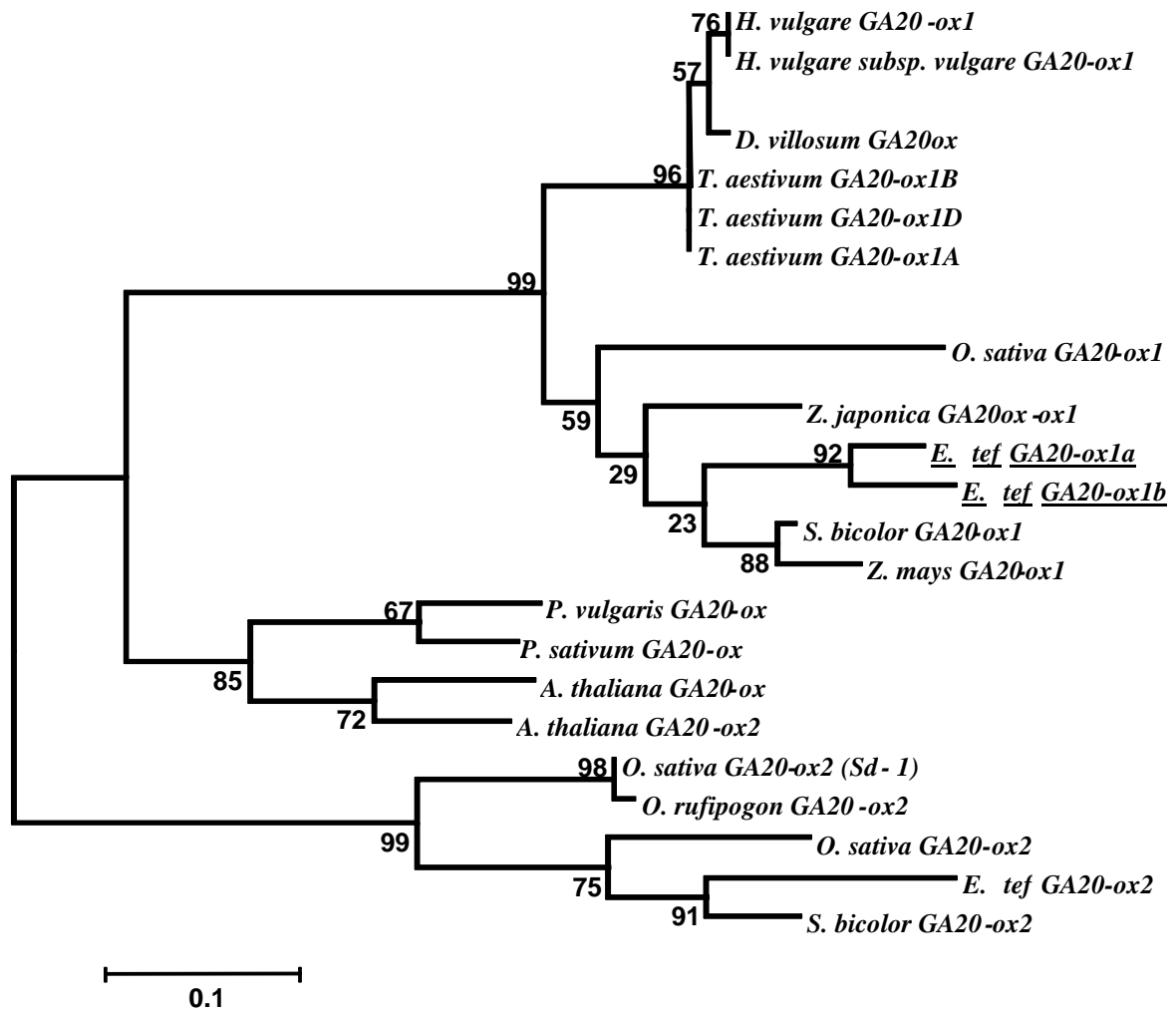
The *EtGA20ox2* partial coding sequence had high polymorphism at the N-terminus when aligned and compared to the other two sequences from *E. tef* or to orthologous sequences from other closely related species. Generally, alignment and phylogenetic relationship further
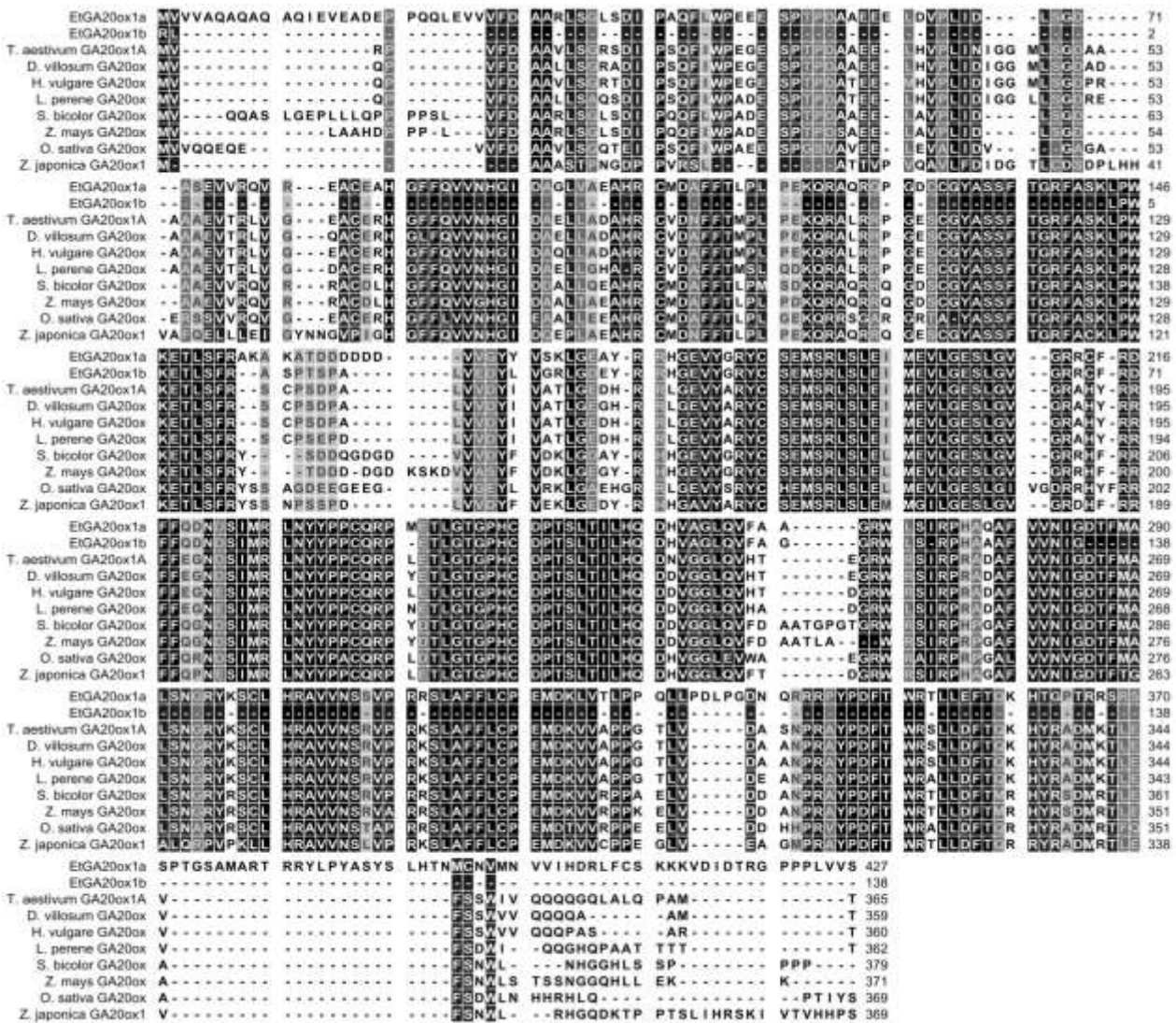
Figure 4. Derived aa sequence alignment of two *E. tef GA20ox* (*E. tef GA20ox1a* and *EtGA20ox1b*) sequences including intron regions with orthologous *GA20ox* gene sequences from *S. bicolor*; (Acc No. XP_002463483.1), *Z. mays;* (Acc No. ACF83905.1), *O. sativa*; (Acc No. P93771.2), *T. aestivum;* (Acc No. 004707.1), *H. vulgare*; (Acc No. AAT49058.1), *L. perenne*; (Acc No. AAG43043.1), *Z. japonica*: (Acc No. ABG33927.1) and *D. villosum*; (Acc No. ACU40946.1). Conserved motifs, NYYPXCQKP (227-236) and LPWKET (143-150) and the putative Fe^{2+}- binding consensus regions (245-247) are shown overlaid with dotted bar (▪▪▪▪). Identical and similar regions in relation to *EtGA20ox1* are shown by dark and light shades respectively whereas number indicates the position of the aa within the predicted protein sequence. Identity with other closely related cereals range from 54 to 69%.

