

**CLONING AND SEQUENCING OF A β -GLUCOSIDASE cDNA FROM
Sorghum bicolor (L.) MOENCH AND ANALYSIS OF EXPRESSION
IN SEEDLINGS**

by
Muzaffer Cicek

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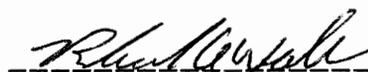
APPROVED:



Dr B W Shirley, Chairman



Dr C. L. Rutherford



Dr R. Walker

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**Cloning and sequencing of β -glucosidase From *Sorghum bicolor*
(L.) Moench and analysis of expression in seedlings**

by

Muzaffer Cicek

Asim Esen, Chairman

Biology

(ABSTRACT)

A full-length β -glucosidase encoding cDNA is isolated and sequenced from *Sorghum bicolor* (L) Moench. Using 5'- and 3'-end specific probes derived from the cDNA clone, the multiplicity of β -glucosidase genes and their expression in different tissues were studied. Southern blotting data showed that β -glucosidase is encoded by a small multigene family. Northern analysis data indicated that mRNA corresponding to the cloned gene is present at high levels in the node and mesocotyl 2 regions of the seedling and at low levels only in the zone of elongation region in roots. Other seedling parts such as mesocotyl 1, root sections adjacent to the seed and coleoptile sections do not have any detectable expression. The amino acid sequence data show 72% sequence identity between maize and sorghum β -glucosidase precursor proteins. In view of high sequence similarity between maize and

sorghum β -glucosidase, immunoblotting analysis was performed with maize-anti- β -glucosidase serum. The immunoblotting results supported the results of Hosel et al., (1987) with respect to the occurrence of two distinct β -glucosidases being present in sorghum.

***Dedicated to my Parents
Makbule and Mirza Cicek***

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CHAPTER 1

Introduction

β -glucosidases (β -D-glucoside glucohydrolases; EC 3.2.1.21) catalyze the hydrolysis of aryl and alkyl β -glucosides as well as glucosides with a carbohydrate moiety (Reese, 1977). These enzymes occur ubiquitously in plants, fungi, mammals and microorganisms (Woodward and Wiseman, 1982). In mammals, β -glucosidase (glucocerebrosidase) is present in the lysosome (Beutler, 1992). Under normal conditions, the substrate glucosylceramide is cleaved to ceramide and glucose by this enzyme (Beutler, 1992). Deficiency in activity of the lysosomal β -glucosidase in humans is known as Gaucher disease, which results from mutations in the gene encoding the enzyme. Seven different mutations have been shown to result in altered human β -glucosidase forms without catalytic activity. A landmark experiment with human β -glucosidase has been performed by Ohhassi et al. (1992). These investigators were able to transduce and express the human β -glucosidase (glucocerebrosidase) gene in mouse hematopoietic stem cells by using a recombinant

retroviral vector. This accomplishment points out the feasibility of using gene therapy for treating Gaucher disease.

Microbial β -glucosidases have also been the subject of much study by various researchers. *Agrobacterium fecalis* β -glucosidase was one of the earliest bacterial enzyme to be purified and characterized, as was the cloning and expression of its gene in *E.coli* (Day and Withers, 1986; Wakarchuk et al., 1986). The characterization of the wild-type *Agrobacterium* enzyme indicated that it was a dimer of 50 kD monomers (Day and Withers, 1986). *Agrobacterium* β -glucosidase has a high specificity for cellobiose; this has recently been documented further by mapping the residues at the catalytic center and determining the mechanism of catalysis (Trimbur et al, 1992). β -glucosidases of the cellulolytic fungi have been the subject of numerous investigations by various research groups. They have potential applications for developing novel carbohydrate foods, alcohol-based fuels and other commercial products from cellulose. Particularly, glucose production from the most abundant biological macromolecule, cellulose, can be achieved by the extracellular cellulase complex from some fungi such as *Trichoderma*. The cellulase enzyme complex isolated from *Trichoderma reesei* comprises at least three different enzymes that together hydrolyze cellulose to oligosaccharides

and glucose (Fowler 1993). Of these, the endoglucanases and cellobiohydrolases synergetically hydrolyze cellulose into small cellooligosaccharides, mainly cellobiose. Subsequently, β -glucosidase hydrolyzes cellobiose to glucose. The β -glucosidase gene *bgl1* from *Trichoderma reesei* was cloned and sequenced by Barnett et al. (1991). Recently, Fowler (1993) has studied β -glucosidase null strain of *Trichoderma reesei* to explore its role in the cellulase enzyme system in the hydrolysis of cellulose and induction of the other cellulolytic enzyme components. This investigator found that extracellular β -glucosidase is required for the induction of the other cellulase enzymes.

In plants, β -glucosidases play a significant role in defense against some pathogens and herbivores by releasing hydroxamic acids, coumarins, thiocyanates, terpenes, and cyanide from their corresponding glucosides (Niemeyer, 1988; Hruska, 1988; Jones, 1988; Poulton, 1990 and Oxtoby, 1991). Plant β -glucosidases also function in the hydrolysis of conjugated phytohormones (e.g., glucosides of giberellins, auxins, abscisic acid, and cytokinins) (Schliemann, 1984; Wiese and Grambow, 1986; Nowachi et al., 1980; Brzobohaty et al., 1993). One of the important functions of β -glucosidases in plants is the hydrolysis of the cyanogenic glucosides (cyanogenesis). In this case, the enzyme and substrate

occur in different compartments but come into contact with each other after injury to cells or tissues by pathogens or herbivores. As a result, the cyanogenic glucoside is hydrolyzed, releasing the toxic HCN (Conn, 1981; Jones, 1988; Poulton, 1990 and Oxtoby, 1991).

Substrate Specificity

β -glucosidases display broad specificity with respect to both the aglycone and the glycone moieties of their substrates. The natural substrates include the steroid β -glucosides and β -glucosyl ceramides of mammals, cyanogenic β -glucosides of plant secondary metabolism, and oligosaccharide products released from digestion of the cellulose of plant cell-walls (Clarke et al., 1993). In fact, β -glucosidases from every source have a similar specificity for the glycone (glucose) portion of the glucoside. However, the cyanogenic diglycoside (R)-amygdalin (the gentiobioside of (R)-mandelonitrile) that accumulates in black cherry seeds and other stone fruits has a disaccharide as the glycone part of the substrate (Poulton, 1993).

In plants, there are a vast number of aglycones, e.g., plant hormone glucosides, hydroxamic acids, flavanols and mandelonitrils conjugated to glucoside (Smit and van Staden 1978; Campos

et al 1993; Cuevas et al 1982; Niemeyer 1988; Conn 1993). Thus β -glucosides may have different functions depending on the aglycone moieties of their physiological substrates. The aglycone moieties of glucosides are implicated in a number of developmental and growth-related functions in plants (Selmar et al., 1987). Hosel and Conn (1982) have emphasized the importance of the aglycone in determining the specificity of β -glycosidases. The most common function of the aglycones is their involvement in plant defense mechanisms, as in the case of cyanogenic glucosides (Poulton, 1990).

The Mechanism of Catalysis by β -Glucosidase

All β -glucosidases have a general mechanism for the hydrolysis of the β -glycosidic linkage between an anomeric carbon and a glycosidic oxygen. There are two stereochemically different hydrolytic mechanisms proposed for cleavage of the beta linkage between the glycone and aglycone parts of a β -glucoside. They differ with respect to retention and inversion at the anomeric center of the reduced sugar residue. β -glucosidases, together with most cellulases and xylanases, are known to hydrolyze their substrate while retaining the anomeric configuration of the sugar moiety (Sinnot, 1990; Clarke et al., 1993). The retaining mechanism

involves the acidic catalysis which protonates the substrate and leads to the formation of a transition state oxocarbenium ion. The orientation of the catalytic group is thought to be complementary to the anomeric configuration of the sugar moiety, such that the acidic site (i.e. the carboxyl group) interacts with the site where glycosidic oxygen is found (Legler, 1990). At the end of the cleavage, the anomeric carbon of the monosaccharide reacts with a water molecule to generate β -D-glucopyranose. This mechanism strongly resembles the double displacement mechanism proposed for lysozyme (Koshland, 1953; Kempton and Withers, 1992; Clarke et.al., 1993) (Figure 1). The inverting mechanism of the anomeric configuration is based on the single displacement by a nucleophilic water molecule (Koshland, 1953). The difference between retention and inversion of the anomeric configuration is that only one transition state occurs in the inversion step of the single displacement mechanism. The final product in the inverting mechanism is α -D-glucopyranoside (Sinnot, 1990). Withers and Street (1989) proposed the double displacement mechanism for β -glucosidase catalysis based on Nuclear Magnetic Resonance (NMR) studies. They substituted the hydroxyl group at C-2 of the sugar by electronegative fluorine to destabilize the adjacent positive charge at the transition state. This resulted in decreased rates of glycosyl-enzyme formation and hydrolysis. They also used

a reactive leaving group, dinitrophenolate, incorporated into the aglycone group so that the reaction can be followed spectrometrically. This incorporation brought about considerable acceleration of glycosyl-enzyme formation without affecting the rate of glycosyl-enzyme hydrolysis. In contrast, the 2-glycoside-fluorine resulted in retardation of glycosyl enzyme intermediate formation and hydrolysis. The glycosyl enzyme intermediate was trapped easily in a transition state, thereby allowing the mechanism of β -glucosidase catalysis to be examined. Withers and Street (1989) also suggested that a covalent glycosyl enzyme intermediate is formed during the formation of oxocarbenium ion in the transition state.

