BARLEY *XYLOGLUCAN ENDOTRANSGLYCOSYLASE* (XTH) EXPRESSION PATTERNS

by
Didem Uçak

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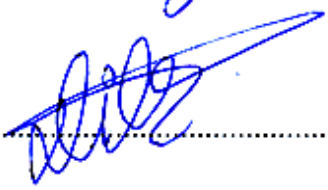
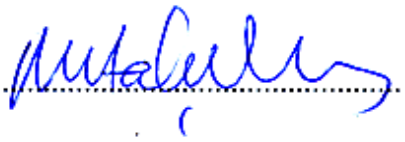
EXPRESSION PATTERNS

APPROVED BY:

Assist. Prof. Dr. Andrew J. Harvey ..... 
(Supervisor)

Assist. Prof. Dr. Dilek Telci ..... 

Prof. Dr. Mustafa Çulha ..... 

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This thesis is dedicated to

Kaan, Dilara, Hakan & Ervin ...

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ABSTRACT

BARLEY *XYLOGLUCAN ENDOTRANSGLYCOSYLASE* (XTH) EXPRESSION PATTERNS

Plant cell wall which strongly influenced by nature is deeply involved in the regulation of the life cycle, such as germination, vegetative growth, reproductive growth and senescence. Xyloglucan is a major non-cellulosic polysaccharide found in both monocot and dicot species where it plays an important role in the structure and strength of plant cell walls. Xyloglucan chains are capable of hydrogen-bond to cellulose, so in the cell wall they may tether adjacent microfibrils and so control cell expansion. Xyloglucan's partial digestion with cellulase can also produce spesific oligosaccharides. The plant xyloglucan endo-transglycosylase (XET) genes are a subfamily of the glycoside hydrolase (GH) family 16 in the CAZy classification. Although the biological functions of XETs have not yet been unequivocally demonstrated, it is believed that they have an important function in the loosening of cell walls during cell elongation by its modification of xyloglucan polysaccharides. Both monocot and dicots species have large numbers of XET genes but the function of their encoded enzymes on cell wall structure is not well known.

In this study we aimed at getting answers for all these unknown questions. We evaluated the expression levels of these genes in different day coleoptiles and different tissues of barley during germination and growth in order to determine their importance for cell wall structure. The reasons for our choice of barley as the model system is that it's germination process is easily studied and there is already some pre-existing knowledge of barley's XET genes and their expression levels, which will be expanded upon in this current study. These datas were compared with other microarrays studies for XTH1, XTH2, XTH3, XTH4, XTH7, XTH8, XTH11, XTH12 genes. The results in this thesis confirms microarray studies for XTH2, XTH3,. XTH4, XTH7, XTH8, XTH11 genes.

ÖZET

ARPA *XYLOGLUCAN ENDOTRANSGLYCOSYLASE* (XTH) ANLATIM ŞEKİLLERİ

Bitki hücre duvarı çimlenme, vejetatif büyüme, çoğalma ve hücre yaşlanması gibi hücre siklusu üzerinde etkisi vardır ve çevreden etkilenir. Ksiloglukan monokotillerde ve dikotillerde bulunan, selülozik olmayan ana polisakkarittir. Bitki hücre duvarının yapısında ve sağlamlığında rol oynar. Ksiloglukan zincirleri selüloz ile hidrojen bağı kurabilirler, böylece hücre duvarı yapısındaki bitişik mikrofibrilleri bağlayabilir ve hücre büyümesini kontrol edebilirler. Bitki ksiloglukan endo-transglikosilaz (XET) genleri CAZy sınıflandırmasında glikosid hidrolaz (GH) 16 ailesinin altailesi olarak yer alırlar. XET'lerin biyolojik görevleri net olarak gösterilememiş olsa bile hücre uzamasında ksiloglukan polisakkaritlerini değiştirerek hücre duvarı gevşemesinde önemli görevleri olduğu düşünülmektedir. Monokotillerde ve dikotillerde fazlaca XET geni bulunmaktadır ancak bu genlerce şifrelenen enzimlerin hücre duvarı üzerindeki fonksiyonu tam olarak bilinmemektedir.

Bu çalışmada tüm bu cevabı bilinmeyen soruları cevaplamayı hedefledik. Hücre duvarı yapısı açısından önemlerini değerlendirebilmek için farklı günlerdeki arpa koleoptillerindeki ve çimlenme sırasındaki farklı arpa dokularındaki anlatım seviyelerini ölçtük. Arpayı model sistem olarak seçmemizin sebebi çimlenme sürecinin kolay çalışılabiliyor olması ve arpa XET genleri ve bunların anlatım düzeyleri hakkında bilgi bulunmasıdır. Bu çalışmada var olan bilgiler genişletilmiştir. Elde ettiğimiz datalar XTH1, XTH2, XTH3,. XTH4, XTH7, XTH8, XTH11, XTH12 genleri için yapılmış mikroarrey çalışmaları ile karşılaştırılmıştır. Bu çalışmadaki sonuçlar XTH2, XTH3, XTH4, XTH7, XTH8, XTH11 için mikroarrey sonuçları ile örtüştüğünü göstermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

Ara*f*	Arabinofuranose
BLAST	Basic Local Alignment Search Tool
CAZY	Carbohydrate Active enzyme
CMC	Carboxymethylcellulose
CslF	Rice cellulose synthase–like gene
CslH	Barley cellulose synthase-like gene
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EST	Expressed sequence tag
EXGTs	Endoxyloglucan transferases
EXGTs	Endoxyloglucan transferases
Fuc*p*	Fucopyranose
Gal*p*	Galactopyranose
GAX	Glucuronoarabinoxylan
GH16	Glycoside hydrolase family 16
Glc*p*	Glucopyranose
HEC	Hydroxyethyl cellulose
HGA	Homogalacturonan
HPLC	High Performance Liquid Chromatography
HvGAPDH	Barley glyceraldehyde-3-phosphate-dehydrogenase
cDNA	Complementary DNA
MLG	Mixed linked glucan
MLG	Mixed-linkage β-glucan
NCBI	National Center for Biotechnology
PCR	Polymerase chain reaction
Q-PCR	Quantitative PCR
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
RNA	Ribonucleic acid

Tm	Temperature
Tm-NXG1	Nasturtium (Tropaeolum majus) endo-xyloglucanase loop mutant
TXG	Tamarind xyloglucan
XEH	Xyloglucan *endo*-hydrolase
XET	Xyloglucan *endo*-transglycosylase
XGOs	Xyloglucan derived oligosaccharides
XTH	Xyloglucan endotransglycosylase/hydrolase
XTHs	Xyloglucan endotransglycosylases
Xyl*p*	Xylopyranose

1. GENERAL INTRODUCTION

1.1. PLANT CELL WALL

1.1.1. General Introduction

Plant cell walls are dynamic structures that change through the life of the cell [1] and they have diverse biological functions that includes regulation of cell volume, determination of cell shape and protection of the protoplast [1].

Generally plant cell walls consist of cellulose, pectin, structural proteins and hemicelluloses [2, 3]. Cellulose microfibrils are the strengthening components in the plant cell wall. Hemicelluloses are shaping agents of the cell wall due to its noncovalent binding ability to the surface of the cellulose microfibrilles. Xyloglucan is the main hemicellulose of higher plants which binds tightly and noncovalently to cellulose microfibrils to form cross-links between them [4-6]. It has a backbone of β-(1→4)-D-glucose and its side chains consist of xylose, galactose and fucose residues [7-6].

In this century, a thorough understanding of wall polysaccharides would provide opportunities to genetically manipulate agro-industrial processes such as paper production, food quality and texture, malting and brewing, bioethanol production, dietary fiber, and ruminant digestibility [8].

1.1.2. Structure of Plant Cell Walls

The plant cell wall can be divided into three zones called the middle lamella, and primary and secondary walls [1] as showed in Figure 1.1. The middle lamella, which holds the cells together, is mostly made of pectic polysaccharides. The growth of the middle lamella follows the completion of cell division. Generally between neighbouring primary cell walls, the middle lamella act as a bonding agent.

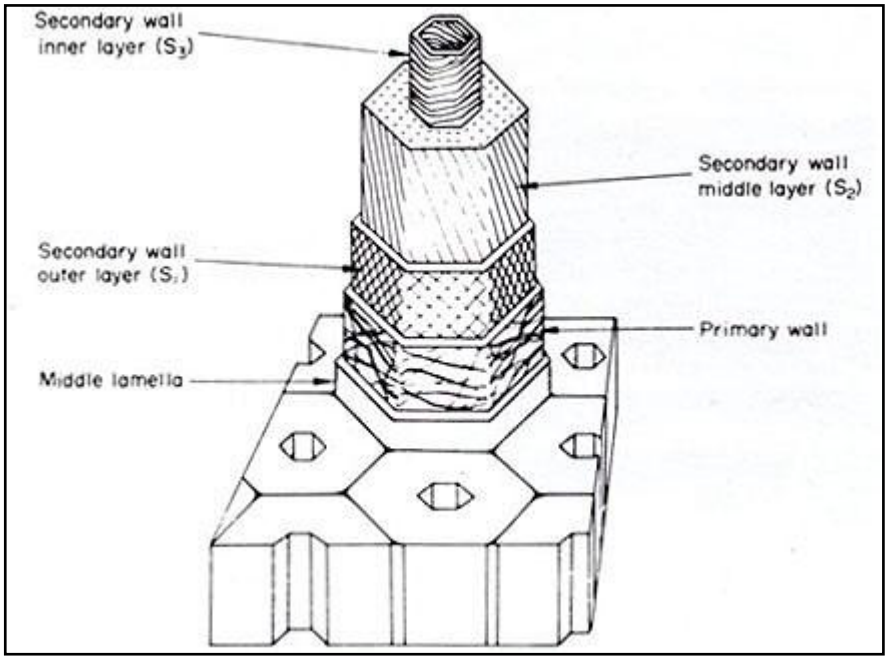

Figure 1.1. Plant cell wall structures, secondary cell wall [9]

Primary cell walls are deposited by the growing cells. Secondary cell walls are deposited in mature such as tracheids, fibers and vessels, after growth has completed. Secondary cell walls contain substances such as lignin, suberin and cutin in addition to elevated levels of cellulose, which supports cells mechanical strength [1] Figure 1.2 show a schematic drawing of a primary plant cell wall structures.

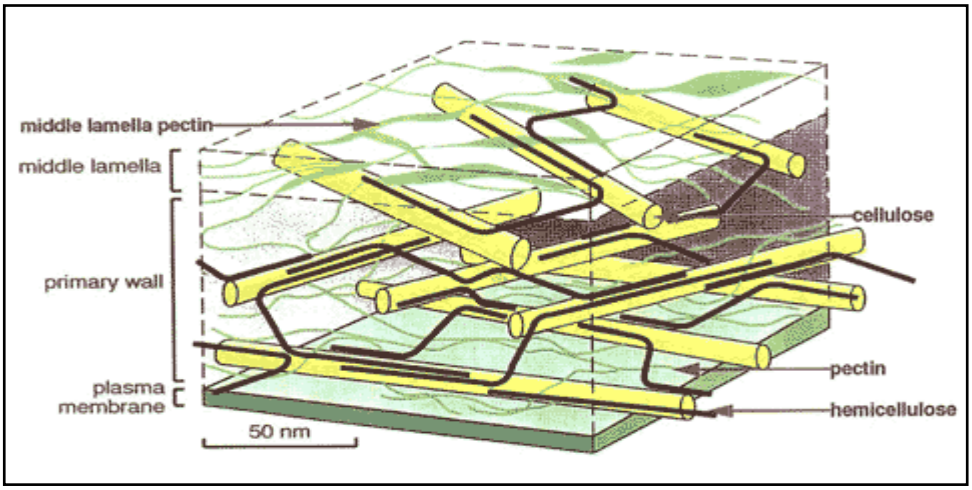

Figure 1.2. Plant cell wall structures, primary cell wall [10]

1.1.3. Structure and Metabolism of Plant Cell Walls

There are two phases in plant cell walls: a cellulosic microfibrillar phase embedded in a matrix phase [7, 11].

The matrix phase of the primary wall is involved in most physiological functions of cell walls, and is composed of about ten types of non-cellulosic polysaccharides and structural glycoproteins. Phenolic compounds such as lignin are major components of the matrix in the secondary wall, in addition to polysaccharides and glycoproteins. The composition of matrix polysaccharides in dicotyledons is different from that of monocotyledon species [7, 11]. The matrix composition among commelinoids including *Gramineae*, which differs from other monocotyledons or dicotyledons [1, 4]. The cell walls of dicotyledons and non-commelinoids are called type I walls, and the cell wall of commelinoids (typically of the *Gramineae*) are type II walls. The molecular size and the amounts of the matrix polysaccharides are similar. Xyloglucans in dicotyledons and (1→3,1→4)-β-glucans in *Gramineae* are similar molecules that have an important role in responses to environmental signals and in regulation of the life cycle. In some specific functions of cell walls, other matrix polysaccharides are also involved. By the active metabolic turnover of their components the physiological functions of the cell walls are sustained [12].

In the apoplast, the synthesized cellulose and matrix polysaccharides may be either degraded or modified. The complete degradation of polysaccharides from the chain end causes a decrease in their levels in the apoplast, whereas the cleavage of polysaccharides in the mid region brings about a decrease in their molecular size. Cross-linking and molecular grafting between polysaccharides may lead to an increase in molecular size. Additionally, the structural modification of polysaccharides induces a change in their chemical properties. The symplast, during the metabolic turnover of the cell-wall polysaccharides, not only supplies the apoplast with their components, including enzymes, but also regulates the apoplastic environment through influencing the activity of proton pumps and ion channels [12, 13].

1.1.4. Physiological Functions of Plant Cell Walls

Plant cell wall is called the apoplast when it is familiar with physiological reactions. The apoplast is the place where the plant cell first interacts with environmental signals such as water, temperature, light, gravity etc. The outer epidermal cell wall acts as the frontier between the plant and the outer environment. Hence, they play an essential role in plant response to the environment. The properties of lant cell wall vary due to response to the outer environment [12].