Figure 5. Derived protein sequence alignment of *EtGA20ox2* sequence with orthologous GA20ox gene sequences from other grass species namely *S. bicolour*; (Acc No. XP_002441117.1), *Z. mays*; (Acc No. ACN25832.1), *O. sativa and O. rufogen,* (Acc No. AAT44252.1 and BAK39011.1), *T. aestivum;* (Acc No. 004707.1) *H. vulgare*; (Acc No. BAK04752.1), *P. sativum*; (Acc No. AAF29605.1) and *A. thaliana* (Acc No. NP_199994.1). 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.

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Figure 6. Molecular phylogenetic analysis of the putative *E. tef* GA20ox sequences (*E. tef GA20ox1a, 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.

showed high identity with *GA20ox2* sequences from species such as *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 sequence regions surrounding the

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functional domains were compared (Fig. 5 and 6). Further, sequences *EtGA20ox1a* and *EtGA20ox1b* were similar to *GA20ox1* sequences from sorghum, maize, zyocia *spp.* and rice. Close similarity of these genes in *E. tef* also imply redundancy of function in the tetraploid *E. tef*.

EtGA20ox1a transcription

The two genotypes Gea Lammie (short phenotype) and DZ-01-196 (tall phenotype) showed differences in transcript abundance for *EtGA20ox1a* in different tissues. In both genotypes, *EtGA20ox1a* was transcribed in germinating hypocotyls, leaf, stem and inflorescence but with higher transcription in young stem and leaf (Fig. 7) but with slight differences between the two genotypes. Transcription was lower at later stages towards maturity and no strong differences were observed, except after 10 weeks when the short genotype had a higher transcript amount in the stem tissue.

Transcript amount was significantly higher at 5th week after emergence corresponding to the period of rapid stem elongation growth before heading (inflorescence emergence). The transcript amount decreased gradually after the 5th week, except in the inflorescence (7th week i.e. early heading stage) in DZ-01-196. However, *EtGA20ox1* transcript abundance was generally higher in Gea Lammie than in DZ-01-196 (Fig. 7) which is possibly caused by interference in GA biosynthesis. However, the amount of bioactive endogenous GA measured in Gea Lammie was lower than in DZ-01-196 (Table 3). Biochemical analysis showed that both the GA_3 amount and the amount of most intermediates of GA in Gea Lammie were reduced by almost half in DZ-01-196. A low amount of bioactive GA was found in the short genotype despite relatively high transcription of *GA20ox.*

Table 3. Quantification of GA forms from internode samples at stem elongation of *E. tef* cultivars DZ-01-196 and Gea Lammie.

Genotype	$GA_1^§$	GA_{29}	$GA_3^§$	GA_{15}	$GA_4^§$	GA_8	GA_{20}	GA_{19}	GA_{34}	GA_9	GA_{53}
DZ-01-196	5.71^{**}	2.25	3.17	0.0	0.0	9.36	6.65	19.22	0.18	ND	10.68
Gea Lammie	4.22	1.12	1.07	0.0	0.0	5.35	4.63	10.97	0.3	0	4.28