The formation of enzyme-substrate complex in β -glucosidase catalyzed reactions is not well understood. Two pathways are proposed for the transition state of β -glucosidase in the double displacement mechanism: endocyclic and exocyclic pathways (Figure 2). Both pathways require two amino acid residues in the active-site, one serving as proton donor and the other as nucleophile stabilizer. In the case of the exocyclic pathway, the carboxylic acid of the amino acid protonates the exocyclic oxygen (the glycosidic oxygen) of the substrate to make it a good leaving group. This gives rise to an unfavorable electronic geometry. The alternative pathway, endocyclic, involves the protonation of the

endocyclic oxygen in the formation of the enzyme-substrate complex (Clarke et al., 1993). However, Withers and Street (1988) have proposed that these pathways may not be mutually exclusive and that β -glycosidases may act via either the endo or exocyclic pathway depending on the substrate. After the transition state has been reached, β -glucosidases hydrolyze the substrate while the configuration of the transition state is maintained.

The active-site nucleophile has been identified using 2'- 4'- dinitrophenyl-2-deoxy-2-fluoro- β -D-glucopyranoside, which inactivates the enzyme (Withers and et al., 1990). As mentioned above, these investigators used 2-deoxy-2-fluoro glucosides with a reactive leaving group (dinitrophenolate) to trap the transition state of β -glucosidase. They identified the sequence motif, ITENGA or its variant YITENGA, as being part of the active center. They also discovered that the glutamic acid residue of this sequence was attached to the sugar moiety. In another study, it was shown that glutamic acid 274 was in the consensus region ITELD in the active site of *Cellulomonas fimi* exoglucanase. The ITELD region is functionally similar to the YITENGA region of β -glucosidases and β -galactosidases (Tull and Withers, 1991).

As stated previously, the involvement of a carboxylic acid in the acid catalysis by β -glucosidases has been documented with

site-directed mutagenesis experiments by some researchers. For example, the importance of the amino acid Glu for the catalytic activity in the chicken egg white lysozyme (CEWL) was confirmed by site-directed mutagenesis (Malcolm and et al., 1989). Bacteriophage T-7 lysozyme is also shown to have a catalytic site similar to that of the CEWL lysozyme, even though two enzymes have different amino acid sequences. The positions of Glu 11 and Asp 20 in the T-7 lysozyme correspond to Glu 35 and Asp 52 in the catalytic site of CEWL lysozyme. Thus the catalytic mechanisms were similar on the basis of mutational studies of the active site (Anand et al., 1988; Malcolm et al., 1989).

In the case of human β -glucosidase, the active site interacts with the fatty acid acyl-sphingosyl and glycone moieties of glycosyl ceramide substrates. Dinur et al. (1986) have used Br[³H] which is an active site-directed covalent inhibitor for affinity labeling. They have demonstrated that the Asp 443 is the nucleophile participating in catalysis.

The most recent studies on the active site of *Agrobacterium* β -glucosidase have involved site-directed mutagenesis (Trimbur et al., 1992). In one major study, 43 point mutations at 22 different sites were introduced around the YITENGA region, focusing on the active site nucleophile Glu 358 (Withers and et al., 1992; Trimbur et al., 1992). Furthermore, Trimbur et al. (1992) generated 9 different

mutations at the Glu 358 position, which decreased the activity a minimum of 10,000-fold. In addition, the replacement of Gly 360 also gave rise to drastic inactivation of the enzyme drastically. The results indicated that all five residues around Glu 358 were found to be important for catalysis.

In conclusion, the above studies suggest that the glutamic acid in the conservative ITENG region acts as nucleophile in the acid-base catalysis by all β -glucosidases. Aspartic acid also has been suggested as a candidate in acid-base catalysis (Trimbur et al., 1992). Moreover, when the nucleophilic glutamate was replaced with the shorter aspartate, the activity of the enzyme was reduced 1000-2000 fold. This result suggests that the steric requirement for the amino acid residue in the active site in such reactions is critical for catalysis even though the amino acid residues have similar chemical properties (Trimbur et al., 1992). Inhibitor studies also indicated that Glu 358 plays a nucleophilic role in the active site of *Agrobacterium* β -glucosidase (Withers et al., 1990; Trimbur et al., 1992).

Cyanogenic β -Glucosidases

In most higher plants, cyanogenesis is the catabolism of the cyanogenic glucosides. Many economically important crops, including sorghum, cassava, lima beans and cherries accumulate

cyanogenic glucosides. Hydrolysis of these compounds produces the respiratory poison, hydrogen cyanide (HCN) (Poulton, 1993; Conn, 1981). In general, cyanogenic glucosides are broken down sequentially following tissue damage, which results in disruption of the compartmentation of the substrate and the enzyme. The catabolism of the cyanogenic glucosides by β -glucosidase results in the accumulation of glucose and a hydroxynitrile. Hydroxynitrile is subsequently broken down by a hydroxynitrilelyase to ketone and HCN.

In white clover (*Trifolium repens* L), two related cyanogenic glucosides, linamarin and lotaustralin, are synthesized. The β -glucosidase linamarase is a glycosylated protein (having high-mannose-type N-asparagin-linked oligosaccharides) that hydrolyzes these substrates to glucose and HCN (Hughes, 1993). According to Kakes (1985, 1993), linamarase occurs in the cell walls of the epidermal cells of leaves. Cyanogenic β -glucosidase linamarase in cassava has been studied in detail by various researchers (Yeoh et al., 1992; Mkpogon et al., 1990; Nok et al., 1990; Hughes, 1982,1993). Cassava plant is consumed as a staple food in some countries. It is a highly cyanogenic plant and causes acute cyanide poisoning in human (Poulton, 1989). Hughes (1993) studied the localization and expression of cyanogenic β -gluco-

sidase in cassava and showed that cyanogenic β -glucosidase in cassava is synthesized in the latex vessels of young leaves and transported to the latex vessels (Pancoro and Hughes, 1992) of the petiole and possibly to the vessels of the stem and roots.

Cyanogenic β -glucosides are known as defense compounds against some pathogens and herbivores after being hydrolyzed by β -glucosidases (Poulton, 1990; Jones, 1988; Kakes, 1993). The most cyanogenic glucosides are derived from five hydrophobic protein amino acids (tyrosine, phenylalanine, valine, leucine and isoleucine). In sorghum, the major natural substrate of β -glucosidase is the cyanogenic β -glucoside dhurrin. The pathway of dhurrin biosynthesis is well-documented in sorghum seedlings. Halkier et al. (1989) found that in the seedlings of *Sorghum bicolor* (L) Moench, dhurrin (β -D-glucopyranosyl-oxy-(S)-p-hydroxymandelonitrile) was derived from L-tyrosine (Halkier et al., 1989). In the gramineae L-tyrosine is synthesized via a shikimate intermediate which is a key substance in the synthesis of plant secondary compounds including lignin, flavonoids, anthocyanins, coumarins and phenolic acids (Conn, 1994). Recently, Sibbesen et al. (1995) found that the cytochrome P-450 enzyme, hemethiolate enzyme, catalyzes the committed step (N-hydroxylation of L-

tyrosine to N-hydroxytyrosine) in the biosynthesis of dhurrin in *Sorghum bicolor* (L.) Moench.

The shoots of young sorghum plants contain large amounts (up to 75 $\mu\text{Mol/g}$ fresh wt) of the cyanogenic glucoside dhurrin. Enzymatic hydrolysis of dhurrin occurs after disruption of the sorghum tissue (Conn, 1994) (Figure 3).

Hosel et al. (1987) have purified two dhurrinase enzymes from sorghum seedlings. Dhurrinase I occurs in shoots of seedlings grown in darkness; in contrast dhurrinase II occurs in the green shoots of young seedlings grown in light (Hosel et al., 1987). Sorghum and maize β -glucosidases share a number of characteristics such as subunit molecular weight (60 kD), acidic pH (6.2 and 5.8 respectively), pI (5.5) (Esen, 1992; Hosel et al., 1987) and plastid localization (Kojima et al., 1979; Thayer and Conn, 1981; Esen and Stetler, 1993). However the enzymes exhibit subtle differences with respect to substrate specificity. The maize enzyme hydrolyzes a broad spectrum of aryl β -glucosides, in addition to the abundant physiological substrate DIMBOA-glucoside (2-O- β -D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) (Babcock and Esen, 1994). On the other hand the sorghum β -glucosidase hydrolyzes almost exclusively dhurrin, having little or no activity towards any of the numerous

natural and artificial substrates that maize and many others β -glucosidases readily hydrolyze.