The apoplast consists of the cell coverings, and also it is the field where plant cells first encounter the outer environment. So it is the major site of plant responses to the environment. In the regulation of each stage and the response specific constituents of the cell wall, such as xyloglucans in dicotyledons and (1→3,1→4)- β -glucans in *Gramineae,* act as key components. By the metabolic turnover of these components the physiological functions of plant cell walls are sustained. The components of cell walls are supplied from the symplast, then they are modified or degraded in the apoplast. And so the metabolism of the cell wall is regulated between the symplast and the apoplast [14].

In some stage of plant development, the physical properties of the cell wall change significantly due to their structural modifications. As a result, the cell wall play an important role in the regulation of plant growth and morphogenesis as well as in the regulation of other stages in the plant life cycle [15].

Plant cell wall is called the apoplast when it is familiar with physiological reactions. The apoplast is the place where the plant cell first interacts with environmental signals such as water, temperature, light, gravity etc. The outer epidermal cell wall acts as the frontier between the plant and the outer environment. Hence, they play an essential role in plant response to the environment. The properties of plant cell wall vary due to response to the outer environment [12].

Plant cell walls are dynamic structures that have the capacity to alter during cell division, growth and differentiation to enable cells to adapt to environmental stresses and changing functional requirements. In addition to this, cell walls are also important for intercellular

cohesion and cell-cell communication, and have to be selectively permeable to water, nutrients and growth regulators [16]. The primary cell walls of vascular plants have a structure where cellulosic microfibrils are embedded in a chemically complex matrix consisting many polysaccharides, structural proteins and enzymes [17, 18].

The major non-cellulosic polysaccharides of primary walls for dicotyledonous plants are xyloglucans and pectic polysaccharides. On the other hand, for *Poales* species and related commelinoid monocots, including commercially important cereals and grasses, the predominant non-cellulosic wall polysaccharides are glucuronoarabinoxylans and (1→3,1→4)-β-D-glucans but levels of xyloglucans and pectic polysaccharides are very low [19].

1.1.5. Primary Cell Wall Types

Two different types of primary cell walls exist in flowering plants; type I and type II walls [4]. Type I walls are characteristics of dicots and non-commelinoid monocots. They form a network by interconnecting cellulose microfibrils with xyloglucan polymers [4, 20]. The surface of the cellulose microfibrils are enclosed with xyloglucan polymers that are attached to each other by hydrogen bonds [21-23]. The widely accepted model is that the xyloglucans cross the intervening space between seperate cellulose microfibrils [4, 21]. The cross-linking xyloglucans are also important as being load bearing molecules in the longitudinal axis of an elongating cell [4].

Primary cell walls are mostly made up of carbohydrates, but structural proteins are present at up to 10 % [7, 24]. For type I primary cell walls, on a dry weight basis, percentage composition for different compounds is generally as following; cellulose 30 %, pectin 35 %, xyloglucans 25 % and structural proteins 10 % [2, 3].
Type II walls are characteristic of commelinoid monocotyledons. This group includes species such as rice, wheat, oats and barley. In comparison to type I walls, they have lower amounts of xyloglucan and pectin [4].

Additionally, on a dry weight basis, the percentage composition of different components are as following; cellulose 30 %, glucuronoarabinoxylan (GAX) 30 %, mixed linked

glucan (MLG) 30 %, pectin 5 %, xyloglucan 4 %, structural proteins 0,5 % [2]. Although secondary cell walls are highly rich in cellulose which makes up 50 % to 80 % of the total wall, the primary cell walls generally use equal amounts of cellulose, pectins and hemicelluloses [25].

Most dicots and non-commelinoid monocots contain xyloglucan with a trisaccharide side chain which contains fucosyl, galactosyl and xylosyl residues [26]. Xyloglucan chains are capable of hydrogen-bonding to cellulose, so in the cell wall they may tether adjacent microfibrils and so control cell expansion. Xyloglucan's partial digestion with cellulase can also produce specific oligosaccharides. It is believed that these oligosaccharides have their effect on growth by acting as substrates of xyloglucan endotransglycosylase (XET) [27].

1.1.6. Regulation of Life Cycle

Plant cell walls are strongly influenced by nature and are deeply involved in the regulation of the plants life cycle, such as germination, vegetative growth, reproductive growth and senescence [15]. Seeds at maturity start to germinate by emerging radicles when placed in a suitable environment. The cell walls of some cell types should be degraded during seed germination to help with organ emergence, and to ease the intercellular transport of various hydrolases. In cereal caryopses, (1→3,1→4)-β-glucans and glucuronoarabinoxylans in the endosperm walls are actively degraded during germination [28, 29]. Additionally, the cell wall of the seed contain various types of storage polysaccharides, such as (galacto)mannans, galactans, xyloglucans, and (1→3,1→4)-β-glucans. These storage polysaccharides that are activated during seed germination are used as sources of energy and materials [30, 31]. Although plant cell walls have many functions in regulation of the life cycle, growth regulation has been widely studied.

Due to determination of the size and shape of plant cells directly by the cell wall during their growth, the cells must expand their restraining coverings. Cell wall expansion occurs in a direction to increase the distance in between cellulose microfibrils through loosening of cross-linking matrix polysaccharides. Therefore, the direction of cell growth is

concluded by the orientation of cellulose microfibrils, which is controlled by the orientation of cortical microtubules [32, 33].

Cell wall proteins are capable of inducing cell expansion and they are believed to have an important role in the expansion of the cell wall by breaking of mainly hydrogen-bonds [34]. Added to this; out of various matrix polysaccharides, xyloglucans in dicotyledons and (1→3,1→4)-β-glucans in *Gramineae* play a central role in determining the capacity of the cell wall to expand. The cell wall enzymes involved in their breakdown may contribute to cell wall matrix relaxation [13-36].

Recently, it was shown that a fungal endo-1,4-β-glucanase [37] increases the mechanical extensibility of plant cell walls and the members of xyloglucan endotransglycosylase/ hydrolase (XTH) devote to xyloglucan hydrolysis [38]. So that; these enzymes may be responsible for the latter loosening process. In addition to expansion, they may have an important role in the differentiation of the cell wall during the vegetative growth phase [14]. A typical example is shown in vascular cell development. Xylogenesis is induced *in vitro* in some plants, that provides us much information on the modifications of the cell wall structure during cell differentiation [39].

The cell wall also plays a role in vegetative growth of plants. For example, pollen differentiation is brought about through the breakdown of the callose wall that protects the meiotic cells, the degradation of the inner layer of another wall, and the development, cleavage of the stomium wall [40]. Endo-1,4-β- glucanases are involved in these processes [41]. Also, in pollen–stigma interactions the main point is the metabolic turnover of the cell wall of the papillary cells at the surface of the stigma. In some species, S-locus glycoproteins in the cell wall and their receptor proteins play an important role in the self-incompatibility process [42].

The specific changes occuring in the cell wall during plant senescence are very important because the level of cellulose is low and the activity of endo-1,4-β-glucanase is high in the cell wall when it is compared with neighbouring cells [43, 44]. A decrease in cellulose levels is induced by the loss of rigidity of the cell coverings. Other cell wall proteins, such as polygalacturonases, pectin methylesterases and expansins may cooperate with endo-1,4-

β-glucanase in this step [43]. Fruit softening is also brought up by a selective breakdown of the cell wall. The degradation of xyloglucans is involved in the breakdown of the cell wall leading to fruit softening [45]. XTHs, and endo-1,4-β-glucanase may be responsible for degradation of the cellulose–xyloglucan complex during fruit softening [46].

1.1.7. Environmental Responses

Plants are surrounded by a great variety of environmental signals. After plants are subjected to such signals, the mechanical properties of the cell walls may be altered. The signals may be lead to change the levels and structure of the components of the cell wall, and such modifications are thought to be the cause of the changes in the mechanical properties. Due to the environmental signals, the changes in the cell walls cause a series of events of signal perception and transduction, and are vital for plant response to such signals. So, the cell walls play a principal role in the interaction of plants with the outer world and let the plants to adapt themselves to the environment [12]. Plants, especially terrestrial ones, have evolved under the constant force of gravity and so gravity strongly effects their growth and development. It is difficult to produce a microgravity environment for sufficient duration on Earth so the changes in growth processes and the nature of cell coverings because of gravity have been analyzed under the hypergravity conditions produced by centrifugation [47-52]. Inhibition of elongation growth of plant seedlings and a decrease in the capacity of the cell walls to expand is the result of hypergravity [50]. Hypergravity induces the cell wall to become thicker [50-52]. Hypergravity also increases the molecular masses of xyloglucans in azuki bean and of (1→3,1→4)-β-glucans in maize seedlings where it decreased the activities of xyloglucan hydrolases and of (1→3,1→4)-β-glucanases in these plants [51, 52]. Microgravity cause completely opposite changes, such as growth stimulation, an increase in the mechanical extensibility of the cell coverings, a thinning of the cell coverings, a decrease in molecular size of xyloglucans and (1→3,1→4)-β-glucans and an increase in the activities of xyloglucan hydrolases and (1→3,1→4)-β-glucanases [53]. Moreover, all these parameters varied in proportion to the logarithm of the magnitude of gravity [54]. These results bring to mind that with changing the mechanical properties of the cell coverings, essentially through modifying the metabolism of xyloglucans and (1→3,1→4)-β-glucans, plants respond to the magnitude of gravity. It is also known that hypergravity increases the pH of the apoplast both in azuki

bean and maize seedlings [55, 56]. The apoplast pH is a key factor that regulates the activity of cell covering enzymes in-situ [12, 13]. In fact, the apoplast pH increases because of hypergravity and so the activities of xyloglucan hydrolases and (1→3,1→4)-β-glucanases decrease significantly [55, 56].

Plants are subject to physical, chemical, and biotic stresses. Generally, plant resistance to the stresses is supported by the hardening of the cell wall and cellulose, extensins, and phenolic compounds are involved in such hardening [56, 57].

Added to this, the metabolism of xyloglucans in dicotyledons and (1→3,1→4)-β glucans in *Gramineae* may be responsible for responses of plants to environmental signals [12]. For example, when light suppresses elongation growth of etiolated stem organs by decreasing the extensibility of the cell wall, the metabolism of xyloglucans in pea and azuki bean epicotyls [55, 56] and (1→3,1→4)- β -glucans in rice coleoptiles [59] are suppressed. The metabolic turnover of (1→3,1→4)-β-glucans is stimulated by submergence of rice coleoptiles, with the accompaniment of an increase in the extensibility of the cell wall and growth stimulation [60]. It was reported that the metabolic activities of xyloglucans and (1→3,1→4)-β-glucans are changed parallel with the extension of the cell wall and growth rate in azuki bean epicotyls and rice coleoptiles that were grown under different temperature conditions [61]. So therefore, these signals may be used as gravity-substituting factors [53].

1.2. CELL WALL POLYSACCHARIDES

1.2.1. General Introduction

Plant cell wall polysaccharides include cellulose and a range of non-cellulosic polysaccharides such as xyloglucans and (1→3,1→4)-β-glucans [16]. Molecular interactions between these polysaccharides which are assumed to be non-covalent in nature in most cases are fundamental to cell wall properties.

1.2.2. Structures of primary cell wall polysaccharides

1.2.2.1. Cellulose

Cellulose is the major cell wall polysaccharide which consists of linear polymer of β-(1→4)-linked D-glucose residues forming (1→4)-β-D-glucan [62] and its main function is to ensure the rigidity of the plant cell wall [63]. Cellulose is synthesized at the plasma membrane level and then deposited into the wall [64]. (1→3,1→4)-β-D-glucan which is thought to be synthetised in the Golgi apparatus is associated with cellulose and other noncellulosic polysaccharides in the primary walls of growing cells [65].

1.2.2.2. Pectic Polysaccharides

One of the major components of primary cell walls of all land plants is Pectin. The major pectic polysaccharides; homogalacturonan (HGA), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II); are mostly occur in all primary cell walls. Generally, it is thought that pectin accounts for about one third of all primary cell wall macromolecules, but however lower levels occur in primary cell walls of certain families belonging to the Poales. In non-extendable secondary cell walls, pectin is greatly reduced or absent [66]. HGA is a linear homopolymer of (1→4)-*α*-linked-D-galacturonic acid and appeared to be synthesized in the Golgi apparatus. RG-II is not structurally related to RG-I but contains an HGA backbone. RG-II has a backbone of around 9 galacturonic acid residues that are (1→4)-*α*-linked. The distribution of HGA, RG-I and RG-II within pectin chains is the unknown aspect of pectic network structure. It is suggested that RG-I and RG-II are both attached to HGA. Whether RG-I and RG-II are attached to HGA at reducing or non-reducing end is unknown. The connections within the pectic network seem to be highly complex. RG-I and RG-II are thought to be covalently attached to HGA and possibly to each other to form a pectic network into the cell wall. Pectic network involves in a range of functions related to growth, development and defence [66].

1.2.2.3. Xyloglucan

Xyloglucans are heterogeneous group of polysaccharides which bind to the surface of cellulose microfibrils by hydrogen bonds to form cross-links between them [4-6]. They have a a backbone of β-(1→4)-linked D-glucose and side chains composed of xylose, galactose and fucose [5-7].

Fry *et al.* developed a nomenclature with the letters G, X, S, L, and F referring to the structure shown in Figure 1.3.

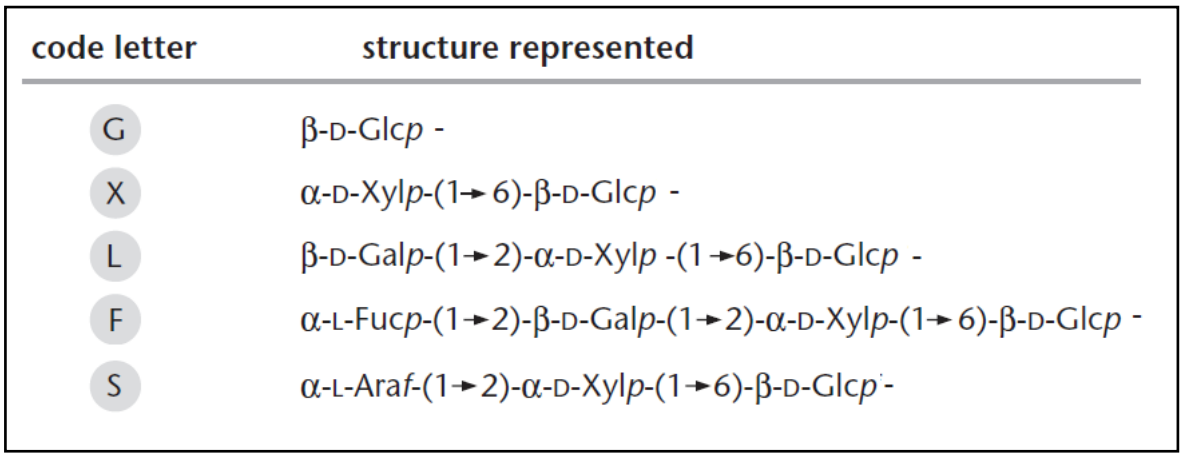

Figure 1.3. Nomenclature for xyloglucan structure with the letters adapted from Fry et al. (1993). Glc*p* is glucopyranose, Xyl*p* is xylopyranose, Gal*p* is galactopyranose, Fuc*p* is fucopyranose, and Ara*f* is arabinofuranose

Four types of oligosaccharides (XXXG, XLXG, XXLG, XLLG) were released from the xyloglucans from the cotyledons of tamarind seeds which were treated with cellulase [65, 66] These xyloglucans contain galactose and have a repeating XXXG core motif are called galactoxyloglucans shown in Figure 1.4 [69].