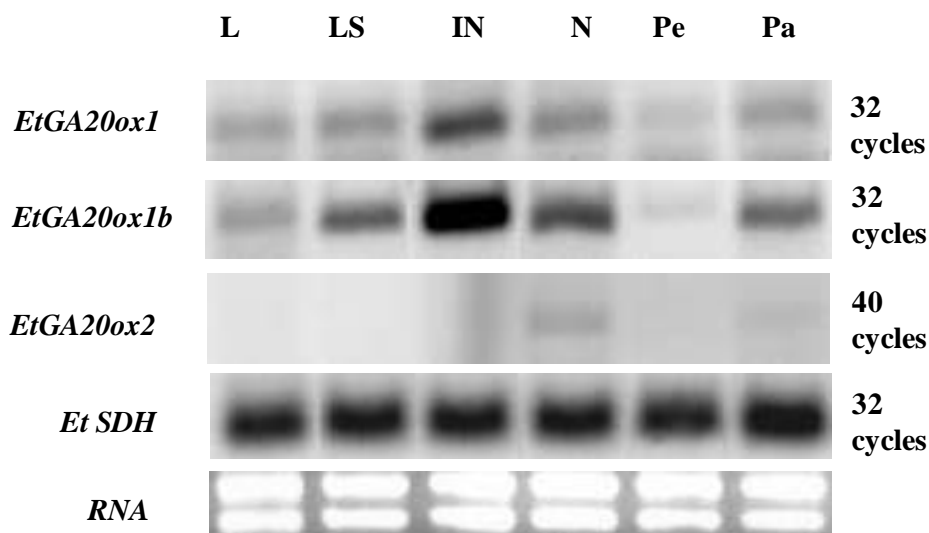ND =not determined; §Bioactive forms of GA; **Values are in ng per g dry weight and a data point represents average of three analyzed samples.
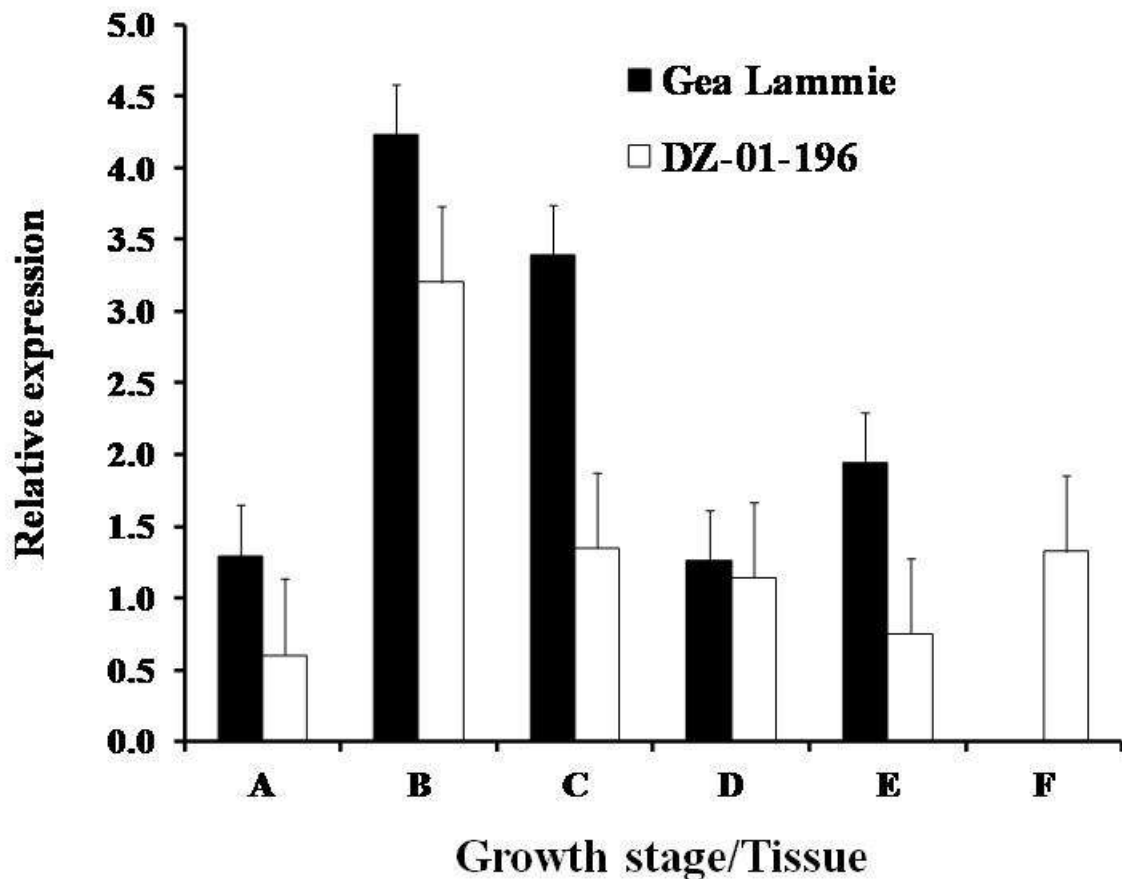
Figure 7. Relative expression of *GA20ox1* in two *E. tef* genotypes Gea Lammie and DZ-01-196 in different plant tissues and growing stages; (A) 3rd week stem + leaf; (B) 5th week stem + leaf; (C) 8th week stem; (D) 8th week leaf (E) Old (10th week) stem and (F) 10 days old inflorescence. The qRT-PCR was repeated three times, and gene expression level was calculated following the expression 2^{-DCt} (Yang *et al*. 2005).