In conclusion, sorghum β -glucosidase is known to function in chemical defense of young plant parts against pests because it catalyses the hydrolysis of toxic cyanogenic glucoside dhurrin. As we mentioned earlier, sorghum and maize β -glucosidases share high structural similarity with each other. It is also known all β -glucosidases isolated from grasses (e.g. maize, sorghum, oat, and rice) are localized in the plastid. In contrast, all β -glucosidases isolated from dicots including *Trifolium repens* (Kakes, 1985), black cherry (Poulton and Li, 1994) and *Brassica napus* (Thangstad et al., 1991; Hoglund et al., 1992) are localized in the cell wall or vacuole (protein bodies). In addition, monocot β -glucosidases are not glycoproteins while dicot β -glucosidases are glycoproteins. Based on these data, which show significant differences between monocot and dicot β -glucosidases, the crucial question is how monocot and dicot β -glucosidases evolved and acquired differences of this magnitude from each other in terms of physiological substrates, posttranslational modification, targeting and function. To answer some of these questions, we decided to clone, isolate and sequence sorghum β -glucosidase cDNA and

study the expression of its gene in different parts of the sorghum seedling.

The objectives of this study follows as:

- 1. Cloning and sequencing of the cDNA coding for β -glucosidase from 2-3 day-old etiolated sorghum seedling.**
- 2. Determining the multiplicity of the gene in the sorghum genome**
- 3. Studying organ-specific gene expression and enzyme activity in etiolated sorghum seedlings.**

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CHAPTER 2

Cloning and Sequencing of a cDNA Coding for β -Glucosidase

(Dhurrinase) from *Sorghum bicolor* (L.) Moench

Cloning and Sequencing of a cDNA Coding for β -Glucosidase (Dhurrinase) from *Sorghum bicolor* (L) Moench (Accession No. U33817)

Muzaffer Cicek and Asim Esen*

**Department of Biology
Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24061-0406**

*** Corresponding author; Fax: 540 231 9307
e-mail: aevatan@vt.edu**

β -glucosidases (EC 3.2.1.21) hydrolyze β -glycosidic linkages between the anomeric carbon and glycosidic oxygen in β -glucosides. These enzymes are found in plants, animals, bacteria and fungi. In plants, β -glucosidases are reported to function in phytohormone metabolism (Smith and van Staden, 1978), defense against phytopathogens and herbivores (Poulton, 1990) and lignification (Hahlbrock and Griseback 1978). Perhaps the most well-documented function of β -glucosidase in plants is the release of HCN from cyanogenic glucosides in tissues damaged by herbivores and pathogens (Poulton, 1990). Therefore, cyanogenic glucosides are considered as secondary plant metabolites functioning in chemical defense.

Sorghum β -glucosidase functions in cyanogenesis, i.e. the hydrolysis of the abundant cyanogenic glucoside dhurrin. Sorghum and Maize enzymes share a number of characteristics such as

subunit molecular weight (60 kD), acidic pH (6.2 and 5.8 respectively), pI (5.5) (Esen, 1992 ; Hosel et al., 1987) and plastid localization (Thayer and Conn, 1981; Esen and Stetler 1993). However, they exhibit subtle differences with respect to substrate specificity. The maize enzyme hydrolyzes a broad spectrum of aryl β -glucosides in addition to the abundant physiological substrate DIMBOA-glucoside (2-O- β -D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) (Babcock and Esen, 1994), while the sorghum enzyme shows extremely narrow aglycone specificity (Conn, 1993), hydrolyzing almost exclusively dhurrin but having little or no activity towards any of the numerous natural and artificial substrates that maize and many other β -glucosidases readily hydrolyze.

We now report the isolation of a near full-length cDNA from sorghum coleoptiles encoding a β -glucosidase (dhurrinase). Using the LASERGENE Megalign program, we found the nucleotide and amino acid sequences of monocot and dicot β -glucosidases to be essentially invariant in certain regions. Based on these invariant regions, we selected a maize β -glucosidase specific oligonucleotide primer (p27, CCGATTCCGTTCTCGGTGAT) derived from the region of the polypeptide containing the peptide sequence ITENG that is universally conserved in all of the known β -glucosidases

that belong to the BGA family. Using primer 27 and an anchor primer ligated to the 3'-end of the first strand cDNA, the 5'-end of sorghum β -glucosidase was amplified by RT-PCR, cloned into the plasmid vector PCR-script SK(+), and the sequence of the insert was determined. Based on new sequence data, a gene-specific primer (p69, TATGTACCCTAAAGGCCTACAC) was designed and used to amplify the 3'-end of the sorghum β -glucosidase cDNA in combination with an oligo-dT anchor primer. The resulting 3'-end PCR product was sequenced.

The near full length dhurrinase cDNA is 1965 bp long and its 1695 bp open reading frame codes for a 565-amino-acid-long precursor protein (Figure 4) which shows 72% sequence identity with maize β -glucosidase. The precursor protein has a 51 amino acid-long plastid targeting N-terminal extension (transit peptide), which shows 78.4% identity to the 54-amino-acid-long transit peptide of the maize β -glucosidase precursor. Like maize β -glucosidase, putative sorghum β -glucosidase has the conserved sequence motifs that are found in the BGA family β -glucosidases.

Table I Characteristics of sorghum β -glucosidase (dhurrinase)

Organism:

Sorghum bicolor (L.) Moench

Gene Product:

β -glucosidase (dhurrinase) (EC 3.2.1.21)

Source and Techniques:

mRNA was isolated from total RNA from etiolated 3-4 day-old shoots by using oligo-dT coated magnetic beads. Oligo-dT-primer (A76) was used for first strand cDNA synthesis by RT-PCR. An oligonucleotide anchor was ligated to the 3'-end of the first strand cDNA using T4 RNA ligase. To amplify the 5'-end fragment, primer 27 (CCGATTCCGTTCTCGGTGAT) was used with the 5' second strand anchor primer. The PCR product was cloned into PCR-script SK(+) for sequencing in both directions. Based on the 5'-end sequence, the gene specific primer 69 (TATGTACCCTA-AAGGCCTACAC) was designed and used in combination with an oligo-dT-anchor primer to amplify and sequence the 3'-end of the dhurrinase cDNA. The resulting 3'-end PCR product was sequenced in both directions.

Sequencing Strategy

Cycle Sequencing (Epicenter Technologies, Madison, WI) using overlapping templates (PCR products) corresponding to the 5'- and 3'- ends of the dhurrinase cDNA.

Methods of Identification:

Comparison of the published maize β -glucosidase cDNA and its deduced amino acid sequences (Brzobohaty et al., 1993, accession no. X74217 ; Esen and Shahid, unpublished, accession no. U25157)

Features of the cDNA:

The 1965 bp long cDNA sequence includes a 69 bp 5' untranslated, a 1695 bp coding, and a 201 bp 3' untranslated region.

Features of the Deduced Protein:

The open reading frame (the 1695 bp coding region) encodes a 565-amino acid-long precursor protein with a calculated molecular mass of 63.8 kD. It has a 51 amino acid-long transit peptide for plastid targeting. The deduced mature protein is 514 amino acid-long with a calculated molecular mass of 58 kD.

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CHAPTER 3

Analysis of β -Glucosidase Expression in *Sorghum bicolor* (L.) Moench Seedlings

**Analysis of β -Glucosidase Expression in *Sorghum bicolor* (L.)
Moench Seedlings**

Muzaffer Cicek and Asim Esen*

**Department of Biology
Virginia Polytechnic Institute and State University
Blacksburg, Virginia 24061-0406**

*** Corresponding author; Fax: 540 231 9307
e-mail: aevatan@vt.edu**

Abstract

A full-length β -glucosidase encoding cDNA is isolated and sequenced from *Sorghum bicolor* (L) Moench. Using 5'- and 3'-end specific probes derived from the cDNA clone, multiplicity of β -glucosidase genes and their expression in different tissues were studied. Southern blotting data showed that β -glucosidase is encoded by a small multigene family. Northern analysis data indicated that mRNA corresponding to the cloned gene is present at high levels in the node and mesocotyl 2 regions of the seedling but at low levels only in the zone of elongation region in roots. Other seedling parts such as mesocotyl 1, root sections adjacent to the seed and coleoptile sections do not have any detectable expression. The amino acid sequence data show 72% sequence identity between maize and sorghum β -glucosidase precursor proteins. In view of high sequence similarity between maize and

sorghum β -gluco-sidase, immunoblotting analysis was performed with maize-anti- β -glucosidase serum. The immunoblotting results supported the results of Hosel et al., (1987) with respect to the occurrence of two distinct β -glucosidases being present in sorghum.