Xyloglucans in the primary walls of most eudicotyledones have a repeting of XXXG core motif and also contain fucose added to galactose and they are referred as fucogalactoxyloglucans which is shown in Figure 1.5 [69].

The proportions of different oligosaccharides vary due to the organ from which the walls were isolated [70]. Especially for the economically important family *Solanaceae* and the species of this family, the xyloglucans structure have been well examined [69]. These xyloglucans have S but no F (S and L = X with α-L-Araf-(1→2)- and β -D-Galp-(1→2)-attached) containing subunits and referred as arabinoxyloglucans showed in Figure 1.6 and have a repeating XXGG core motif [67]. For example; the xyloglucans of primary walls of tobacco (*Nicotina tobacum*) leaves consist of mostly XXGG and XSGG [71]. Less is known about the structures of the xyloglucans of primary walls of monocotyledones when

it is compared with eudicotyledones [69]. Most studies have been done in *Poaceae*. The *Poaceae* family form a part of a large group of monocotyledones[69]. XXG_n is the predominant repeating core motif for some structural studies of *Poaceae* [69]. In an early study of the xyloglucans in the walls of barley coleoptiles, the most abundant oligosaccharide was XXGGG; XXGG and XXGGGG were also found in relatively large proportions [72].

Kato *et al.* also concluded that the predominant repeating unit of barley seeding xyloglucan was XXGGG [69].

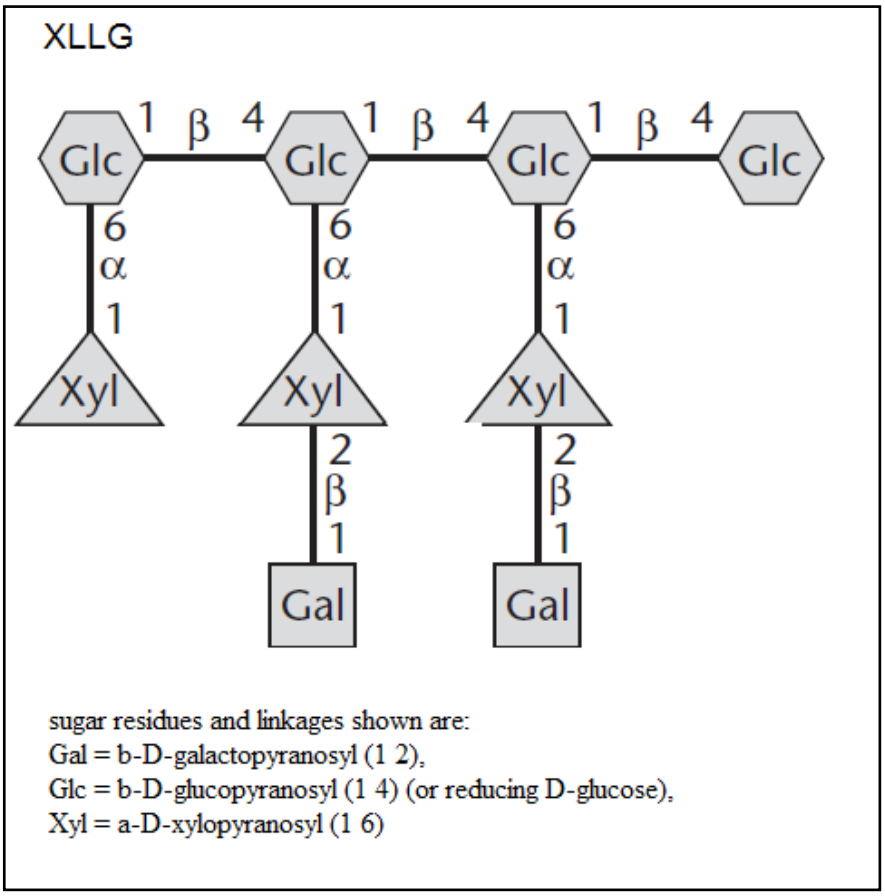

Figure 1.4. Galactoxyloglucan (modified from Fry 1993, see Figure 1.2 for details)

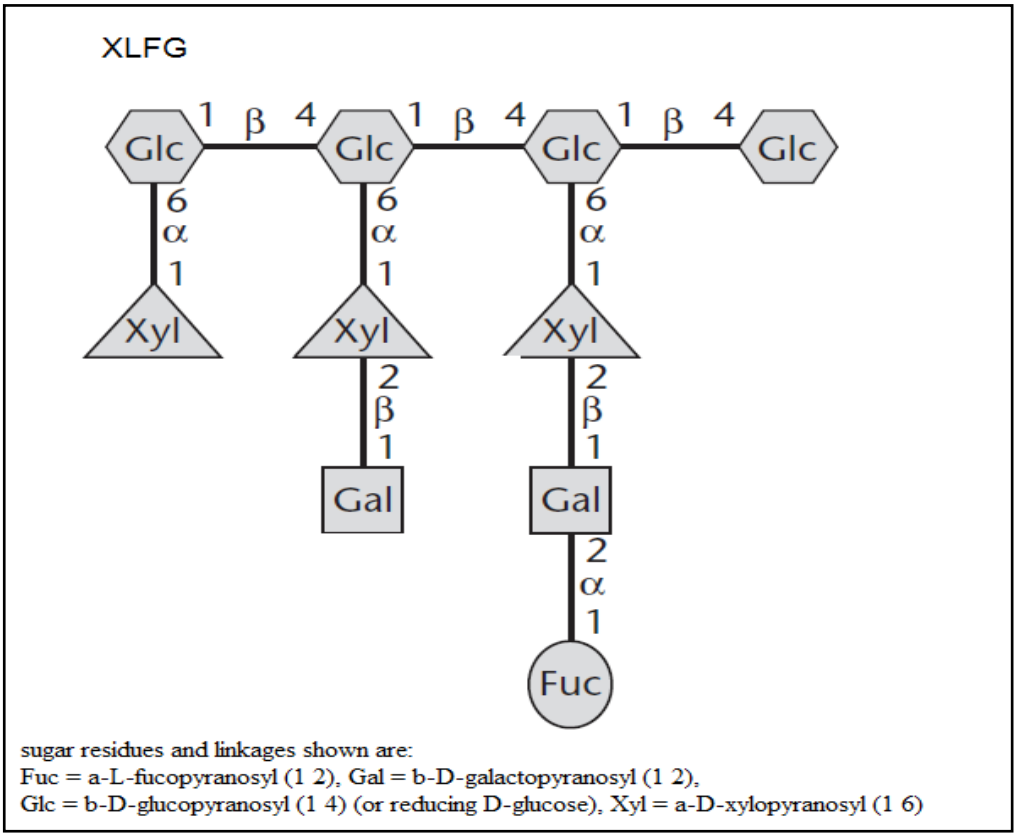

Figure 1.5. Fucogalactoxyloglucans(modified from Fry 1993 see Figure 1.2 for details)

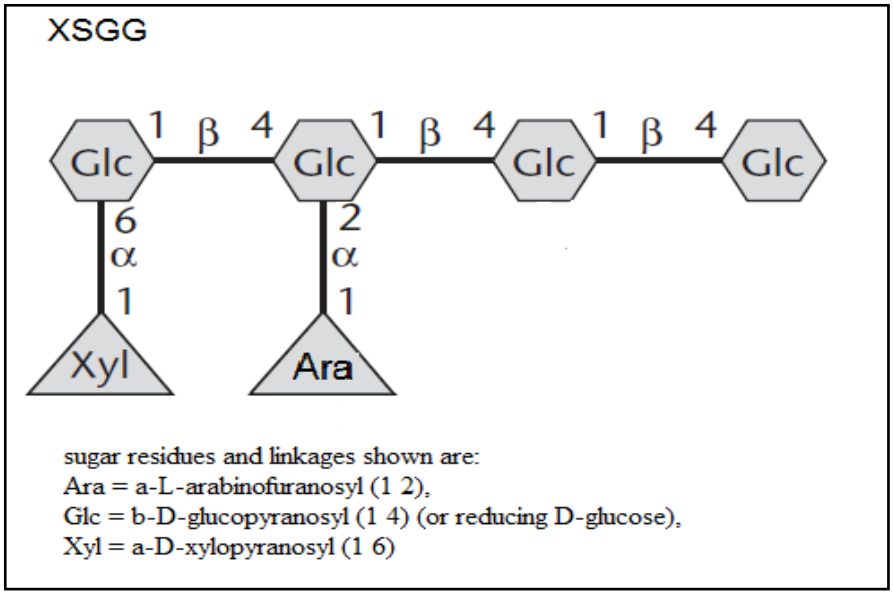

Figure 1.6. Arabinoxyloglucans(modified from Fry 1993 see Figure 1.2 for details)

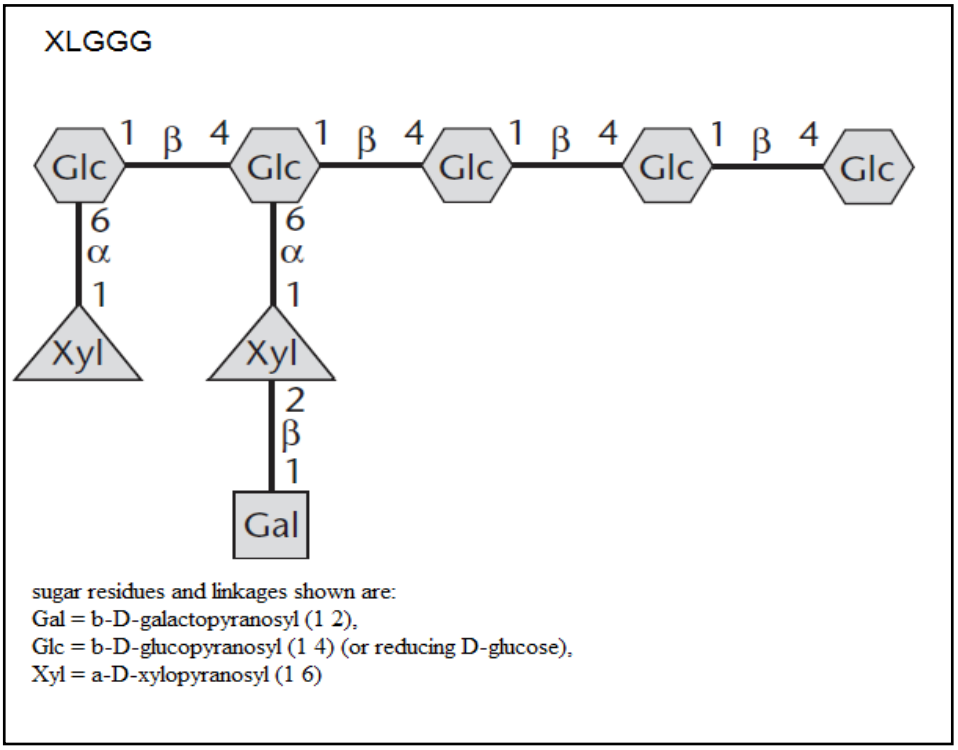

Figure 1.7. Poaceae xyloglucan (modified from Fry 1993, see Figure 1.2 for details)

The primary cell wall of flowering plants consist of a framework of cellulose embedded in a matrix of hemicelluloses, pectins, and structural proteins [4, 7, 15]. Xyloglucan is the major hemicellulosic matrix polysaccharide in the primary cell walls of dicotyledons. It is thought that xyloglucan and xyloglucan endotransglycosylases/hydrolases play an important role in the expansion and/or assembly of plant cell walls [73-75]. Xyloglucans are capable of forming tight non-covalent bonds, using hydrogen-bonds, with cellulose microfibrils [5, 6, 76-78]. Added to this xyloglucan sticks to the microfibril surface or entrappes within microfibrils [22].

1.2.2.4. Mixed Linkage (1→3,1→4)-β-D-glucans

Poales among vascular plants contain an additional hemicellulose known as mixed linkage (1→3,1→4)-β-D-glucans (MLG). A MLG known as lichenan also occurs in some lichens. Table 1.1 shows us the list of the speices surveyed for MLG content in a recent study [79].