Transcription of *GA20ox* homologs

Transcription of the three *EtGA20ox* homologous genes was further studied with leaf, leaf sheath, uppermost two internodes, nodes from these internodes, peduncle and inflorescence in the tall variety DZ-01-196 applying RT-PCR. Both *EtGA20ox1a* and *EtGA20ox1b* transcription was highest in the uppermost internodes followed by nodes (adjoining internodes) (Fig. 8). However, transcription of *EtGA20ox1b* was greater than *EtGA20ox1a* in internodes, nodes, leaf sheath and panicle. In contrast, transcription of *EtGA20ox2,* an ortholog to the rice *sd-1,* was relatively low in the nodes, panicle and upper most internodes when compared to the other two homologous *E. tef* genes.

	L	LS	IN	N	Pe	Pa	
EtGA20ox1							**32 cycles**
EtGA20ox1b							**32 cycles**
EtGA20ox2							**40 cycles**
Et SDH							**32 cycles**
RNA							

Figure 8. 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 32/40 and 32 cycles for *EtGA20ox* and *EtSDH*, respectively. The experiment was repeated three times with similar result. *EtSDH* was used as an internal PCR amplification control.

EtGA20ox copy number

The copy number of *EtGA20ox* gene in *E. tef* was determined with Southern blot analysis where the probe hybridized with four fragments after HindIII digestion of genomic DNA (one is slightly visible) or BamHI digestion. But only one clear fragment, and two hardly detectable fragments, were found after EcoRI digestion (Fig. 9). Therefore, four copies of *EtGA20ox* are possibly present in the allotetraploid *E. tef* genome.
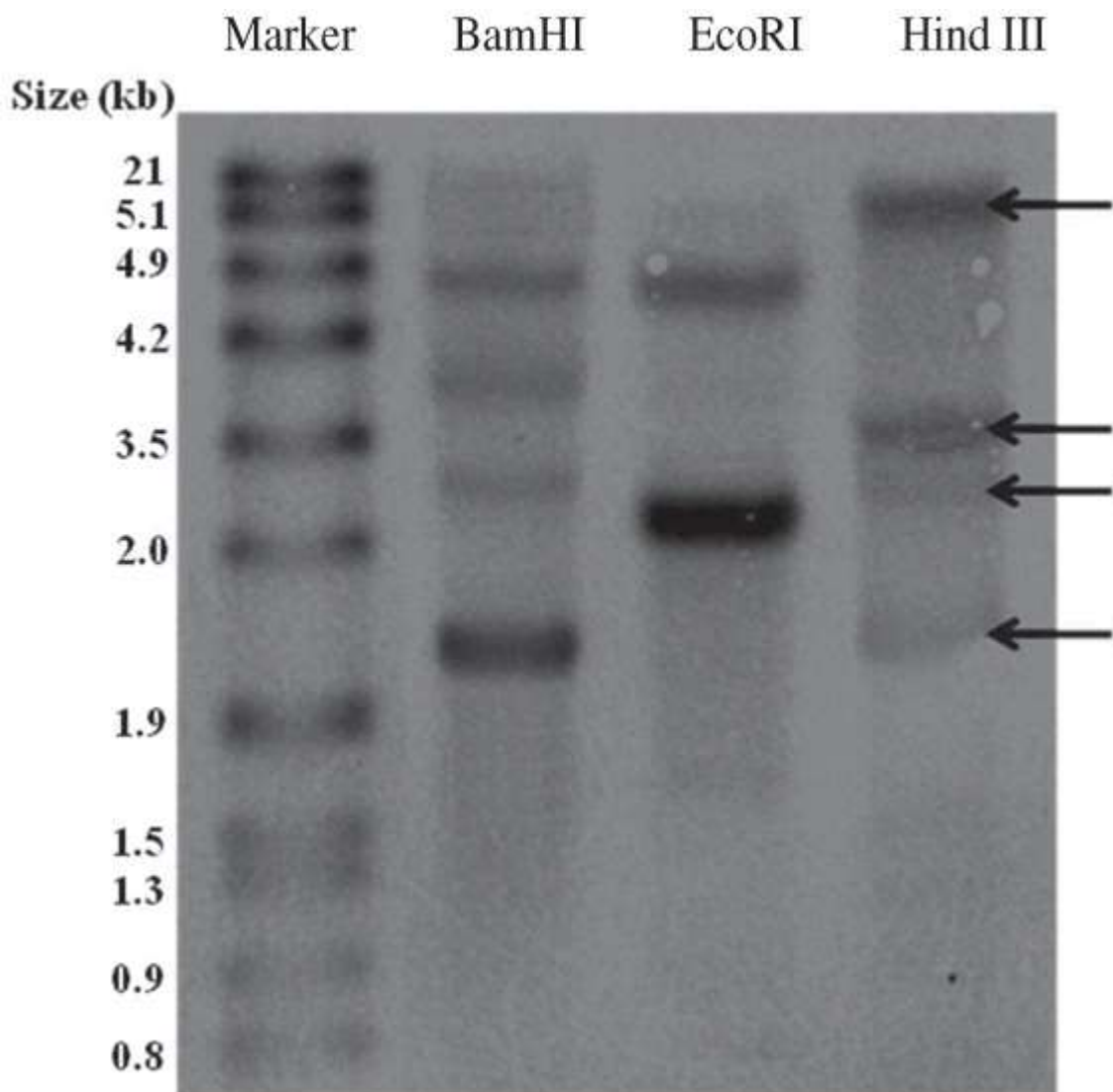
Figure 9. Detection of EtGA20 ox gene copies in the *E. tef* genome after restriction enzyme digest using Southern blotting.