Introduction

β -glucosidases (β -D-glucoside glucohydrolases; EC 3.2.1.21) catalyze the hydrolysis of aryl and alkyl β -glucosides, releasing β -D-glucose and aglycone (Reese, 1977). These enzymes occur ubiquitously in plants, fungi, animals and bacteria (Woodward and Wiseman, 1982). β -glucosidases display broad specificity with respect to the aglycone moiety of their substrates but somewhat narrow specificity for the glycone moiety. The natural substrates include the steroid β -glucosides and β -glucosyl ceramides of mammals, cyanogenic and hydroxamic acid β -glucosides of plant secondary metabolism, in addition to oligosaccharide products released from digestion of the cellulose of plant cell walls (Beutler, 1992; Conn, 1981; Niemeyer, 1988; Cuevas et al., 1992; Clarke et al., 1993). In fact, β -glucosidases from every source have similar specificity for the glycone (glucose) portion of the substrate but differ dramatically in specificity for the aglycone portion. The aglycone specificity of plant β -glucosidases have been well-established by numerous studies (e.g. Babcock and Esen, 1994; Hosel and Conn, 1982; and Hughes and Dunn, 1982). For example, Babcock and Esen propose that a hydrophobic aglycone

group is needed to cleave the β -glycosidic bond between glycone and aglycone residues. However, the cyanogenic diglycoside (R)-amygdalin (the gentiobioside of (R)-mandelonitrile) of black cherry seeds has the disaccharide gentiobiose as its glycone instead of glucose (Poulton, 1993). The aglycones, the active group of glucosides, play important roles in plant defense, development and growth (Selmar et al., 1987; Poulton, 1990). For many years cyanogenic β -glucosides have been known as defense compounds against some pathogens and herbivores because they contain a respiratory poison hydrogen cyanide (HCN) (Hruska, 1988; Poulton, 1993) released by β -glucosidase. In fact, many important crops, including sorghum, cassava, lima beans and cherries, contain cyanogenic β -glycosides (Poulton, 1989). As a result of decompartmentation due to tissue damage, a cyanogenic β -glucosidase and its substrate come into contact with each other and the substrate is hydrolyzed, releasing a toxic aglycone or its derivatives (e.g. HCN) (Selmar, 1993; Kakes, 1985; Hosel et al., 1987) (Figure 3). The most cyanogenic glucosides are derived from five hydrophobic protein amino acids (tyrosine, phenylalanine, valine, leucine and isoleucine) (Conn, 1994). Biosynthesis of the cyanogenic glucoside dhurrin (β -D-glucopyranosyl-oxy-(S)-p-hydroxy-mandelonitrile) has been studied in sorghum seedlings by

Halkier et al (1989). Dhurrin was derived from L-tyrosine (Halkier et al, 1989). L-tyrosine is synthesized from shikimate intermediate via a reaction catalyzed by phenylalanine ammonia lyase (PAL), which is a key substance in the synthesis of plant secondary compounds, including lignin, flavonoids, anthocyanins, coumarins and phenolic acids (Conn, 1994). The shoots of young sorghum plants contain large amounts (up to 75 $\mu\text{Mol/g}$ fresh wt) of dhurrin.

Hosel et al. (1987) purified two dhurrinase enzyme fractions (dhurrinase I and II) from sorghum seedlings. Dhurrinase I occurs in shoots of seedlings grown in the dark; in contrast, dhurrinase II occurs in the green shoots of young seedlings grown in the light (Hosel et al., 1987). Kojima et al. (1979) showed that dhurrinase activity was localized in mesophyll cells of sorghum leaves. In contrast, cyanogenic β -glucosidase linamarase was localized to the cell walls, especially in epidermal cells and cuticle of *Trifolium repens* (Kakes, 1985).

Sorghum and maize β -glucosidases share a number of characteristics such as subunit molecular weight (60 kD), acidic pH (6.2 and 5.8 respectively), pI (5.5) (Esen, 1992; Hosel et al., 1987) and plastid localization (Thayer and Conn, 1981; Esen and Stetler, 1993), yet they exhibit subtle differences with respect to substrate specificity. The maize enzyme hydrolyzes a broad

spectrum of aryl β -glucosides in addition to the abundant physiological substrate DIMBOA-glucoside (2-O- β -D-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one) (Cuevas et al., 1992; Babcock and Esen, 1994). In contrast, sorghum β -glucosidase hydrolyzes almost exclusively dhurrin, with little or no activity towards any of the numerous natural and artificial substrates that maize and many others β -glucosidases readily hydrolyze.

The purpose of this study was to clone and sequence a cDNA encoding β -glucosidase from *Sorghum bicolor* (L.) Moench and compare it to those of maize as part of our research focusing on β -glucosidases structure and function in plants. The cDNA cloned has high similarity (72%) to the two maize β -glucosidase cDNAs because sorghum and maize are taxonomically close to each other, being in the same tribe (Andropogoneae), and partly because of selective constraints on enzyme structure and function.

We suggest that the isolated cDNA clone codes for one of the cyanogenic β -glucosidases in sorghum. We also studied the multiplicity and expression of this clone using 5'- and 3'-end specific PCR-amplified fragments as probes in Southern and northern blot analysis. In addition, organ-specific expression of

the β -glucosidase gene at the protein level was performed by western blot analysis using antiserum maize β -glucosidase.

Materials and Methods

Plant Material

Sorghum bicolor (L) Moench seeds (P-721N) were obtained from Dr Richard Axtell (Purdue University). Seeds were grown in vermiculate in darkness at 25 °C for 2-3 days. Two to three day-old seedlings were dissected into different parts to isolate total RNA and protein, respectively, for northern and western blot analysis. Seedlings were divided into the following parts: coleoptile, node, mesocotyl 1, mesocotyl 2, root 1 and root 2 (Figure 6). Dormant (dry) seeds were soaked in H₂O at 4 °C overnight to remove the embryonic tissues for RNA isolation. In addition, whole seedlings were used for genomic DNA isolation.

Isolation of mRNA

Total RNA was isolated from 2-3 day-old etiolated seedlings using TRIZOL reagent (Life Technology, MD) according to the vendor's instructions. Typically, 2 g frozen seedlings were ground in a chilled mortar and suspended in 40 ml TRIZOL reagent. After precipitation with alcohol, the RNA pellet was washed twice with 70% EtOH (prepared in DEPC treated-dH₂O), resuspended in DEPC

treated-dH₂O, and LiCl was added to the RNA solution to a final concentration of 1 M. The solution was recentrifuged for 15 min at 18,000g at 4 °C. Finally, the resulting pellet was resuspended in 1 ml DEPC-treated dH₂O and the amount of RNA was determined spectrophotometrically. Seventy-five µg total RNA was used for isolation of poly (A)⁺ RNA using oligo-dT magnetic beads (Jacobsen et al., 1990). RNA was incubated with 0.2 ml (1 mg) oligo-dT magnetic beads in 2X binding buffer (20 mM Tris-HCl pH 7.5, 1.0 M LiCl, 2 mM EDTA). The beads were placed in the Dynal-MPC-E magnet for 30 sec. The supernatant was removed, and the beads were washed twice with the wash buffer (10 mM Tris-HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA). Poly (A)⁺ RNA was eluted with 20 µl of 2 mM EDTA, pH 7.5 by heating at 65 °C for 2 min.

Reverse transcription and cloning

Ten µl of eluted mRNA was used as template for reverse transcription to synthesize first-strand cDNA. The reaction mixture included 10 µl mRNA (approx. 2 µg), 1 µl 20 mM anchor-ligated oligo-dT (Perkin Elmer, Lo Jolla, CA), 4 µl 5X first strand buffer (Gibco BRL, MD), 1 µl 0.1 M DDT, 2 µl 10 mM dNTP and 1 µl RNasein (Gibco BRL, MD) and 1 µl Superscript RT II (200 u/µl) (Gibco BRL, MD). The reaction was performed at 48 °C for 2 hrs.

The resulting cDNA was purified by binding to a silica matrix in presence of 6 N NaI (Clontech vendor's instruction). Purified cDNA was dissolved in 10 μ l DEPC-treated dH₂O to use in the 5'- and 3'-RACE experiments. The nucleotide and amino acid sequences of monocot and dicot β -glucosidases were compared using the LASERGENE Megalign program to locate invariant regions. The oligonucleotide primer, p27 (CCGATTCCGTTCT-CGGTGAT) encoding peptide sequence ITENG that is universally conserved in all of the known monocot β -glucosidases was synthesized as antisense primer. The 3'-end of the first strand cDNA was ligated with AmpliFINDER anchor (Clontech, Palo Alto, CA) by T4 RNA ligase (Boehringer Mannheim, Germany) for 5'-RACE. Using the combination of p27 and second strand the AmpliFINDER anchor primer, the 5'-end of sorghum β -glucosidase was amplified by PCR. An aliquot of the PCR reaction was analyzed on a 1% TAE-agarose gel. A product of the expected size was obtained and cloned into the SrfI site of PCR Script SK (+) vector (Stratagene, La Jolla, CA). The construct was transformed into XL1-Blue host cells. The white (positive) colonies were screened by PCR using T3 and T7 primers (Gussow and Clackson, 1989). Of twelve white (positive) colonies selected, six contained an insert of the expected size, all of which were sequenced.