Table 1.1. Plants surveyed for MLG, scale (++++) abundant, (+) detectible, (-) undetectible [79]. Poales MLG consist of an unbranched chain of β-D-glucopyranase residues linked by (1→3) and (1→4) bonds. MLG typically takes the form of G3G4G4G3G4G4G4G3G4G4G3G4G4G3G ... where G is glucopyranase and 3 and 4 indicate (1→3) and (1→4) bonds [79]

Species	Organism	Classification	C4C3C	C4C4C3C
Chara vulgaris	Whole shoots	Alga, Charales	-	-
Nitella opaca	Whole shoots	Alga, Charales	-	-
Anthoceros agrestis	Cell culture	Bryophyte, hornwort	-	-
Pella epiphylla	Thallus	Bryophyte, liverwort	-	-
Pella epiphylla	Seta	Bryophyte, liverwort	-	-
Pella epiphylla	Capsule	Bryophyte, liverwort	-	-
Pleurozia purpurea	Whole shoots	Bryophyte, liverwort	-	-
Sphagnum rubellum	Whole shoots	Bryophyte, moss	-	-
Drepanocladus sp.	Whole shoots	Bryophyte, moss	-	-
Hygrohypnum sp.	Whole shoots	Bryophyte, moss	-	-
Hylocomium splendens	Whole shoots	Bryophyte, moss	-	-
Mnium hornum	Whole shoots	Bryophyte, moss	-	-
Plagiothecium sp.	Whole shoots	Bryophyte, moss	-	-
Rhytidiadelphus loreus	Whole shoots	Bryophyte, moss	-	-
Diphasiastrum alpinum	Whole shoots	Lycopodiophyte	-	-
Huperzia selago	Whole shoots	Lycopodiophyte	-	-
Lycopodium clavatum	Whole shoots	Lycopodiophyte	-	-
Lycopodium phlegmaria	Whole shoots	Lycopodiophyte	-	-
Selaginella apoda	Whole shoots	Lycopodiophyte	-	-
Selaginella willdenovii	Whole shoots	Lycopodiophyte	-	-
Psilotum nodum	Whole shoots	Pteridophyte, Psilotopsida	-	-
Equisetum arvense	Whole shoots	Pteridophyte, Equisetopsida	++	++++
Equisetum fluviatile	Whole shoots	Pteridophyte, Equisetopsida	++	++++
Equisetum scirpoides	Whole shoots	Pteridophyte, Equisetopsida	+	+++
Equisetum sylvaticum	Whole shoots	Pteridophyte, Equisetopsida	+	+++
Equisetum x trachyodon	Whole shoots	Pteridophyte, Equisetopsida	+	+++
Angioptens erecta	Leaves	Pteridophyte, Marattiopsida	-	-
Osmunda regalis	Leaves	Pteridophyte, Polypodiopsida	-	-
Asplenium trichomanes	Leaves	Pteridophyte, Polypodiopsida	-	-
Athyrium filix-femina	Leaves	Pteridophyte, Polypodiopsida	-	-
Polypodium vulgare	Leaves	Pteridophyte, Polypodiopsida	-	-
Polystichum aculeatum	Whole shoots	Pteridophyte, Polypodiopsida	-	-
Pteridium aquilinum	Leaves	Pteridophyte, Polypodiopsida	-	-
Cycas revoluta	Leaves	Gymnosperm	-	-

Table 1.1. Plants surveyed for MLG, scale (++++) abundant, (+) detectible, (-) undetectible [79]. Poales MLG consist of an unbranched chain of β-D-glucopyranase residues linked by (1→3) and (1→4) bonds. MLG typically takes the form of G3G4G4G3G4G4G4G3G4G4G3G4G4G3G ... where G is glucopyranase and 3 and 4 indicate (1→3) and (1→4) bonds [79] (continue)

Larix decidua	Leaves	Gymnosperm	-	-
Iris pseudacorus	Leaves	Angiosperm, nonpoalean monocot	-	-
Avena sativa	Coleoptiles	Angiosperm, Poales	++++	+++
Festuca arundinacea	Cell culture	Angiosperm, Poales	++++	++
Holcus lanatus	Leaves	Angiosperm, Poales	++++	++
Hordeum vulgare	Coleoptiles	Angiosperm, Poales	++++	++
Lolium perene	Leaves	Angiosperm, Poales	++++	++
Zea mays	Cell culture	Angiosperm, Poales	+++	++
Rosa sp.	Cell culture	Angiosperm, eudicot	-	-
Spinacia oleracea	Cell culture	Angiosperm, eudicot	-	-

Poaceae and Equisetum spp. are two types of vascular plants that have different cell walls and both contain (1→3,1→4)-β-D-glucans. In Poaceae, (1→3,1→4)-β-D-glucans are associated with growing cells and are essentially absent in mature tissue. (1→3,1→4)-β-D-Glucans are generally found in expanding cells such as the coleoptiles of cereals [80]. (1→3,1→4)-β-D-Glucans also may be seen in some of the vascular cells of the leaf which supports that MLG plays a structural role in secondary cell walls [80].

Various rice cellulose synthase–like (CslF) genes were shown to produce and deposit (1→3,1→4)- β -D-glucan in their cell walls. The CslF gene family has now been defined in barley [80].

Now there is quite strong evidence that the products of the CslF and barley cellulose synthase-like (CslH) genes are associated with the biosynthesis of (1→3,1→4)-β-D-glucans in the Poaceae, however the locations of polysaccharide synthesis have not been defined unequivocally [80].

1.2.3. *Xyloglucan endotransglycosylase/hydrolase* (XTHs)

Xyloglucan is a major non-cellulosic polysaccharide found in both monocot and dicot species where it plays an important role in the structure and strength of plant cell walls. Plant xyloglucan endotransglycosylase/hydrolase (XTH) genes are a subfamily of the glycoside hydrolase (GH) family 16 in the Carbohydrate Active Enzyme (CAZy) classification [81]. Although the biological functions of XTHs have not yet been unequivocally demonstrated, it is believed that they have an important function in the loosening of cell walls during cell elongation by its modification of xyloglucan polysaccharides.

Wall loosening could be achieved through enzymes hydrolysing portions of the xyloglucan network, therefore weakening the structure surrounding and constraining the cellulose microfibrils [5, 6, 82]. XTHs acting in XET mode cleave and rejoin xyloglucan chains or suitable xyloglucan derived oligosaccharides (XGOs) [83, 79-82] and therefore XTHs are the most obvious candidates for wall loosening [80]. XET activity is often related with growth rate [80, 83-89] and expression of XTH genes is mainly found in elongating tissues [90, 91]. However is also found that measurable XET activity occurs in non-growing regions of plant cell walls during secondary cell wall formation [92, 93]. It could be assumed that other processes, such as peroxidase catalysed cross-linking of wall polymers [94-96] or lignification override cell wall loosening and may be responsible for wall-tightening [97], so as to describe why XET activity is detectable in actively elongating as well as non-elongating cells and tissues. One of XTHs' main functions could be restructuring of cell walls during and even after cell elongation [98]. Multiple XTH isoforms are expressed in different organs of various plant species in response to numerous hormonal, environmental and developmental stimuli [80, 86, 99, 100]. High XTH action is most prominent in the cell elongation zone of all vascular plants, from the primitive plant *Selaginella* up to the most developed angiosperms and at the future site of root hair emergence [101]. The exact roles of XTHs in cell growth are still under debate. It may be assumed that the different gene products are active in different aspects of cell wall metabolism. XTH action is now reported to be localised in either a fibrillar pattern, which is obviously assocaited with the presence of cellulose microfibrils, or in a uniform pattern in different cell walls at the subcellular level. Furthermore, the result of different XTH

isoenzymes having a different function from the ones causing the fibrillar staining pattern may be the uniform pattern. The XET action in root hair side walls points to a role in the integration of newly deposited xyloglucans in non-expanding cell walls. For a study of cell elongation, the most interesting pattern is the fibrillar pattern. This pattern of XET action is thought to be the result of re-localization of the xyloglucans themselves [102].

Cell wall proteins are thought to be involved in controlling cell wall extensibility [103]. This family of proteins include the enzymes known as xyloglucan endotransglycosylases (XTHs) and endoxyloglucan transferases (EXGTs) which break xyloglucan polymers and paste one of the strings to the end of another xyloglucan string [104]. Thus, the tension of load bearing xyloglucan polymers can be released. The endotransglycosylation activity of XTHs is optimum at pH 5.8 [80].

XTHs operate by a transglycosidic mechanism by splitting the β-1,4-linked poly-glucose backbone of xyloglucan molecules and rejoining its newly created reducing ends to the nonreducing ends of other xyloglucan molecules with β-1,4-glycosidic bonds [105]. It is believed that the members of the *XTH* gene family may have a variety of expression patterns in plants. But how the expression patterns of the *XTH* members correlate with their physiological roles is unclear.

XTHs are encoded by large multigene families in plants. They play a pivotal role in the re-modeling of cell wall architecture [104]. *Arabidopsis* has 33 different XTH genes [99]. This number is at least 25 in tomato, where the full genome sequence is not yet available, and one of these genes (*SlXTH5*) shows a high expression levels in ripening fruits [106]. There are at least 29 XTHs in rice (*Oryza sativa*) [12, 107].

The largeest cluster;namely groups I and II, can be subdivided into a number of small clades based on protein sequence at Figure 1.8. The second main branch encompassing fewer sequences coincides with the historical group III, which indicates that group III is not monolithic but can be subdivided into two predominant clades. Bootstrop values indicate that 5 sequences; namely At-XTH1, At-XTH2, At-XTH3, At-XTH11, and Pt-XTH15, form an intermediate between group I/II and group III [132].

None of the enzymes from group III/B has been shown to have endo-xyloglucanase (hydrolase) activity. It is suggested that all members of group III/B and the putative ancesteral group may be predominant, while mixed-function xyloglucanase/XETs (e.g, Nasturtium (Tropaeolum majus) endo-xyloglucanase loop mutant (Tm-NXG1) are found only in group III-A)[132].

The majority of the XTH gene products have very little or no detectable hydrolytic activity and are thus predominant or strict xyloglucan endo-transglycosylases [80]. Ptt-XET16 from the hybrid apsen Populus tremula is one such example that has undetectable xyloglucanase activity; xyloglucan endo-b-1,4-glucanase [XEG] or xyloglucan endohydrolase [XEH] [132]. The three-dimensional structure of this enzyme has been solved, xyloglucanase activity and gene sequence have been conclusively linked for only one member of GH16, Tm-NXG1 from *Tropaeolum majus* (nasturtium), which is a predominant endo-xyloglucanase that can also perform xyloglucan endo-transglycosylation at elevated concentrations of acceptor substrates as can be observed in Figure 1.8 [132].

In various plant species, XTH activities have been detected [82, 104]. For example, in *Arabidopsis thaliana AtXTH27* plays an essential role in the generation of tracheary elements in rosette leaves [115] and different isoforms of XTH act in different processes of root development [101, 124]. Some XTH genes may have essential role in the formation of cell wall architecture during the pistil and anther development and self-pollination. Recent studies have shown that a large number of cell wall genes such as glycosyl hydrolases, glycosyltransferases and structural proteins work at different stages of anther development [116]. But the major function of XTHs in plants is during the cell wall elongation as XTHs act at the microfibril–matrix interface and maintain thickness and integrity of cell wall [114].

The biological functions of XTHs have not been demonstrated unequivocally [119]. It may have functions in covalent cross-linking of different classes of polysaccharides in the cell wall [117], a process that has been coined ´hetero-transglycosylation' [118] and is added to cross-linking of different xyloglucan molecules to reinforce the cell wall [80]. A recent study showed that a purified XET from barley can covalently link tamarind xyloglucan (TXG), hydroxyethyl cellulose (HEC), other cellulosic substrates, and (1→3)(1→4)-β-

glucans[117]. This data also proves the earlier suggestions that some XETs might be active on the more abundant matrix phase polysaccharides of cell walls such as in the Poaceae where it is (1→3)(1→4)-β-D-glucans, rather than the assumed substrate of xyloglucan. Large number of XTH genes and their high expression levels in many tissues with relatively low abundance of xyloglucans in barley cell walls supports this suggestion [72].

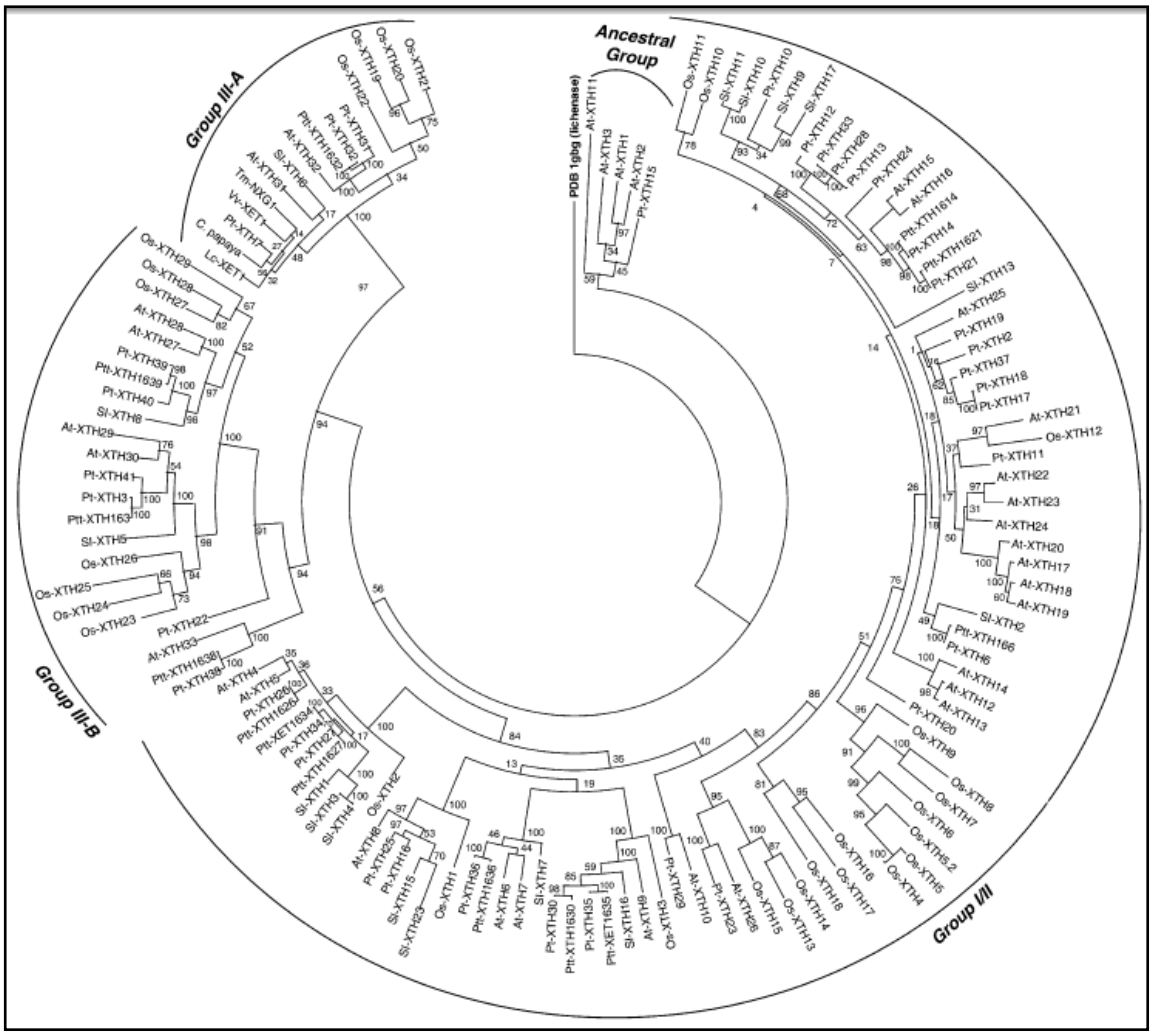

Figure 1.8. Unrooted Phylogenetic Tree of 130 Full-Length XTH Gene Products and Bacillus licheniformis Lichenase [132]

The composition and structure of plant cell walls vary between cell/tissue types and from species to species. While much is known about the cell wall polysaccharide content of most species of plants, little is known about the enzymes that synthesize and modify them.