In vitro enzymatic activity of *GA20ox1a*

To confirm whether the isolated cDNA clone encodes a functional *GA20ox*, the cDNA was subcloned into the restriction sites BamHI and HindIII of the vector pET32a to create a fusion with the open reading frame of the LacZ alpha peptide. The fusion was then introduced first into cells of *E. coli* DH5-α and then into *E. coli* B21 cells for protein expression. Expressed *EtGA20ox1* protein catalyzed the conversion of the [^{14}C]-labeled gibberellin precursor GA_{12} to GA_9 which could be detected by full-scan GC-/MS. In some
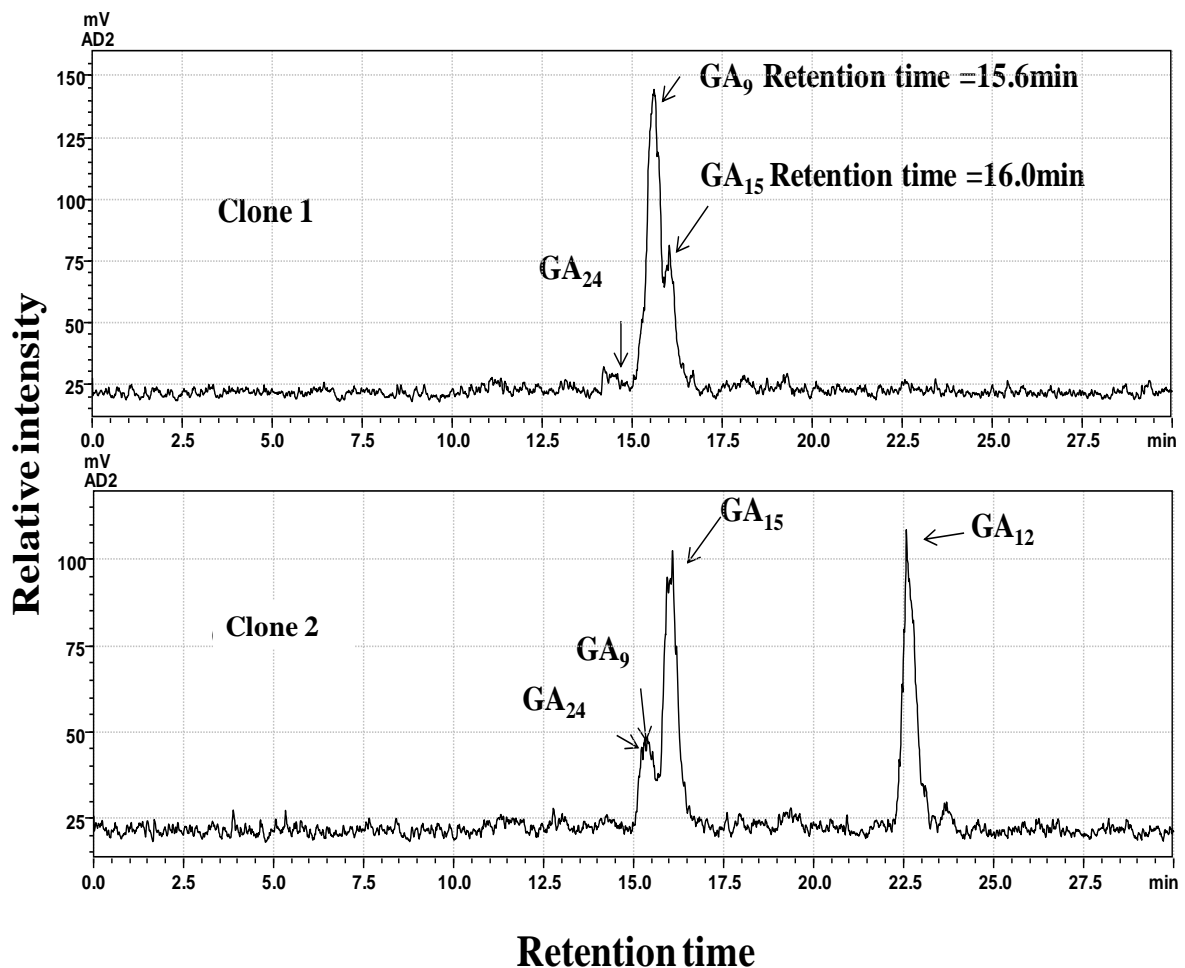
Figure 10. Radiochromatograms after HPLC of *E. tef GA20ox1* activity products after incubation with a ^{14}C-labeled GA_{12} as a substrate. Clone1 and 2 represent complete and partial conversion respectively of GA_{12} substrate to intermediates.