Based on 5'-end sequence data, the 3'-end of a sorghum β -glucosidase cDNA was amplified using a second strand anchor primer, A76 (GGCCACGCGTCGACTAGTA) and gene specific primer, p69 (TATGTAACCCTAAAGGCCTACAC). The resulting 3'-end PCR product was treated with SAP (Shrimp Alkaline Phosphatase) (USB, Cleveland, OH) and Exonuclease II (USB, Cleveland, OH) to dephosphorylate and digest free oligonucleotides and was sequenced directly without cloning using the procedure of cycle sequencing (Epicenter Technology, WI). The sequences of sorghum β -glucosidase cDNA and its putative protein product were compared with sequences in the GenBank database to confirm their identity.

Isolation of total RNA for Northern blot analysis

Typically, 2 g of each sorghum seedling part were placed in 3 ml extraction buffer (4 M guanidium thiocyanate, 0.5% sarcosyl, 0.1 M 2-mercapto ethanol in 25 mM Na-citrate) immediately after dissection and stored at -80 °C. The frozen tissues were ground in a chilled mortar and additional 17 ml extraction buffer was added. To the suspension, 20 ml water-saturated phenol, 4 ml 2 M NaAc pH 4.5, and 8 ml chloroform isoamyl alcohol (49/1: v/v) were added, and the mixture was vortexed for 1 min at room temperature. The mixture was kept on ice for 15 min and then centrifuged at 18000g

for 20 min at 4 °C. The aqueous phase was transferred into a DEPC-treated 50 ml-nalgene tube by passing through a layer of Miracloth (Calbiochem, Lo Jolla, CA) which had been soaked in extraction buffer. One volume of isopropanol was added to the resulting filtrate and incubated for 3 hrs at -20 °C to precipitate the RNA. The mixture was centrifuged at 18000g for 30 min at 4 °C and the resulting pellet was air-dried and resuspended in 2 ml DEPC-treated dH₂O. One volume RNA loading buffer was added and the sample was then stored at -80°C. Approximately 10 mg RNA was loaded onto 1.2% formaldehyde-agarose gel with MOPS-NaAc-EDTA buffer.

Probe synthesis

The second strand AmpliFINDER anchor primer and p27 were used to amplify of 5'-region of the cDNA in a standard PCR reaction, while the 3'-region was amplified using primers p69 and A76. The resulting PCR products were excised from a low melting agarose gel (1%) and purified using the Magic™ PCR* Preps system according the procedure provided by the vendors (Promega, Madison, WI). The purified PCR products were labeled with α -[³²P]dATP using random hexamers (Ambion, TX) and exonuclease free Klenow enzyme (Ambion, TX) at 37 °C for 1hr. The 5'-end

(1446 bp) and 3'-end (609 bp) fragments were used as probes for northern and Southern blot analysis.

Northern and Southern blot analysis

According to EtBr staining intensity on gels, approximately 10 µg total RNA from various plant parts were heated to 65 °C for 15 min and loaded onto a 1.2% formaldehyde agarose gel. Electrophoresis was at 5V/cm for 6 hrs in gel. A Nytran membrane (Schleicher & Schuell, NH) and gel were soaked in 10X SSC (1.5 M NaCl, 0.15 M Na-Citrate pH 7.0) for 10 min. RNA was transferred to the membrane in 10X SSC overnight (Sambrook et al. 1989). The RNA was attached to the membrane by UV-crosslinking and the membrane allowed to dry for a few minutes. The membranes were prehybridized in Church buffer (1 mM EDTA pH 8.0, 0.5 M NaHPO₄ pH 7.2, 7% SDS) at 65 °C for 2 hrs (Church and Gilbert, 1984) and hybridized with corresponding ³²P[dATP] labeled 5'-end fragment in 10 ml Church buffer overnight at 65 °C. The membranes were washed once in 1X SSC with 0.1% SDS at room temperature for five minutes, twice in 1X SSC/0.1% SDS at 65 °C for 20 min, and then once in 0.1X SSC/0.1%SDS at 65 °C. The washed membranes were dried and exposed to Kodak X-ray film (X-OMAT) with intensifying screens at -80 °C for 12 hrs.

Genomic DNA was isolated from 3-4 day-old seedlings as described by Dellaporta (1993). Approximately 10 μ g DNA was digested with Sall, PvuII, Apal, XhoI, BamHI and XbaI for 24 hrs and loaded onto a 0.8% agarose gel with Tris-Acetate-EDTA buffer. After electrophoresis at 1.5 V/cm for 18 hrs, the DNA was depurinated by soaking in 0.25 M HCL solution, the gel was then rinsed with dH₂O. The DNA was denatured in 0.5 M NaOH-1.5 M NaCl solution for 20 min and then neutralized in 0.5 M TrisHCl-1.5 M NaCl pH 7.0 for 30 min before transfer. In the meantime, a Nytran membrane was wetted in dH₂O and briefly soaked in 10X SSC. Transfer was performed in 10X SSC overnight. The DNA was attached to the membrane by UV-crosslinking. Southern blots were probed separately with ³²P[dATP]-labeled 5'- and 3'-end PCR products. Hybridization conditions were similar to those for Northern analysis except that the Church buffer included 1% (w/v) BSA. Washes were: once in 1X SSC/0.1% SDS at room temperature, twice in 1X SSC/0.1% SDS at 65 °C for 20 min, once in 0.5X SSC/0.1% SDS at 65 °C for 15 min. The membrane was exposed to Kodak X-ray film (X-OMAT) with intensifying screens at -80 °C for 3 days.

PAGE and Western blotting

Frozen sorghum seedling parts, 1 g per sample, were ground

in a chilled mortar, extracted in 3 ml 50mM Tris-HCL, pH 7.8, and centrifuged at 18,000g at 4 °C for 10 min. The resulting supernatant was mixed with sample buffer (50mM Tris-HCL pH6.8, 100 mM 2-ME., 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min (Laemmli, 1970). Samples were loaded onto 12% SDS gels and electrophoresed at 60 mA for 4 h. After electrophoresis, gels were soaked in 1X blotting buffer (Tris-Glycin-SDS) and the proteins transferred to PVDF membrane (Milipore Corporation, Bedford, MA) (Pluskal et al., 1986). Immunodetection was carried out using anti-maize β -glucosidase serum (R-681) as described by Mohammad and Esen (1989).

Results

Sequence and structure of β -glucosidase in *Sorghum bicolor*

Poly (A)⁺ RNA was isolated from 2-3 day-old etiolated seedlings. Reverse transcription-PCR amplified the expected PCR product of 1446 bp length. The resulting fragment was cloned in and sequenced. Based on the 5'-end sequence, a gene-specific primer p69 was synthesized to amplify the 3'-end fragment. The p69 and A76 primer pairs amplified a 609-bp PCR product. The sequences of two PCR fragments had a 90-bp overlap. The nucleotide and deduced amino acid sequence of the cDNA are given in Figure 4. The dhurrinase cDNA which likely to be full-

length as it was isolated by anchor-PCR is 1983 bp long and has 1695 bp open reading frame which codes for a 565-amino-acid-long precursor protein. The deduced precursor protein sequence shows 72% sequence identity with the maize β -glucosidase precursor proteins (*Glu 1* and *Glu 2*). The predicted mature protein is of 514 amino acid-long and its N-terminus has a 51-amino-acid-long extension (transit peptide) for plastid targeting. The predicted transit peptide sequence shows 78.4% identity to the 54-amino-acid-long transit peptide of the maize β -glucosidase precursor (*Glu 1*). According to Hosel et al. (1987), there are two β -glucosidases (dhurrinases) in sorghum, each shows high specificity for the cyanogenic β -glucoside dhurrin. Of these, dhurrinase I was isolated from shoots of etiolated seedlings. Since our cDNA was synthesized from mRNA isolated from etiolated seedling of comparable age to those of Hosel et al. It is very likely that this cDNA corresponds to dhurrinase I.