Although the types and abundance of polysaccharides in plant cell walls have been generally well studied, there are questions about the molecular interactions between these polysaccharides within the plant cell wall. It is assumed that the different polysaccharides are held in place through extensive intermolecular hydrogen bonding rather than through covalent interactions in most wall models [17, 120].

1.3. EXPRESSED SEQUENCE TAGS (ESTs)

EST databases contain sequence data and other information on 'Expressed Sequence Tags from a number of organisms. These sequences provide a resource to help evaluate gene expression.

The genome sequence of two plant species, that represent model dicot and monocot species with relatively small genomes, namely *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000) and *Oryza sativa* [124, 125] are essentially complete. The genomes of other agronomically important cereal species, such as barley and wheat, are substantially larger, up to more than 100-fold that of the arabidopsis genome. Focused analysis of expressed genes provides a basis for the discovery of genes and their functions even for large-genome species and is an alternative to full genome sequencing.

Expressed sequence tags directly represent transcribed portions of the genome and avoid the highly repetitive DNA that makes up the bulk of genomic DNA. EST sequencing projects for many plants including cereals are important. Furthermore, ESTs can be a valuable resource for the creation of high-throughput expression analysis using complementary DNA (cDNA)-array technology [131].

Plant growth and development which is regulated by global changes in the expression of specific complements of genes is in turn characterized by tissue and cellular differentiation [131]. In addition to the array hybridization technology, computational analysis of the ESTs from various cDNA libraries provides an alternative for expressional profiling of genes as transcript abundance could be inferred from EST frequency [129, 130]. Large amounts of EST data exist in public databases for many species. The EST-count method has advantages over hybridization strategies using arrayed cDNA clones or oligonucleotide

chips, where sequence assembly of ESTs can differentiate closely related gene family members [127, 128].

EST collections represent significant contributions to international efforts in the structural and functional analysis of the barley genome. One obvious possibility to use these ESTs together with bioinformatic approaches is the systematic development of large numbers of genetic markers, as they are required for the construction of high-density transcript maps where large size of barley genome is given [131]. At the functional level, collections of barley ESTs have already been used as a resource for the construction of cDNA arrays and contributed to the development of a 22K probe array described below [126].

1.4. MICROARRAY STUDIES

A Deoxyribonucleic acid (DNA) microarray is an array of microscopic spots of DNA on a solid suface which is used to measure the expression levels of a large number of genes. Each DNA spot contain a specific DNA sequence that is known as a probe.

DNA microarray analysis has become the most widely used source of genome-scale data in the life sciences becausebecause microarray expression studies are producing massive quantities of gene expression and other functional genomics data that provides key insights into gene function and interactions within and across metabolic pathways [133, 134]. As opposed to genome sequence data that has standard formats for presentation and widely used tools and databases; much of the microarray data generated so far remains inaccessible to the broader research community [137].

Comparing gene expression data is considerably more difficult because microarrays do not measure gene expression levels in any objective units. Furthermore, most measurements report only relative changes in gene expression using a rarely standardized reference. In fact, different microarray platforms and experimental designs produce data which are normalized in different ways that makes questions in comparison and integration of these data [135, 136].

For major crop plants, publicly available, standard microarray platforms for parallel expression analysis have been limited. A publicly available barley microarray, the 22K Barley1 GeneChip probe array, was created as a model for plants without a fully sequenced genome. Array content was derived from the worldwide contribution of 350,000 high-quality ESTs from 84 cDNA libraries, in addition to 1,145 barley (*Hordeum vulgare*) gene sequences from the National Center for Biotechnology Information nonredundant database. The result was a microarray chip with 21,439 non-redundant exemplar sequences, that are thought to belong to unique genes [126].

1.5. AIM OF THE STUDY

It is known that the cell wall composition and the levels of component polysaccharides varies with respect to the growth phase, cell type, and cell position [62]. The rapid formation of a cross-linked protein network acts together with the deposition of lignin and callose, strengthening the cell walls and forming a new physical barrier to microorganisms in response to pathogen attack, can be a given as a simple example [63].

Both monocot and dicots species have large numbers of XTH genes but the function of their encoded enzymes on cell wall structure is not well known. This has special relevance in species such as barley where the levels of xyloglucan, the expected substrate of these enzymes, is found only at low levels.

In this study we are aiming at getting answers for some of these unknown questions. We are planning to evaluate the expression levels of these genes in different tissues of barley during germination and growth in order to determine their importance for cell wall structure. Most plant species including rice, cotton, wheat and barley have large numbers of XTH genes. We used barley as the model plant because its germination process is easily studied and there is already some pre-existing knowledge of barleys XTH genes and their expression levels, which will be expanded upon in this current study.

In this thesis we are taking a part attention to the catalytic activity of barley xyloglucan xyloglucosyl transferase, a member of the GH16 group of glycoside hydrolases, for the *in vitro* formation of covalent linkages between xyloglucans and cellulosic substrates, and

between xyloglucans and (1→3,1→4)-β-D-glucans. Because many studies raised that there is a possibility that xyloglucan endotransglycosylase (XET) could link different polysaccharides *in vivo*, and so may have an important function in increasing cell wall strength, flexibility and porosity.

We expect to find a result about the importance of different members of this gene which may help us to understand its functions in plant cell wall by comprising the expression levels of different *XTH* members in different barley tissues.

Previously; we made a cDNA library from different barley tissues to compare expression levels of most *XTH* members of barley by Quantitative PCR (Q-PCR).

One of our goal is to to compare Q-PCR results with microarray analysis identified by Affymetrix probe ID. Microarray analysis is available for 14 XTH genes. However, the problems at HPLC purification step and designed primer step made it somewhat difficut to analyze expression profiles confidently for all XTH genes of barley.

2. MATERIALS

2.1. CHEMICALS

Sloop Barley seedlings were obtained from Australia. Agarose, Dnase Inactivation Reagent (Ambion TURBO DNA-free™,Cat. No. AM1907), cDNA synthesis kits (SuperScript® III First-Strand Synthesis SuperMix, Cat. No: 18080-400), Trizol Reagent, DNA ladder, PCR Purification Kit (Quick Gel Extraction and PCR Purification Combo Kit,Cat. No.K220001) were purchised from Invitrogen and Deoxyribonucleotide triphosphate (dNTP) and Taq DNA polymerase were obtained from SibEnzyme. Tris, boric acid, ethidium bromide, chloroform, isopropanol, ethanol, triethylammonium acetate were purchised from Sigma-Aldrich (Taufkirchen, Germany), Ethylene diamine tetra acetic acid (EDTA), acetonitrile were purchased from Merck (New Jersey, USA).

2.2. LABORATORY EQUIPMENT

During the whole experiment shaker (BS-T, Sartorius, Aubagne, France), waterbath (OLS200, Grant, Cambridgeshire, England), vacuum machine (GmbH, Ilmvac,Ilmenau, Germany), agarose gel instruments (Bio-rad, California,USA), UV spectrophotometer (Mutiskan Spectrum, Thermo, Massachusetts, USA), microcentrifuge (Sigma-Aldrich, Taufkirchen, Germany), autoclave (Hirayama, Saitama, Japan), laminar flow (Hfsafe-1200, Heal Force, Shanghai, China), growth chamber (Digitech, Ankara, Turkey) were used.

3. METHODS

3.1. SAMPLE PREPARATION

In this thesis 13 samples from different tissues of barley *(Hordeum vulgare* cultivar Sloop*)* at different developmental stages were used, 8 of which were selected because they correspond to 8 of the tissues used in the the Affymetrix Barley1 microarray chip experiments. Barley seedlings were all grown at the same light, moisture and temperature conditions (17°C for 16 hours with light for day and 12°C for 8 hours with no light for night temperature). The samples used in this study are shown in the Table 3.1.

Table 3.1. Information of barley cultivar Sloop samples that were used to make cDNA libraries which corresponded to the tissues used in the microaray studies. The tissue of the organism used to get target cell type was written for Sloop variation growth timetable

Target cell type	Part of organizm
Coleoptile day 1	Germinating seed
Coleoptile day 2	Germinating seed
Coleoptile day 3	Germinating seed
Coleoptile day 4	Germinating seed
Coleoptile day 5	Germinating seed
Coleoptile day 6	Germinating seed
Root	10cm seedling stage
Crown	10cm seedling stage
Leaf	10cm seedling stage
Bracts	1-2days before anthesis
Pistil	1-2days before anthesis
Anthers	1-2days before anthesis
Caryopsis	10 DAP

For coleoptile samples, seeds were germinated in 20×40×15 cm plates on two layers of 2mm filter paper completely wetted with distilled water. Six plates were prepared, each plate contained 50 seeds, with one plate dedicated to each stage of coleoptile development.

Plates were placed into the growth cabinet with temperature conditions of 17°C for 16 hours and 12°C for 8 hours as the day / night temperature cycles.

For root, crown and leaf samples, 3 seeds were planted in a single pot containing vermiculite without compost. Twenty pots (13×13 cm each) were placed into a growth cabinet with 17°C for 16 hours and 12°C for 8 hours as the day / night temperature cycles.. Sampling was done randomly 12 days after planting when the average length of the seedling shoots was 10 cm as seen in Figure 3.1.f. Seedlings were then carefully removed from the vermiculite to avoid damaging the roots. Vermiculite trapped in the seedling was washed off with distilled water. Ten seedlings per sampling were collected.

Bracts, anthers, and pistils were collected 1-2 days before anthesis. The lemma and palea, collectively known as bracts, were carefully removed from the rest of the floret. Anthers and pistil were also collected from the florets. Seven spikes were collected, with three florets from the center of each spike being used per sample.

One to two caryopses, 8-10 mm long were collected from the middle part of the spike 8-10 days after hand-pollination. Ten caryopses from seven spikes were collected per sampling. Representative images of all the tissues used to make cDNA libraries are shown in Figure 3.1.

3.2. cDNA PREPARATION

3.2.1. Ribonucleic acid (RNA) Extraction

The Trizol (Invitrogen) RNA extraction method was used for all tissue samples. 50-100 mg from each sample was analysed. First the tissue was ground in 500 µl of Trizol with plastic pestle in a 1.5 ml eppendorf tube. After grinding, 500µl of Trizol was added to make up a 1 ml total volume, mixed to homogeneity, then incubated at room temperature for 5 minutes.

An addition of 200 µl of chloroform followed by shaking the tubes vigorously for 15 seconds precipitated the RNA. After 3 minutes at room temperature, the samples were centrifuged at 15.980 g for 15 minutes at 4°C.

The supernatant was then transfered to a fresh 1.5 ml eppendorf, with care being taken to exclude any interphase material. Next, 500 µl isopropanol was added to the eppendorf and mixed by pipetting. After 10 minutes at room temperature, the samples were spun at 15.980 g for 10 minutes at 4°C. The supernatant was then removed with a pipettor and discarded, and 1ml of 75 % ethanol was added and the tube spun at 9960 g for 5 minutes at 4°C. The supernatant was removed with a pipettor and the pellet was dried in air for 5 minutes and resuspended in 50 µl of sterile water.

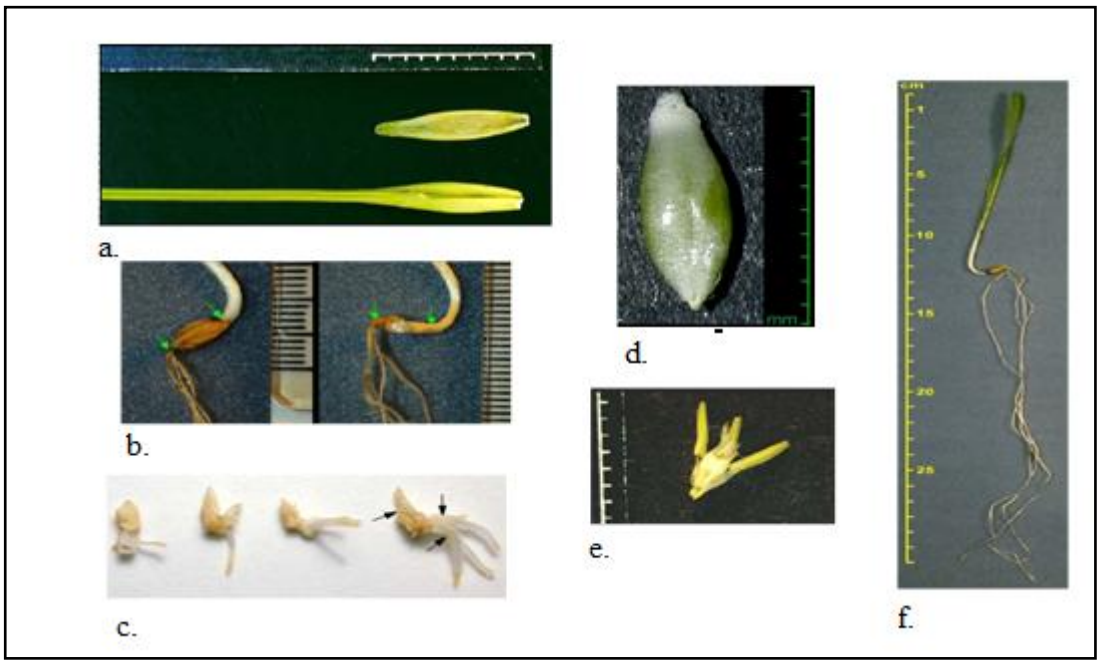

Figure 3.1. All the samples that are used to make cDNA library. a. Bracts (lemma and palea(bracts) were removed from the rest of the floret. The awn was cut off leaving about ¼ of the original length., b. Crown (10 cm seedling stage, root and stem with the first and second leaves about 7 mm from the root were cut off. The remaining piece of tissue was named crown), c. Coleoptile (coleoptile were cut off the remaining embrio and collected separately), d. 10DAP(caryopsis 10 mm long were colected from the middle part of the spike, e. Anther & Pistil (Anthers and pistil were collected separately from the lemma and palea(bracts), f. Leaf & Root (10 cm seedling stage)
(microarray analysis identified by Affymetrix probe ID)

3.2.2. Treatment to Remove DNA & Confirmation of Quality

From each sample, 10 µl was gently combined with 1 µl buffer and 1 µl DnaseI, and the mixture was incubated at 37°C for 30 minutes. After 1,2 µl Dnase Inactivation Reagent was added and mixed well, the mixture was incubated at room temperature for 2 minutes. The tube was then spun at 10000 g for one minute. The clear aqueous phase was used for analysis.