clones, only a partial conversion of the substrate was found, possibly due to sub-optimal reaction conditions. Intermediates of the reaction pathway, GA_{24} and GA_{15}, were also detected by HPLC analysis (Fig. 10). Optimum activity was found by complete conversion of GA_{12} into $GA_9 > GA_{15} > GA_{24}$.

Discussion

In this study, full and partial coding sequences of three GA biosynthesis genes from *E. tef GA20ox* have been isolated, characterized and their expression analyzed for the first time. The putative *EtGA20ox* genes were similar to genes of other cereal species (sorghum, maize, rice, wheat, barley) and also to the grass species *Zyocia japonica*. The putative *E. tef GA20ox* genes belong to the GA 2-oxoglutarate-dependent dioxygenase (2-ODD) gene family. This was confirmed by our heterologous expression assay producing final and intermediate products of GA 20-oxidase activity.

In a first step, we characterized the three isolated *E. tef* GA 20-oxidase DNA sequences. The three *E. tef* GA 20-oxidases (named *EtGA20ox1a, EtGA20ox1b and EtGA20ox2* with current not finally confirmed accession numbers JX894241, JX894242 and JX894243, respectively) have characteristic conserved amino acid residues, namely the 2-oxoglutarate co-factor, LPWKET motif, which is very likely involved in GA substrates binding (Xu *et al*. 2002), and the NYYPXCQKP motif for common co-substrate binding (Mrao *et al*. 2020). The conserved H and D residues are involved in Fe^{2+} binding at the active site of isopenicillin N synthase (Hedden and Phillip 2000 and Xu *et al*. 1995). *EtGA20ox2* has further a 120 bp intron *23 aa* downstream of the LPWKET domain. In comparison, the rice *sd-1* has three exons and two introns (Spielmeyer *et al*. 2002) where the first intron is 102 bp long after a 557 bp long

coding region and a 1471 bp long intron after a 981 bp coding region in the second exon region. The three *E. tef* sequences have also polymorphic regions due to changes in amino acid type and length arising from nucleotide deletions or substitutions between their conserved domains. The amino acid length in the most polymorphic coding region was 78, 73 and 69 for *EtGA20ox1a, EtGA20ox1b* and *EtGA20ox2*, respectively. The highest polymorphism between the closely related sequences, *EtGA20ox1a* and *EtGA20ox1b,* was in the region between the conserved domains. Based on our Southern analysis, a fourth copy might also exist in *E. tef*. But this copy has not been yet cloned and we have also no evidence yet if this copy is very similar or divergent to the already cloned copies. In the tetraploid *E. tef* each of the above genes are further expected to exist in two copies in the genome but more detail analysis might also reveal if *EtGA20ox1a* and *EtGA20ox1b* are haplotypes of the same gene.