Southern blot analysis and distribution of β -glucosidase transcripts in different organs of *Sorghum bicolor*

Southern blot analysis of *Sorghum bicolor* genomic DNA digested with five different restriction enzymes which do not cut the cDNA was performed probing with 5'- and 3'-end specific ^{32}P [dATP]

labeled probes (1446 bp and 609 bp, respectively). The result revealed a number of bands annealing with probe sequences and thus suggesting a multigene family encoding β -glucosidase. In the case of Sall, Apal and XhoI (Lane 1, 3 and 4, Figure 7a) digests showed one strongly hybridizing band (~4.2 kb, ~5.3 kb and ~3.8 kb respectively) when probed with 5'-end of cDNA fragment (1446 bp). In contrast, additional bands were detected in Apal and XhoI digests with the 609 bp fragment corresponding to the 3'-end (Figure 7b). These results suggest that either the genotype used (P721N) for DNA isolation is heterozygous or that an additional restriction site is present in an intronic region of 3'-end of dhurrinase I gene, having two alleles differing by an Apal site. Based on Southern analysis, the β -glucosidase gene appear to belong to a small gene family.

Northern analysis (Figure 8) showed that the size of the sorghum β -glucosidase transcripts is about 2 kb, consistent with the size of the isolated β -glucosidase cDNA clone (1.983 kb). The data clearly indicate that sorghum β -glucosidase expression is spatially regulated, highest steady-state levels of mRNA being observed in the node section (which includes the shoot apex and primary leaves) of the seedling, followed by the mesocotyl 2, which is adjacent to the node section. The mesocotyl half adjacent to the

germ did not have any detectable β -glucosidase mRNA in the 2 kb region of the blot (Figure 8). Similarly the coleoptile, the upper half of the primary root (root 2) and mesocotyl 1 parts contained no detectable levels mRNA while lower half (root 1 which includes root tip) contained a low level of mRNA.

Immuno blot analysis

Immunoblotting was performed with anti-maize β -glucosidase due to high similarity between sorghum and maize β -glucosidase amino acid sequences. Immunoblotting of the extracts from the following organs was performed: coleoptile, node, mesocotyl 2 and 1, root. Two immunoreactive bands (57 and 62 kD) were detected (Figure 9). The coleoptile section showed one immunoreactive band (~62 kD), while the node section showed two immunoreactive bands (~62 kD and ~57 kD respectively). In contrast, the mesocotyl 1 and 2 sections showed only lower size immunoreactive band (~57 kD). We also observed that the young green leaves had only the ~62 kD immunoreactive band (data not shown). This suggests that there are two different sorghum β -glucosidases and their expression is regulated spatially in different organs of the seedling. The node section might be the key section for β -glucosidase activity. These results corresponded to these of Hosel et al. (1987)

who found two cyanogenic β -glucosidase proteins (Dhurrinase I and II) in sorghum. Dhurrinase I is expressed in shoots of etiolated sorghum seedling. In contrast, dhurrinase II is expressed in green leaves of light-exposed 6 day-old sorghum seedling. The native form of both enzyme is tetrameric. The monomer weight of the dhurrinase I and II is 57 kD and 62 kD respectively. Based on computer analysis, the molecular weight of the putative protein product of the clone is 58 kD. This corresponds well to dhurrinase I in terms of molecular weight and amino acid similarity with maize β -glucosidase.

Discussion

Cyanogenic β -glucosidases have been implicated in plant defense, releasing toxic aglycones and derivatives (e.g. HCN) from their substrates. In the case of sorghum, two β -glucosidases involved in cyanogenesis were isolated and characterized by Hosel et al. (1987). They also determined their substrate specificity and spatial expression of the two enzymes in sorghum seedlings. However gene structure for these enzymes had remained to be understood. The cDNA we isolated and cloned by RT-PCR appears to correspond to one of the two enzymes characterized by Hosel et al. (1987). The size of the cloned cDNA is similar to those of

β -glucosidases that belong to member of the β -glucosidase family BGA (Beguin, 1990) and codes for a preprotein with a 51-amino-acid-long N-terminal extension for plastid targeting. The predicted transit peptide sequence shows 78.4% identity to the transit peptide of the maize β -glucosidases (*Glu 1* and *Glu 2*). The putative cleavage site of the transit peptide is postulated to lie between Arg⁵¹ and Ala⁵². Taking this into account, the cleavage site of the transit peptide would coincide with Arg⁵¹ in all known β -glucosidases from grasses (Figure 5). Comparison of the deduced amino acid sequence of β -glucosidase with maize *glu1* and *glu2* shows high similarity (72%).

Both cDNA fragments (1446 bp and 609 bp) were used as probes to determine the multiplicity of the β -glucosidase genes in sorghum by Southern blot analysis. The Southern data suggest that β -glucosidase is encoded by a small gene family in Sorghum, supporting the data of Hosel et al. (1987) who showed the existence of two isoforms of the enzyme (Dhurrinase I and Dhurrinase II).

Northern analysis demonstrated clear organ-specific expression of β -glucosidase in sorghum. It is apparent that sorghum β -glucosidase is expressed in the node section of the seedling

at the highest level, followed by the mesocotyl 2 and the zone of elongation in the root (Figure 8). In contrast, the coleoptile contained no detectable level of transcript. A β -glucosidase was recently described from maize seedlings and its organ specific distribution was shown by Northern analysis (Brzobohaty et al., 1993). Brzobohaty et al. found the largest amount of the transcript were in the root and mesocotyl sections. The distribution of β -glucosidase in various organs of maize seedling does not coincide well with that of sorghum in the node, mesocotyl and root portions of the seedling, even though maize and sorghum β -glucosidase amino acid sequences show high similarity because both taxa are members of the same tribe (Andropogonea) in the subfamily Poaceae.

β -glucosidase expression in the node, mesocotyl 2, mesocotyl 1, and root portions of sorghum seedling was also studied by western blotting using anti-maize β -glucosidase sera. This was possible because sorghum and maize β -glucosidases showed immunological cross-reactivity, sharing common antigenic sites because they have the amino acid sequence similarity. As stated in the results section, antibodies react specifically with two polypeptides of 57 and 61 kD (Figure 9), which correspond well to data of Hosel et al., (1987). It should be noted that these

two immunoreactive dhurrinases are organ-specific in terms of expression. Particularly, the node section, which contains actively dividing cells and tissue (shoot apex and primary leaves), has the highest level of dhurrinase mRNA and protein (Figure 7). The abundant expression of dhurrinase I in 2-3 day-old etiolated seedlings (Hosel et al., 1987) and its size (57 kD) suggest the cDNA cloned from the seedling of the same age as those used by Hosel et al. (1987) codes for dhurrinase I. However, when leaves of the light-grown seedlings were used, only the larger immunoreactive band (62 kD), presumably dhurrinase II, was observed (data not shown). This suggests that the two dhurrinases genes differ both temporally and spatially with respect to regulation, making them an interesting model for study of gene regulation.

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FIGURES

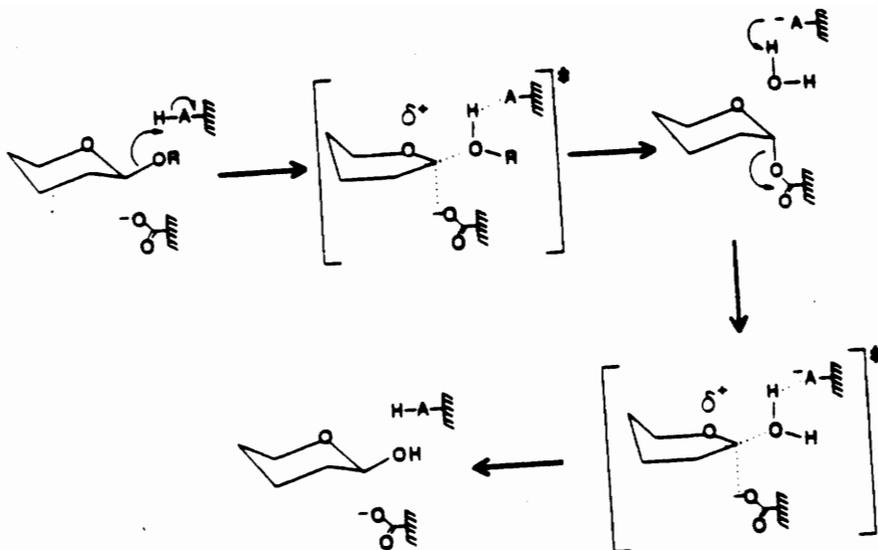


Figure 1. Generally accepted endocyclic pathway of the double displacement mechanism proposed for the retaining β -glucosidases

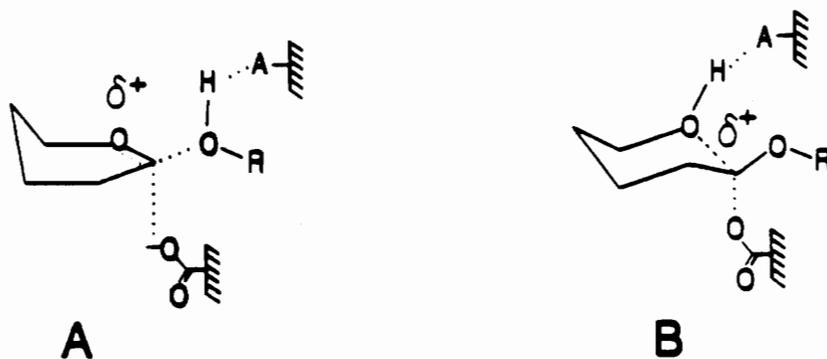


Figure 2. Proposed transition states of β -glucosidase catalysed hydrolysis by the (A) endocyclic, and (B) exocyclic pathways for the double displacement mechanism leading to the formation of the covalent enzyme-substrate intermediate (Clark et al., 1993).