After this step each sample was checked both by being run on a non-denaturing agarose gel (1,6 %) with detection by ethidium bromide on a UV lightbox, and by spectrophotometer reading at 260/280nm. This common confirmation step was important since it gave us valuable information about the integrity of our sample.

As judged from the agarose gel, good quality RNA was measured at 260 nm and 280 nm to quantitate and then used to make cDNA for Q-PCR purposes.

3.2.3. cDNA Synthesis

The following protocol is adapted from the Superscript II cDNA Synthesis Kit (Invitrogen) protocol and produces cDNA that is suitable for real time PCR experiments. A standard volume of 2µl for each sample was used for the synthesis. Firstly 2µl of RNA template was mixed with 1 µl of the oligo(dT) primer and 1 µl of 10mM dNTP mix in a 0.5mL eppendorf tube and was incubated at 65°C for 5 minutes.

Samples were then placed on ice for 2-3 minutes and then each sample was mixed gently with the Master RT mix. The volumes of buffers is given in Table 3.2

The mixture was incubated at 50°C for 50 minutes. To terminate the reaction, the mixture was heated at 85°C for 5 minutes and put on ice. Finally 1µl of RNAse H was added and the sample was incubated at 37°C for 20 minutes and stored at -20°C.

Table 3.2 Volume of buffers in 10 µl Master RT mixture for cDNA synthesis reactions

Master RT mixture	Volume
10 x RT Buffer	2µl
25 µM $MgCl_2$	4µl
0.1 M DTT	2µl
RNase out	1µl
Superscript III	1µl

3.3. DESIGN PRIMERS FOR TARGET XTH GENES

The programs that were used to design and validate primers for Q-PCR are Primer3 and NetPrimer. Due to a High Performance Liquid Chromatography (HPLC) purification step required for template-standard purification, the PCR product could be in the range of 80-400bp but ideally should be between 150-300bp.

There are several parameters within the Primer3 program that were changed to optimise the primer design process (product size range, primer Tm values, Max Self Complementarity etc.)

NetPrimer was used to predict primer dimers and hairpin loops.

Primers to target individual barley XTH genes were checked by using the Primer Basic Local Alignment Search Tool (BLAST) online software of National Center for Biotechnology (NCBI) and validated primers were ordered from Invitrogen to be synthesized at 50 nmoles. The primers designed for this study are listed below in Table 3.3.

Table 3.3. Designed Primer List for control and all known barley XTH genes

Target XTH Gene	Forward Primer	Reverse Primer
HvHSP70	CGACCAGGGCAACCGCACCAC	ACGGTGTTGATGGGGTTCATG
HvActin	GTCTTTCCCAGCATTGTAGG	CGACACGGAGCTCATATAGAA
HvGAPdH	GTGAGGCTGGTGCTGATTACG	TGGTGCAGCTAGCATTTGAGAC
HvCyclophilin	CCTGTCGTGTCGTCGGTCTAAA	ACGCAGATCCAGCAGCCTAAAG
HvTubulin	AGTGTCCTGTCCACCCACTC	AGCATGAAGTGGATCCTTGG
HvXET1	GACGCAAGTGATGATGATGATG	GCAAAAATGTACATAGACGGGAG
HvXET2	GCCGCAACTGATCCATCATCAC	ACATACATAACCGATCGATCAC
HvXET3	AGCAAGGTAGCATTGGACCGTC	GAAGAACAGAACGAGATGAGAC
HvXET4	AAGCTCCGCTGATGAGAATGGG	ACTTGCATAAGTACGTACATGG
HvXET5	GTCAGGAAGGAGCACACCATC	GCAATCAGAAGAATAATAAAG
HvXET6	CGCCAGCAAGTAGAATGTC	ATCCATATACGTAGGCATAGAAA
HvXET7	GCCCTTCCGTTCCAGTGATG	CAGTACGTGTCACAAATAGCAATAA
HvXET8	GACCGAGGTTCGTTCATCTATC	ACCCGCCGCATGAAAAGTC
HvXET9	CTTCCCCGCCGAGTGCT	GGAATCAAGAACAAATAGACCAACAA
HvXET10	GCCCTTCCGTTCCAGTGATG	TCAAACGAGCTGTGCGTATTGTC
HvXTH11	TGGACCGCGTATCCGTTGAT	TGGGCGTAAATGACGAACAGAA
HvXTH12	AGCATTGGACAGCCCGTGTA	CGTGAGCGTGATCGTGACAAA
HvXTH13	GGCATTCACCCATTGATT	GTACTATTGTTGTATCCGAAAGG
HvXTH14	TGGGCCAGGAGGAACTACAT	CAATCCGTCTCGGCAACAA
HvXET15	GCAGCAACAGCAAGTGAAGG	TGTTCCAAAAGAATGGGCTAAA
HvXTH16	TGTTTTGTTCCGTCGGCCATA	TTGCTACGCCTACACGTCACATC
HvXET17	GCGAGTGAGCGACGAATGT	CACCCGACCTACGAAGAAGT
HvXTH18	TCCTAGCTCTGATCCATCCACAC	TCTACAAACAATCGCCCACACA
HvXTH19	CATGGGAAGGAATCTTGAT	CAAGGCAGAGATAGCAAAC
HvXET20	GCACCGAGGATGTACAGAG	GGATGGTCCAAGGAGACAATA
HvXTH21	TTTCGTCGGTGTGTTTGCTTGT	AGGCATTCCAGCAGCGGTAG
HvXET22	GCTGGATGACGCTCAACT	GCTACGCAGGAACGAACT
HvXTH23	GGCCATGAAGATCCTTTCGTTTT	GGGAGGCCGGAATCAGAAAT
HvXTH24	CTGCGCTATACGATGATCCATGA	TCGATCAGGCCAGAAGAGAATG
HvXET25	GCTGCCTGATCGTGGAA	CGGGGAGCAACTAGAATC
HvXTH26	GGATCGGACGGACCACAAG	CACACAAAACCAAACAGATCCATCA
HvXET27	CAGCTACACTACTTAGACTGGTTG	AAATGATGTGTTATTATGTGTCTTG

3.4. HPLC STUDIES

PCR products (100 µL each) was produced for HvXTH1, HvXTH2, HvXTH3, HvXTH4, HvXTH7, HvXTH8, HvXTH9, HvXTH11, HvXTH12, HvXTH13, HvXTH14, HvActin, HvTubulin, and HvGapdH. These HvXTH genes were chosen because they are all represented on the Affymetrix Barley1 microarray chip and could be used to compare Q-PCR and microarray experiments. Four independent 25µL PCR reactions were combined, and purified by HPLC on an Agilent Varian PLRP-S 1000 Å, 5µm, reverse phase column (Agilent Technologies, Palo Alto; CA). Chromatography was performed by using a gradient of Buffer A and Buffer B, where Buffer A contains 100 mM triethylammonium acetate with 0.1 mM EDTA, and Buffer B contains 100 mM triethylammonium acetate, 0.1 mM EDTA, and 25 % acetonitrile. The gradient was applied at a flow rate of 0.2 mL/ min at 40 ºC as follows: 0 to 30 min. with 35 % (w/v) buffer B, 30 to 31 min. with 70 % (w/v) buffer B, 31 to 40 min. with 35 % (w/v) buffer B and after 40 min. with 35 %(w/v) buffer B. The DNA was detected using a UV/Vis diode-array spectrometer at 260nm. The purified products were quantified by comparison of the peak area with the areas of a pUC19/HpaII digest of known amount. From these data, an average value for nanograms per unit area of a peak was calculated. This value was used to determinate the mass of the purified PCR product. The product was dried, and then dissolved in water to produce a 20 ng/µL stock solution. An aliquot of this solution was diluted to produce a stock solution containing 10^9 copies of the PCR product per µL. A dilution series was prepared from the 10^9 copies covering 10^7, 10^5, 10^3, and 10^1 for each gene. Three replicates of each of the four standard concentrations were used with every Q-PCR experiment, together with one no-template control. A pUC19/HpaII standard digest produces 13 different fragments, but due to two fragments having identical sizes (34 bp), two fragments separated by only 1 base pair (110 bp and 111 bp), and the two largest fragments (489 bp and 501 bp) co-eluting, only 10 peaks were observed in the chromatogram which are showed in

Table 3.4. Expected pUC19/HpaII digest fragments for each peak. This result represents the standard for each HPLC study. In this study, the 5th, 6th, 7th and 8th peaks for each injection were used to calculate the average units of peak area per ng of DNA

	Fragment Position on Plasmid (bp)	Fragment Size (bp)	Fragment Peak Number
1	2234-48	501	10th peak
2	525-1013	489	10 th peak
3	1377-1780	404	9 th peak
4	83-413	331	8 th peak
5	1992-2233	242	7 th peak
6	1187-1376	190	6 th peak
7	1014-1160	147	5 th peak
8	414-524	111	4 th peak
9	1882-1991	110	4 th peak
10	1850-1881	67	3 rd peak
11	49-82	34	2 nd peak
12	1781-1814	34	2 nd peak
13	1161-1186	26	1 st peak

3.5. Q-PCR STUDIES

The SYBRgreen real time pcr method was used to detect the expression levels. All Q-PCR experiments were done using a BioRad iCycler Q-PCR detection system. c-DNAs were mixed with primers and SYBR Premix Ex Taq (INVITROGEN) in a final volume of 20 µl.

In this thesis Q-PCR experiments were performed in duplicate; one with HPLC purified PCR products and one without HPLC purification.

The barley glyceraldehyde-3-phosphate-dehydrogenase (HvGAPDH) gene was chosen as the house-keeping gene for normalization of the data.

Table 3.5. Q-PCR conditions of primers per reaction where annealing Tm changes at second step of cycle two per reaction

Q-PCR PROTOCOL		
Cycle: (1X)		
Step 1:	93.0 °C	for 03:00
Cycle 2: (40X)		
Step 1:	93.0 °C	for 03:00
Step 2:	58.0 °C	for 00:30
Step 3:	72.0 °C	for 00:40
Cycle 3: (1X)		
Step 1:	72.0 °C	for 10:00
Cycle 4: (90X)		
Step 1:	58.0 °C	for 00:12
Cycle 5: (1X)		
Step 1:	4.0 °C	hold

Q-PCR conditions of primers per reaction is shown on Table 3.5. Annealing Tm changes at second step of cycle two per reaction is shown on Table 3.6.

Table 3.6. Q-PCR annealing Temperature (Tm) conditions of primers per reaction. Annealing Tm changes at second step of cycle two per reaction

Target Gene	Annealing Tm
HvXTH1	58 Cº
HvXTH2, HvXTH3, HvTubulin, HvActin	51 Cº
HvXTH8, HvXTH7, HvXTH11, HvXTH12	53 Cº
HvXTH4, HvXTH13	49 Cº
HvGapdH	50 Cº

4. RESULTS and DISCUSSION

4.1. RESULTS

4.1.1. Sample Preparation Results

All samples were prepared by following the instructions given in the materials & method in section 3.1. Barley cultivar Sloop seedings were prepared by following the timetable in Table 4.1.

Table 4.1. Barley(*cultivar Sloop*) sample preparation timetable for this study. Growth initiation and sampling dates are reported to get each c-DNA which is expected to be the same as Microarray studies

Target cell type	Growth start date (post-imbibition)	Sampling date
Coleoptile day 1	14/03/2010	15/03/2010
Coleoptile day 2	14/03/2010	16/03/2010
Coleoptile day 3	14/03/2010	17/03/2010
Coleoptile day 4	14/03/2010	18/03/2010
Coleoptile day 5	14/03/2010	19/03/2010
Coleoptile day 6	14/03/2010	20/03/2010
Root	08/07/2010	20/07/2010
Crown	08/07/2010	20/07/2010
Leaf	08/07/2010	20/07/2010
Bracts	27/08/2010	22/09/2010
Pistil	27/08/2010	22/09/2010
Anthers	27/08/2010	22/09/2010
Caryopsis	27/08/2010	04/10/2010

4.1.2. cDNA preparation Results

RNA extraction was performed as stated in the materials & method in section 3.2.1 with an additional step to remove any contaminating DNA for all tissue types and samples.

4.1.2.1. RNA extraction & Treatment to remove DNA results

For coleoptile samples the average values were shown in Table 4.2 for determination of the Total RNA levels.

Table 4.2. Average values of each coleoptile tissues from which the c-DNA was prepared

	AVERAGE LENGTH	AVERAGE WEIGHT	Total RNA Concentration (ng/ml)
COLEOPTILE DAY 1	1.5 mm	84,3 mg	1225
COLEOPTILE DAY 2	1,9 mm	80 mg	1383
COLEOPTILE DAY 3	2.9 mm	99 mg	517
COLEOPTILE DAY 4	3.2 mm	128 mg	259
COLEOPTILE DAY 5	3.1 mm	100,05 mg	146
COLEOPTILE DAY 6	2.9 mm	99,5 mg	75

The purity of each sample was analyzed both by running on a normal agarose gel (1.6 %) and also by spectrophotometer reading showed at Table 4.3 in order to confirm that there was no degradation, and to determine the total RNA concentration

Agarose gel results for each tissue are shown in Figure 4.1 where a sharp doublet of bands was expected (28S and 18S rRNA subunits).