When transcription of the three *EtGA20ox* genes was determined in a next step using semi-quantitative RT-PCR, differential transcription was found in different plant tissues in the tall variety DZ-01-196. *EtGA20ox1a* and *EtGA20ox1b* are transcribed in germinating hypocotyls, young stem, leaf as well as immature (emerging) inflorescence, but with differences in relative transcript abundance. Highest transcription is in the uppermost internodes followed by nodes. In contrast, *EtGA20ox2* transcription is relatively lower in nodes and internodes when compared to transcription of the other two genes. *EtGA20ox1a* or *EtGA20ox1b* seemingly promotes internode elongation and panicle growth acting redundantly and with some involvement in reproductive growth of the panicle. Such overlapping transcription has also been found in Arabidopsis. In Arabidopsis six homologous genes have been identified with *AtGA20ox1* and *AtGA20ox2* acting redundantly promoting elongation in the hypocotyl and internode, flowering time, and elongation of anther filaments (Rieu *et al*. 2008). In

Arabidopsis, *GA20ox1* also contributes to internode elongation (Galun 2010). In rice, among the four GA 20-oxidase homologous genes identified, *OsGA20ox2* (*SD-1*) is a dwarfing gene with a role in height control (Spielmeyer *et al*. 2002, Monna *et al*. 2002). In our study, *EtGA20ox1b,* but not *EtGA20ox2,* appeared to be an ortholog of the rice *sd-1* gene and possibly a functional *E. tef* equivalent expressed in stem. *EtGA20ox1b* might be therefore a potential candidate for future development of a semi-dwarf *E. tef* genotype. Further, in rice, among the four known *sd-1* homologous genes (*GA201ox1 - 4*), transcripts of *OsGA20ox1* were detectable predominantly in reproductive tissues (Oikawa *et al*. 2004) i.e. in the epithelium of the scutellum, whereas *OsGA20ox2* is expressed in the growing shoots, as well as in the epithelium (Yamaguchi 2008). Oikawa *et al*. (2004) also reported involvement of *OsGA20ox1* in stem elongation with silencing of *OsGA20ox1* by RNAi in rice resulting in a semi-dwarf phenotype. The possibility that *EtGA20ox1b* regulation in *E. tef* affects plant growth, similar to rice *sd-1 (OsGA20ox2),* requires further investigation. A*n* analysis of a loss-of-function mutant or RNAi silencing for each *EtGA20ox* genes and their detailed expression analysis might also provide more specific information about the roles of each gene in *E. tef* plant growth and development.

EtGA20ox1a, which seems to be more involved in stem tissue elongation, was used in a next step to measure tissue specific transcription between *E. tef* varieties Gea Lammie and DZ-01-196. The results from the two genotypes were variable with higher transcription abundance for *EtGA20ox1a* in Gea Lammie than in DZ-01-196. However, higher transcription was not related to any higher level of biologically active GA. In the short *E. tef* variety Gea Lammie the amount of bioactive GA was lower than in the taller genotype despite more transcripts. An increased transcript amount with reduced sensitivity to exogenous GA application has been previously reported in GA response pathway mutants (Olszewski, *et al.* 2002). The

relation between GA amounts *GA20ox* expression therefore requires further investigation to determine the exact relationship between *GA20ox* expression and actual GA amounts.

In a final step, we analyzed heterologous *EtGA20ox1a* expression and $^{14}GA_{12}$ substrate conversion to the alcohol and aldehyde intermediates GA_{15}, GA_{24} and GA_9 (Hedden and Kamiya 1997; Yamaguchi 2008) with further GA_9 conversion by *3β*-hydroxylation to the bioactive GA_4 (Junttila *et al.* 1992; Rood and Hedden 1994). All these products were detected after expression of *EtGA20ox1a* in *E. coli* with GA_{12} as substrate. This indicates that a true *EtGA20ox* has been isolated and characterized from *E.tef*.

In summary, this study has contributed with new genomic information to better understand the specific roles of *GA20ox genes* in *E. tef* and their association with physio-morphic variations such as plant height (semi-dwarfism), lodging resistance and yield. GA genes related to *E. tef* plant height isolated in this study will further allow studying function and expression in contrasting phenotypes for allele identification. In particular, *EtGA20ox1b,* predominantly expressed in stem tissue, might be a prime target for stem height regulation to develop a semi-dwarf phenotype more resistant to lodging either through plant transformation or mutation. The obtained sequence information will thereby facilitate selection of mutants affected in stem height through TILLING or Eco-TILLING for marker-assisted breeding for lodging resistance in *E. tef*.

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