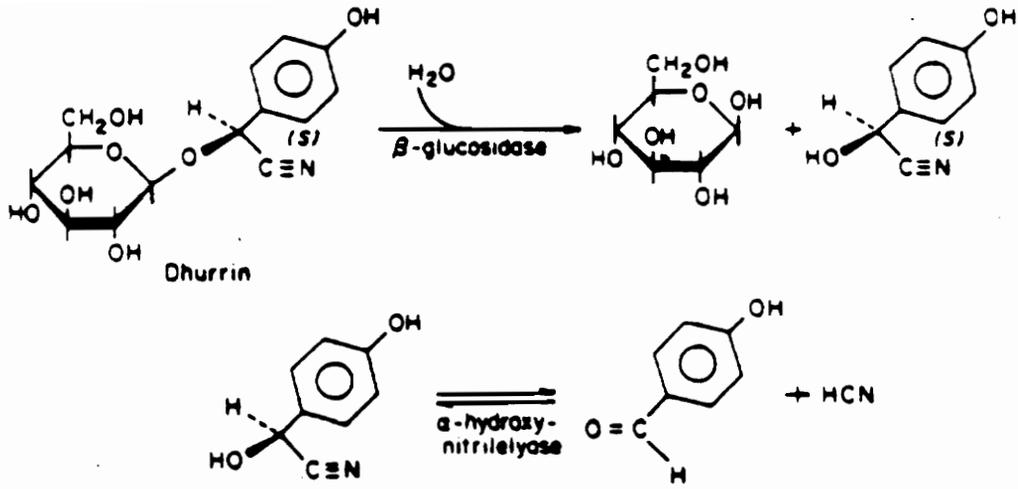


Figure 3. Enzymatic hydrolysis of the cyanogenic glucoside dhurrin which occurs after disruption of sorghum tissue (Conn, 1994).

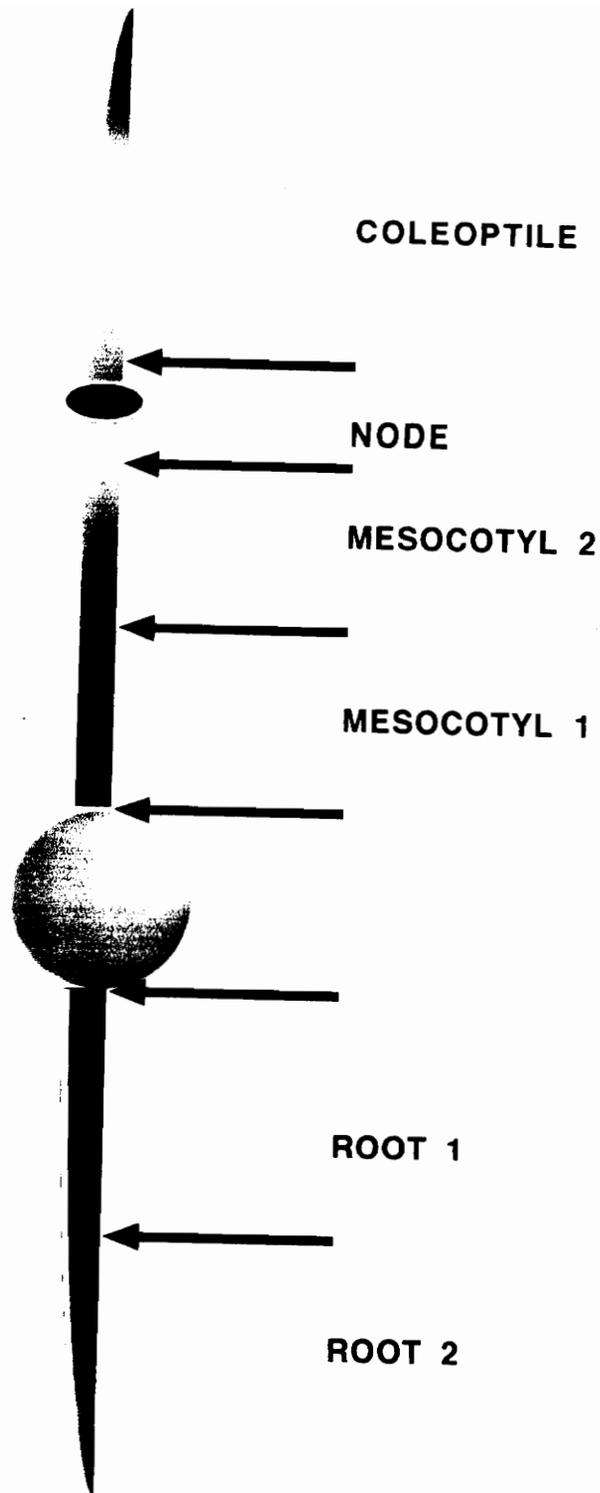


Figure 4. Schematic representation of the sorghum seedling parts for the spatial and temporal expression analysis.

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 ACCESSION U33817
 NID g1127574
 KEYWORDS .
 SOURCE sorghum.
 ORGANISM Sorghum bicolor
 Eukaryotae; mitochondrial eukaryotes; Viridiplantae;
 Charophyta/Embryophyta group; Embryophyta; Magnoliophyta;
 Liliopsida; Poales; Poaceae; Sorghum.
 REFERENCE 1 (bases 1 to 1983)
 AUTHORS Cicek,M. and Esen,A.
 TITLE Cloning and Sequencing of a cDNA Coding for Beta-Glucosidase (Dhurrinase) from Sorghum bicolor (L) Moench 1 (Accession No. U33817) (PGR95-097)
 JOURNAL Plant Physiol. (1995) In press
 REFERENCE 2 (bases 1 to 1983)
 AUTHORS Cicek,M. and Esen,A.
 TITLE Direct Submission
 JOURNAL Submitted (13-AUG-1995) Asim Esen, Biology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0406, USA
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Figure 6. Nucleotide and deduced amino acid sequences of the β -glucosidase encoding cDNA clone.

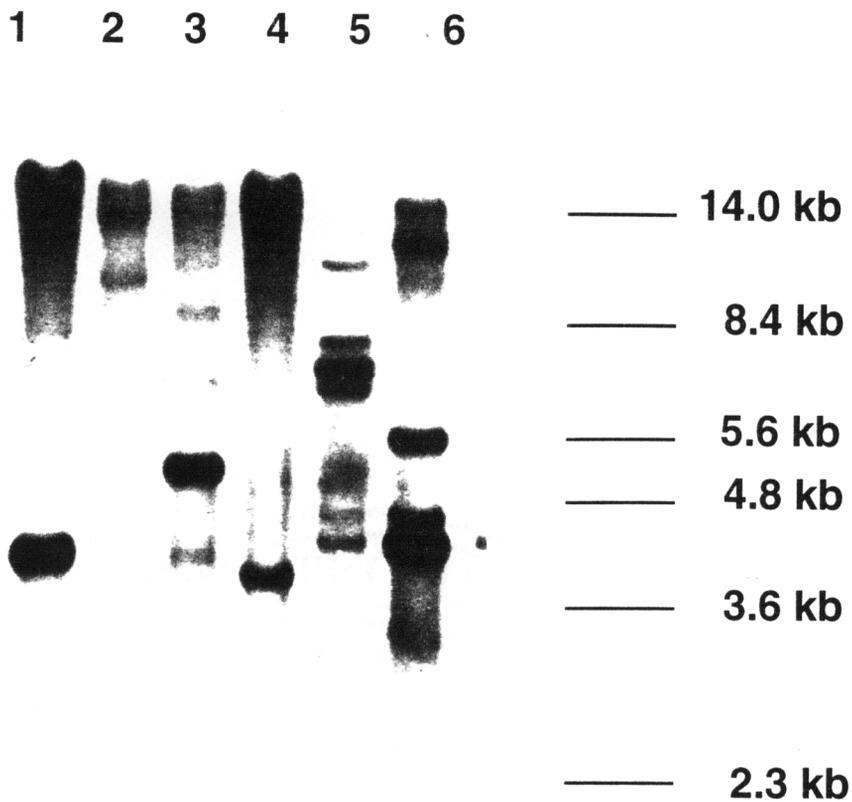


Figure 7a. Genomic Southern analysis of sorghum DNA. Nuclear DNA of *Sorghum bicolor* (L.) Moench was digested with Sall (Lane 1), PvuII (Lane 2), Apal (Lane 3), XhoI (Lane 4), BamHI (Lane 5), XbaI (Lane 6) and subjected to Southern analysis. 5'-end PCR-amplified fragment (1446 bp) was radiolabelled and used as hybridization probe.