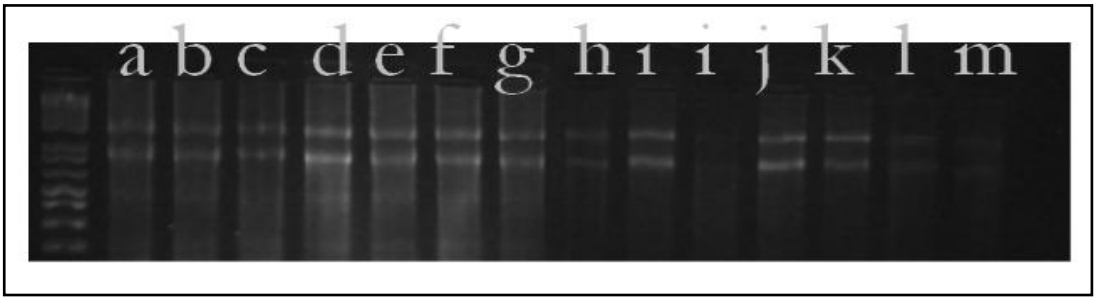

Figure 4.1. Agarose gel electrophoresis of each RNA sample after the RNA extraction step. The samples are as follows; a- Coleoptile day 1, b- Coleoptile day 2, c- Coleoptile day 3, d- Coleoptile day 4, e- Coleoptile day 5, f- Root, g- Crown, h- Coleoptile day 6, ı- Leaf, i- Pistil, j- Bracts, k- 10 DAP, l- Anther

Table 4.3. Spectrophotometer results of each RNA sample after the RNA extraction step

Target cell type	Concentration (ng/ml)	A260/A280	A260/A230
Coleoptile day 1	982	1.972	1.564
Coleoptile day 2	1516	2.016	2.043
Coleoptile day 3	486	1.898	1.687
Coleoptile day 4	358	1.904	1.078
Coleoptile day 5	226	1.982	1.712
Coleoptile day 6	120	1.939	1.332
Root	584	2.116	2.042
Crown	1358	2.089	1.991
Leaf	662	1.505	1.351
Bracts	242	1.921	1.198
Pistil	95	41.0	-1.185
Anther	392	1.620	0.544
Caryopsis	322	1.602	2.127

Table 4.4. Results for each primer that is designed for each target gene

Target XTH Gene	Basic Tm	PCR Results	PCR Length	Used for HPLC Study	Q-PCR Results	Microarray Data equivalent
HvHSP70	51 °C	No band	108 bp	Not used	Non-specific product	No
HvActin	50 °C	Expected band	201 bp	Used	Non-specific product	No
HvGAPdH	50 °C	Expected band	198 bp	Used	Q-PCR product	No
HvCyclophilin	53 °C	Expected band	122 bp	Not used	Q-PCR product	No
HvTubulin	50 °C	Expected band	248 bp	Used	Q-PCR product	No
HvXET1	51 °C	Expected band	202 bp	Used	Q-PCR product	Yes
HvXET2	49 °C	Expected band	247 bp	Used	Q-PCR product	Yes
HvXET3	51 °C	Expected band	213 bp	Used	Q-PCR product	Yes
HvXET4	49 °C	Expected band	238 bp	Used	Q-PCR product	Yes
HvXET5	45° C	Expected band	256 bp	Used	Non-specific product	Yes
HvXET6	50° C	Expected band	102 bp	Not used	NA	Yes
HvXET7	53° C	Expected band	191 bp	Used	Q-PCR product	Yes
HvXET8	53° C	Expected band	120 bp	Used	Q-PCR product	Yes
HvXET9	53° C	Expected band	132 bp	Used	Q-PCR product	Yes
HvXET10	53° C	Expected band	154 bp	Not used	NA	No
HvXTH11	53° C	Expected band	151 bp	Used	Q-PCR product	Yes
HvXTH12	53° C	Expected band	167 bp	Used	Q-PCR product	Yes
HvXTH13	45° C	Expected band	176 bp	Used	Non-specific product	Yes
HvXTH14	51° C	Expected band	227 bp	Used	NA	Yes
HvXET15	49° C	No band	173 bp	Not used	Non-specific product	Yes
HvXTH16	50° C	No band	157 bp	Not used	NA	No
HvXET17	53° C	Expected band	205 bp	Not used	NA	No
HvXTH18	53° C	Expected band	152 bp	Not used	NA	No
HvXTH19	45° C	Expected band	146 bp	Not used	Non-specific product	Yes
HvXET20	52° C	Multiple Bands	222 bp	Not used	NA	No
HvXTH21	52° C	Multiple Bands	140 bp	Not used	NA	Yes
HvXET22	49° C	Good result	156 bp	Not used	NA	No
HvXTH23	53° C	Wrong size	189 bp	Not used	NA	No
HvXTH24	53° C	No band	168 bp	Not used	NA	No
HvXET25	49° C	No band	152 bp	Not used	Non-specific product	Yes
HvXTH26	54° C	Multiple Bands	108 bp	Not used	NA	No
HvXET27	49° C	No band	162 bp	Not used	Non-specific product	Yes

4.1.2.2. cDNA results

Each template is checked after the cDNA preparation step. Figure 4.2 shows agarose gel electrophoresis results of some barley genes.

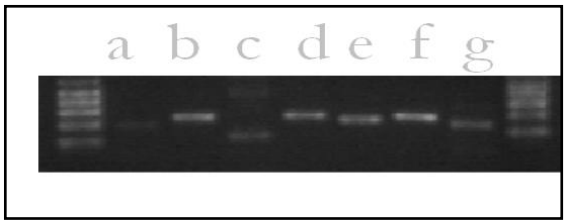

Figure 4.2. Agarose gel electrophoresis of cDNAs for some tissues. a. HvActin, b. HvTubulin, c. HvHsp70, d. HvXTH4, e. HvXTH3, f. HvXTH2, g. HvXTH9

4.1.3. Designed primers for target HvXTH genes

We designed 27 primer pairs in order to study the expression levels of 27 Barley XET genes. The success of the primers at optimum Tm for each gene is shown in Table 4.4.

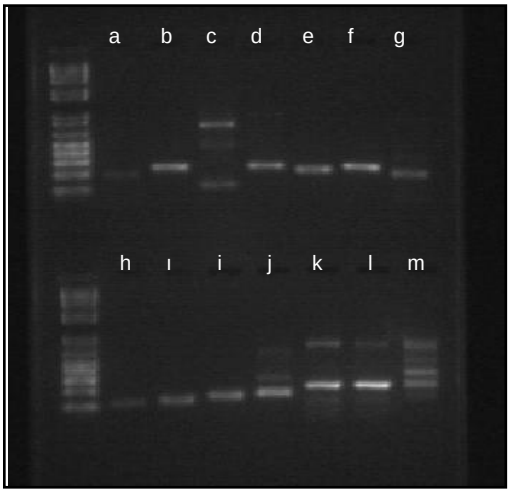

Figure 4.3. Agarose gel results of samples used at HPLC purification step a-HvActin, b-HvTubulin, c-HvCyclophylin, d-HvXTH2, e-HvXTH3, f-HvXTH4, g-HvXTH7, h-HvXTH8, ı-HvXTH9, i-HvXTH11, j-HvXTH12, k-HvXTH14, l-HvXTH14, m-HvXTH19

4.1.4. HPLC Study Result

In our study, we calculated the average value of three pUC19/HpaII HPLC injections. For each injection we have 10 peaks which are also showed at Table 3.4. HPLC injection result for pUC19/HpaII digestion is also shown on Table 4.5.

Table 4.5. Average area value results for pUC19/HpaII digestion that indicates our standart

PEAK	1st. Inj.	2nd. Inj.	3rd. Inj.	Average Value for 2 µL Injection	Average Value for predicted 30 µL Injection
5	89593	95130	48294	77671	1165065
6	124911	132870	86385	114722	1720830
7	164617	173822	86774	141737	2126055
8	227854	241957	110306	193372	2900580

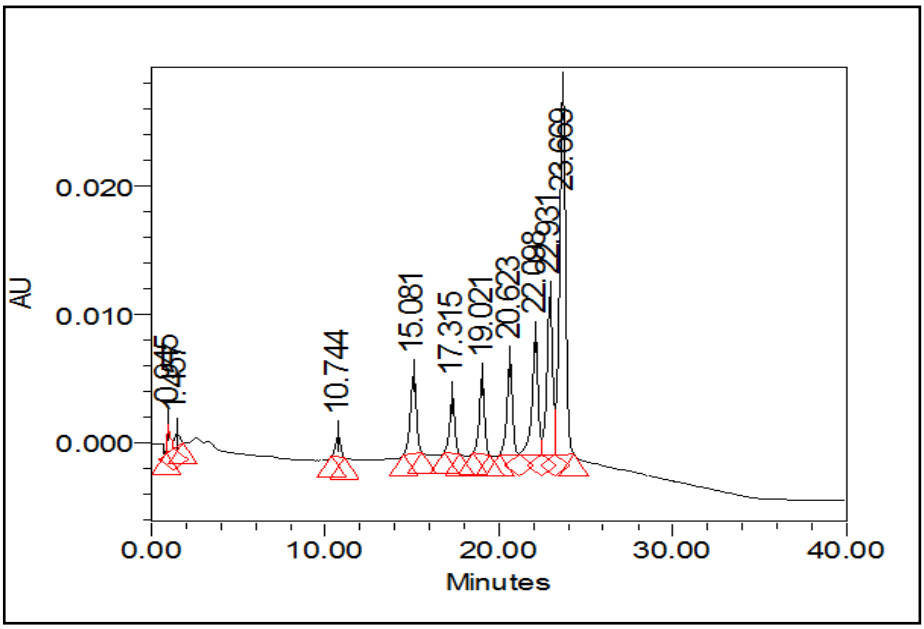

Figure 4.4. Chromatogram of pUC19/HpaII digestion that is used as the standard for each PCR product

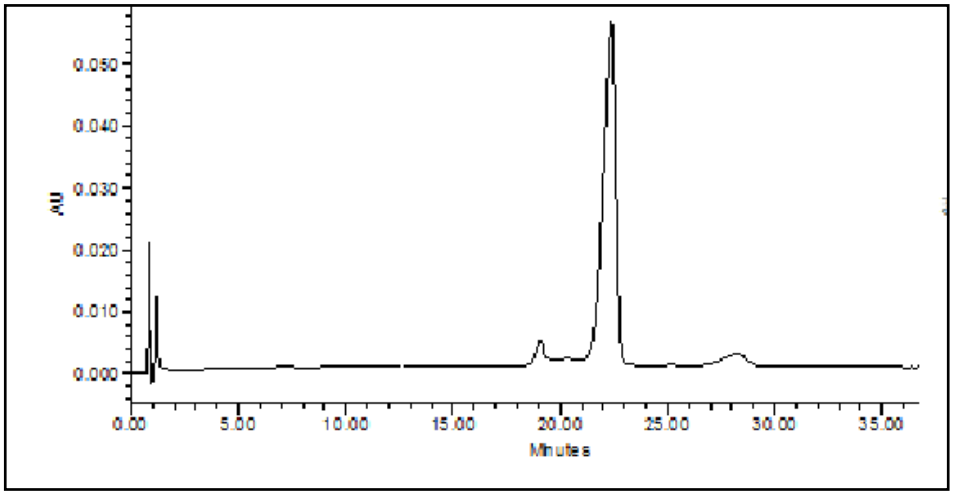

Figure 4.5**.** Chromatogram of HvXTH3 where the expected single peak was at 22.391 minute. The sample was started to collect between 21,5 and 21 minutes to get purified HvXTH3 standard

All the calculations were done according to the units/peak area method that matches with the standard. As an example, the method used for HvXTH1 is described below.

The peak area was 1609151 for HvXTH1. For this gene, we matched the data with our standard. Calculated amount (for 30 µl) was 507,61 ng. The results for all genes is shown in Table 4.6. We multiplied this data by two because we did two 30 µl HPLC injections and collected both peak fractions. For HvXTH1 the dried amount of DNA was 1015 ng (507,61 ng * 2 = 1015 ng). After that we dissolved the DNA in 50,75 µl of dH_20 to give a final product concentration of 20 ng / µl.

For the next step we used the http://molbiol.edu.ru/eng/scripts/01_07.html web site to get a calculation for 10^9 copies of template per µl. We added 89,9 µl dH_20 to have 10^9 copies for the HvXTH1 template stock. Dilution series to make up 4 standarts (10^7, 10^5, 10^3, 10^1 copies) were set up from this template stock.

The results and calculations for all HPLC injections are summerized in Table 4.6.

Table 4.6. The results and calculations for all HPLC injections that will be used in the Q-PCR amplification step

	HPLC	Q-PCR Prod. Length	Peak area (mv*sec)	Calculated Amount(dried out)
HvXTH1	Yes	202 bp	1609151	1015 ng
HvXTH2	Yes	247bp	1669236	1053 ng
HvXTH9	Yes	132bp	758733	449,7 ng
HvXTH8	Yes	120bp	265027	157,08 ng
HvXTH4	Yes	238bp	1051031	663,10 ng
HvXTH3	Yes	213bp	2160082	1363 ng
HvXTH7	Yes	191bp	400313	237 ng
HvXTH12	Yes	167bp	1306467	774,3 ng
HvXTH13	Yes	177bp	462387	274,06 ng
HvXTH11	Yes	151bp	2191872	1391,78 ng
Hv GapdH	Yes	198bp	960272	609,74 ng
Hv Actin	Yes	201bp	574091	364,52 ng
Hv Tubulin	Yes	248bp	2794243	1774 ng
HvXTH14	Yes	227 bp	4596848	2880 ng

4.1.5. Q-PCR Results

As was mentioned in the methods chapter, Q-PCR studies were done in two parts. Firstly, Q-PCR studies were done with all target primers and control genes. The results are summarized in Table 4.7.

These results indicate that 3 out of 5 of the control genes could be used as reliable housekeeping genes. For HvHsp70 and HvActin no reliable Q-PCR results were obtained, so they were discarded from further study. Our next target was to use these 3 control genes for purification step by HPLC. But for some unknown reasons only HvGapdH gave the expected HPLC results. Thus normalization was processed only with respect to HvGapdH levels, but the Ct levels for HvTubulin and HvCyclophilin, the other two control genes, co-

varied with HvGapdH and provided a high level of confidence for normalization. It can be concluded from these data that Q-PCR is sensitive method to detect expression level Results are showed at Table 4.7.