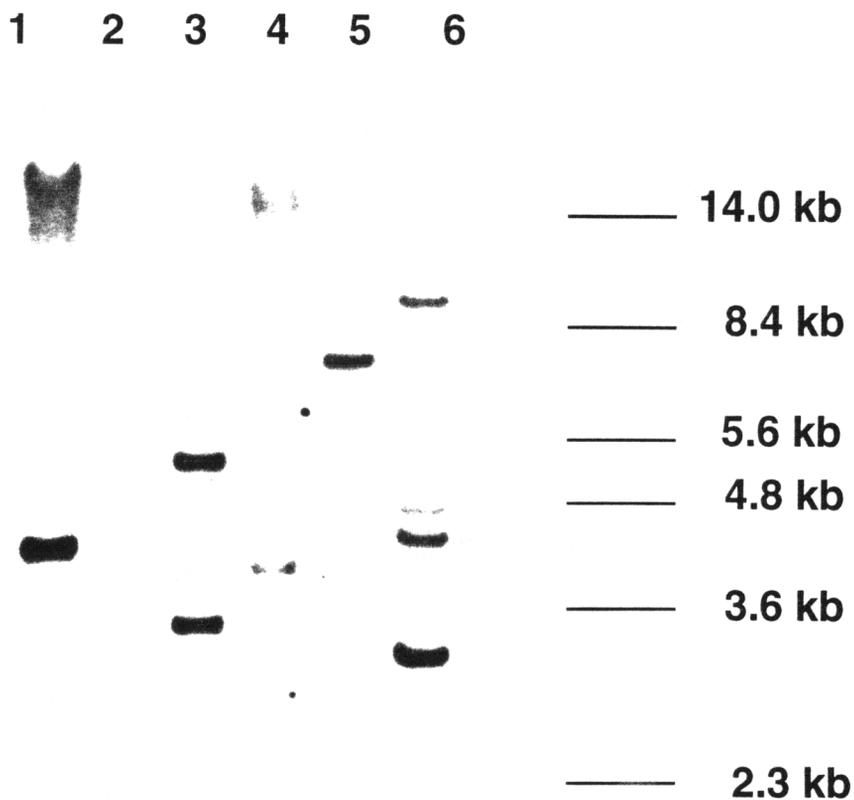


Figure 7b. Genomic Southern analysis of sorghum DNA. Nuclear DNA of *Sorghum bicolor* (L.) Moench was digested with Sall (Lane 1), PvuII (Lane 2), ApaI (Lane 3), XhoI (Lane 4), BamHI (Lane 6), XbaI (Lane 6) and subjected to Southern analysis. The 3'-end PCR-amplified fragment was used as a hybridization probe.

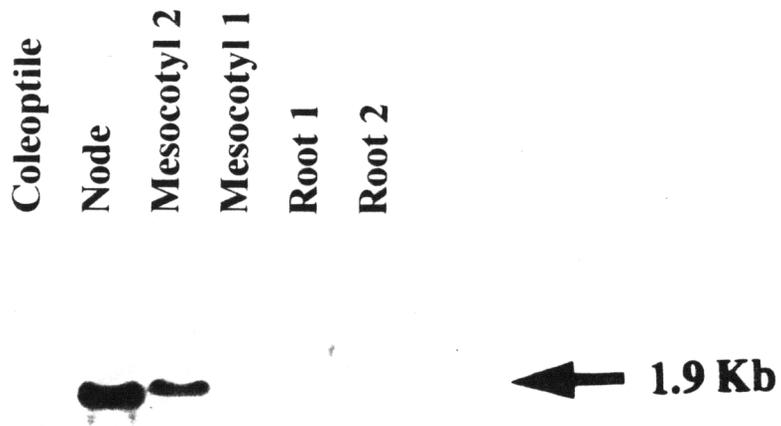


Figure 8. Northern blot analysis of total RNA from different organs of *Sorghum bicolor* (L.) Moench (3-day-old) using a 5'-end specific PCR-amplified fragment (1446 bp) of the β -glucosidase cDNA.

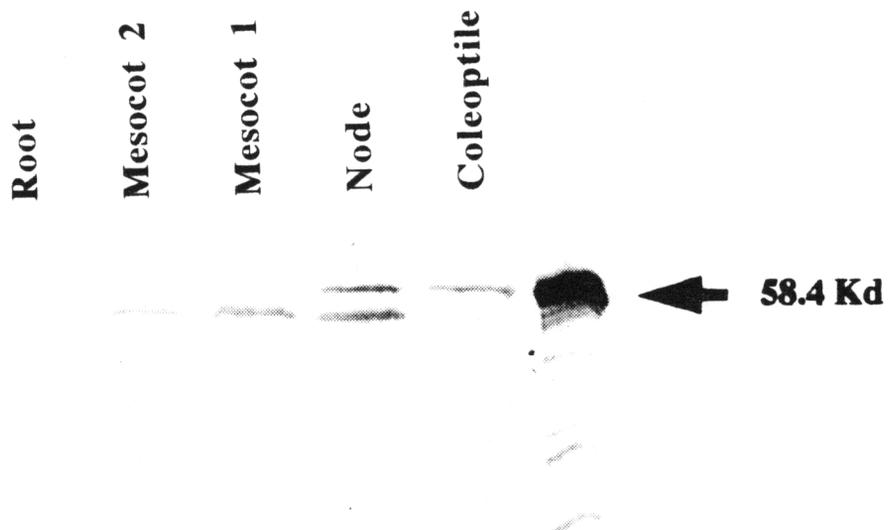


Figure 9. Detection of sorghum β -glucosidase isoforms on Western blots. Proteins from different organs of etiolated 3-day-old seedlings were prepared and subjected to immunoblot analysis. Immunoblots were developed with anti maize β -glucosidase serum. Arrowhead indicates molecular mass of immunoreactive bands.

Curriculum Vitae
of
MUZAFFER CICEK
750 Hethwood Blvd.# 700-J
Blacksburg, VA 24060
Phone: (540) 951-0142 (H)
231-8951 (W)

Personal Details

Place of Birth : Ankara, TURKIYE

Marital Status : Married

Nationality : Turkish

Academic Degrees

January 1994- present

**Msc, Biology Department, Virginia
Polytechnic Institute and State University,
Blacksburg, Virginia.**

Thesis Topic

**Cloning and sequencing of a β -glucosidase
cDNA from *Sorghum bicolor* (L.) Moench
and analysis of expression in seedlings.**

1986-1990

**Bsc, Dept of Food Science and Technology,
Faculty of Agriculture Ankara University,
Diskapi-Ankara 06110 TURKIYE (TURKEY)**

English Language Courses

**Intensive language course for five months,
Middle East Technical University, Ankara,
TURKIYE, 1993.**

**Intensive English course, Michigan State
University & University of Pennsylvania for
five months, 1993.**

Training Programs

Studies in Plant Biotechnology, Wye College University of London, U.K., summer 1991.

Protein purification and characterization techniques, Hacettepe University, Dept of Food Engineering, Ankara, TURKIYE, summer 1992

Scholarships

Scientific & Technical Research Council of Turkiye awarded for the studies in Wye College, University of London, U.K., summer 1991.

Turkish Ministry of Education awarded in the field of Biotechnology to pursue Master of Science degree, 1994-present.

Publications

Cicek M and Esen A

Cloning and Sequencing of a cDNA coding for β -glucosidase (Dhurrinase) from *Sorghum bicolor* (L.) Moench (Accession No. U33817). Plant Gene Registrar PGR 95-097. Plant Physiology Vol.109, No.4, pp 1497.

Muslim C, Cicek M, and Esen A

Partial purification and characteriation of two rice (*Oryza sativa* L.) β -glucosidases. (submitted to Plant Physiology)

Teaching Experience

General Biology Labratory, Biology Dept, Virginia Tech. Blacksburg. Period; 1995-96. Fall and Spring semesters

Seminars attended

New Dimentions in Genetic Analysis, 1994 national seminar series sponsored by Perkin-Elmer, NIH, Washington DC., May 19,1994.

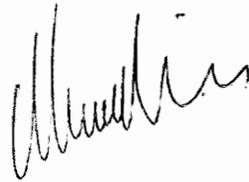
Characterizing Expressed Proteins & Structural Analysis of Recombinant Proteins, 1994 national series sponsored by Perkin-Elmer, Rockville, Maryland, October 12, 1994.

Membership of Professional Societies

American Association for the Advancement of Science

Social activities

President of Virginia Tech Turkish Student Association (1995-96)

A handwritten signature in black ink, appearing to read "Alkan" or similar, written in a cursive style.