Q-PCR results showed us copy numbers for each gene. The calculations are done sensitively and normalized to form Table 4.9. This also provided that transcript levels can be carefully normalised by using HvGapdH. As a sample, HvXTH2 Q-PCRmelt curve graph is given in Figure 4.6

Table.4.7. Q-PCR Results- The Ct(cycle threshold) Values. Results for control and XTH genes in barley. Values are non-normalized and were calculated from the dilution series of template DNA created individually for each gene

	Hv Tubulin	Hv Gapdh	Hv Cyclophylin	Hv XTH 1	Hv XTH 2	Hv XTH 3	Hv XTH 4	Hv XTH 7	Hv XTH 8	Hv XTH 9	Hv XTH 11	Hv XTH 12	Hv XTH 19
Coleoptile Day 1	19,1	19,1	19	28,8	25,75	23,4	19	34,4	31,7	23,9	26,2	25,7	26,15
Coleoptile Day 2	19	19,85	19,9	24,2	23,4	18,9	18,5	28,5	30,8	23,3	22,6	23	28,8
Coleoptile Day 3	23	24,2	20,8	23,85	22,9	21,5	19,8	29,8	28,7	27,2	25	25,3	35,8
Coleoptile Day 4	24	24	24,15	32	28	23,4	28,6	32,8	27,9	30,7	27,6	28,3	37,1
Coleoptile Day 5	19,3	20	20,95	28,8	27,5	22	22	29	30,3	24,3	23	24,5	33,6
Coleoptile Day 6	25,7	26,75	24,5	32,9	31	24,5	30,5	33	30,2	29,3	28,2	26,2	32,6
Bracts	30	30,3	30,6	37,25	34,6	25,4	33,1	37,8	35,1	33,3	31,4	28	28,3
Root	22,5	22,15	21,7	27,6	24,1	24,7	25,3	30	27,9	26,6	29,3	28,6	28,5
Caryopsis	20,6	17,2	17,8	24,4	24,6	24	25,9	26,6	36	25,3	25	26,3	24,4
Anter	23	19,5	23,5	32,8	22	26,6	31,9	33,7	32,25	29,6	30	30,5	35,3
Pistil	26,3	26,5	22	30,2	33	26,4	29,5	34	31,5	29,3	27	28,8	26,5
Crown	20,3	21	18	26,7	26	22,5	24,5	28,1	23,6	25,7	23,3	25,6	22,9
Leaf	25	24,5	25,5	29	28,95	24,6	27	36	33,5	29,1	27,9	27	28,5

Table 4.8. Raw Q-PCR Results for control and XTH genes in barley. Values are non-normalized and were calculated from the dilution series of template DNA created individually for each gene

	HvGapdH	HvXTH 1	HvXTH 2	HvXTH 3	HvXTH 4	HvXTH 7	HvXTH 8	HvXTH 11	HvXTH 12
Coleoptile Day 1	100000	6309	794	229	354813	4466	19	794	5011
Coleoptile Day 2	19952	2512	2512	69183	4570881	190546	316	10000	39810
Coleoptile Day 3	501	5012	1258	10000	151356	100000	31	3981	10000
Coleoptile Day 4	630	251	158	125	851	13803	56	501	501
Coleoptile Day 5	15848	1585	316	630	1348962	190	1000	15848	10000
Coleoptile Day 6	316	630	18	63	19952	21877	177	398	3162
Bracts	3	794	6	31	398	912	19	39	1584
Root	1995	501	1737	28	15848	100000	1258	199	1000
Caryopsis	1584893	794	2511	173	199526	524807	1584	5011	5011
Anter	316227	1258	50	18	12589	7943	1258	50	79
Pistil	100	501	14	11	19952	7943	50	630	630
Crown	316227	630	1000	4466	562341	100000	11220	15848	3981
leaf	31	562	100	26	35481	3162	22	251	794

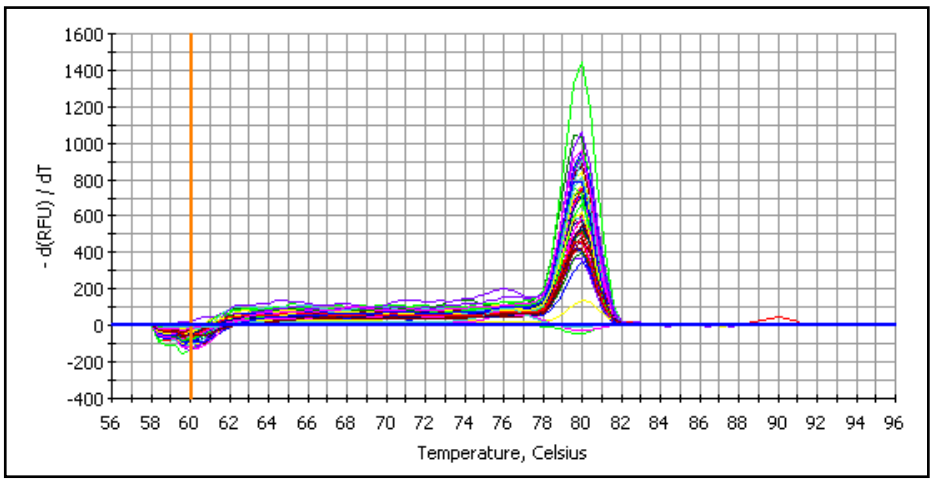

Figure 4.6. HvXTH2 Q-PCR melt curve graph

Table 4.9. Normalised Q-PCR Results for control and XTH genes in barley. Values were calculated from the dilution series of template DNA created individually for each gene and then normalized with respect to HvGapdH

	HvGapdH	HvXTH 1	HvXTH 2	HvXTH 3	HvXTH 4	HvXTH 7	HvXTH 8	HvXTH 11	HvXTH 12
Coleoptile Day 1	3625720	228746	28800	8306	12864540	161940	688	28800	118170
Coleoptile Day 2	3625720	456486	456486	12572101	830630440	34626446	57466	1817221	7234489
Coleoptile Day 3	3625720	36271673	9110616	72369660	1095359094	723696600	228832	28810868	72369660
Coleoptile Day 4	3625720	1444533	912127	724511	4898406	79442645	323610	2883311	2884404
Coleoptile Day 5	3625720	362617	72347	144121	308617076	43594	228782	3625934	2287810
Coleoptile Day 6	3625720	7228492	208823	723996	228932331	251019392	2040380	4566571	36283362
Bracts	3625720	959607280	7626098	38215092	481145156	1102231027	23567182	48113307	1915455896
Root	3625720	910519	3158284	51214	28803902	181740360	2287984	362620	1817403
Caryopsis	3625720	1816	5746	397	456452	1200591	3625	11465	11465
Anter	3625720	14423	574	208	144342	91074	14434	574	910
Pistil	3625720	18164857	512314	406805	723426134	288001091	1817204	22842021	22876843
Crown	3625720	7223	11465	50134	6447558	1146556	128640	181716	45645
Leaf	3625720	65730789	11695870	3076013	4149851400	369856157	2618705	29356633	92903804

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4.2. DISCUSSION

XTHs play complex and critical roles in growth and development in various plant species. In this thesis, the expression patterns of various HvXTH genes was investigated to elicudate where and when HvXTHs appear in action. These results may give clues about physiological processes for specific barley XTH genes.

The expression patterns revealed by Q-PCR in a wide range of barley tissues are suggestive of functional links among individual XTH genes.

We first analyzed the RNA levels of each coleoptile. RNA levels of each barley coleoptile was similar to that found by Gibeaut *et al.* [139]. Gibeaut et al. mentioned that for barley coleoptiles the most rapid growth phase was day 3, which is supported by our results. The total RNA curve was also similar with our study.

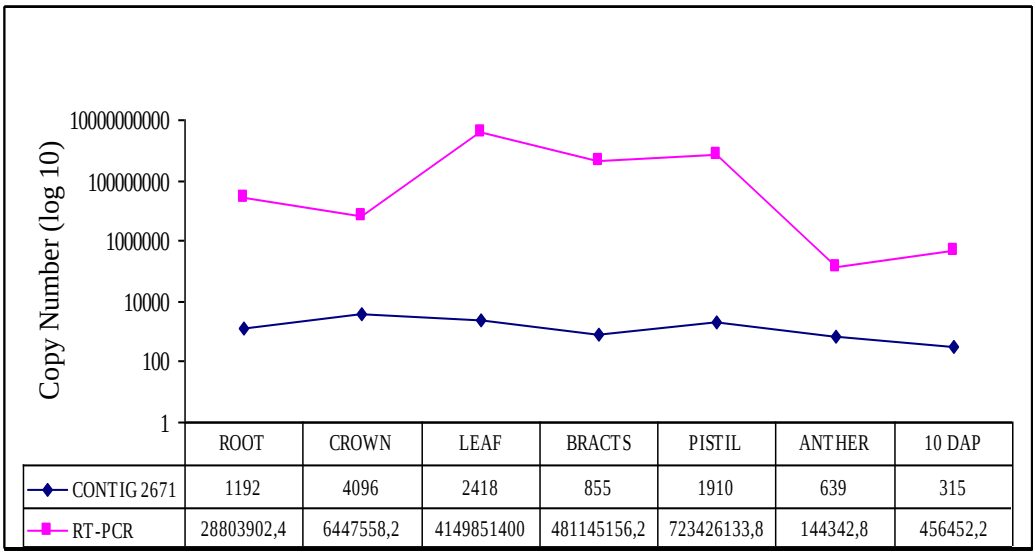
In many studies, it is reported that XTH activity levels are high in rapid growing tissues. Fry *et al*. reported that in both grasses and dicot systems, the highest XTH activity levels were associated with regions of cell elongation. In our study the highest XTH activity is reported for young coleoptiles, root and leaf tissues all of which are undergoing rapid elongation.

In this study, we also compared our normalised HvXTH genes with microarray sequences identified here by their Affymetrix probe ID. For each gene, the BLAST program from www.plexdb.org database was used for selection of the corresponding probe and the results are given in the appendix. (Appendix A)
The values of the microarray results from different barley tissues were added into the appendix (Appendix B). All comparable result tables are added (3 at Discussion and 5 at Appendix C.)

For HvXTH12, in a direct comparison between Q-PCR and microarray results, we observed similar expression levels for most of the tissues. However for roots the results were quite different. (Appendix C).

The results are also similar for HvXTH7. For some unknown reason, the pistil tissue results did not match between the two studies, with high levels of HvXTH7 detected by Q-PCR but a low result being reported by microarray. This is curious given that the levels are relatively similar for the other HvXTH genes studied. Barley HvXTH7 expression levels are high for root, bracts and leaf in both studies (Appendix C). The differences may be indicative of varietal difference, where cultivar Morex, a six-rowed barley, was used for the microarray experiments and cultivar Sloop, a two-rowed variety, was used for the Q-PCR work.

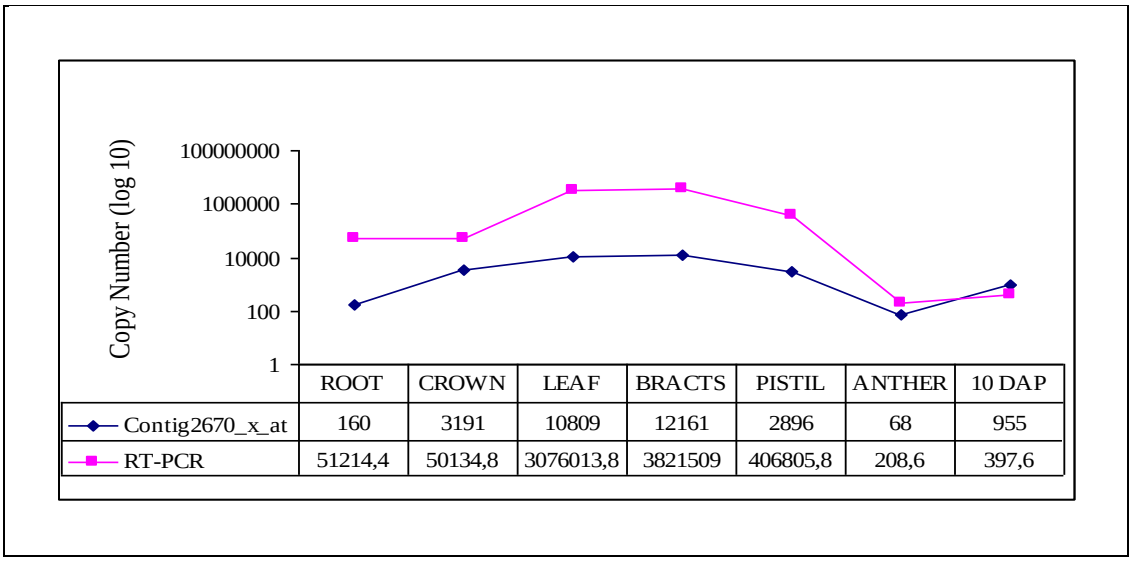
XTH expression levels are quite similar for HvXTH12 in both studies (Appendix C). This gene is expressed at high levels at pistil, leaf and bracts in all studies. Hrmova *et al.* suggest that covalent linkages between different polysaccharides do occur in plant cell wall [117]. Hrmova et al. showed that an XET from barley seedlings can catalyze the formation of covalent linkages between xyloglucans and cellulosic substrates and between xyloglucans and (1→3)(1→4)-β-D-glucans. The high expression levels of HvXTH11 and HvXTH8 could be for wall rigidity and strength due to the covalent linkages as Hrmova *et al.* suggest [117].



	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
CONTIG 2671	1192	4096	2418	855	1910	639	315
RT-PCR	28803902,4	6447558,2	4149851400	481145156,2	723426133,8	144342,8	456452,2

Figure 4.7. Comparison of expression levels of HvXTH4 in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels

Our Q-PCR results are comparable with the microarray data for HvXTH4 like in Figure 4.7, because expression levels are nominally the same and the levels are obviously high for crown, leaf, pistil and root in two studies. However the different values could be because of the variations in the two studies. HvXTH4 expression levels are very low for anther and 10 DAP caryopses in both studies. In our study, the highest level was identified for leaf however this was found to be crown by the icroarraymicroarray study. This could be because of margin of error in the sampling protocol. In our study, the highest leaf, coleoptile and crown expression levels are detected at HvXTH4. HvXTH4 appears to have fundimental functions during elongation. Gibeaut *et al.* mentioned that (1→3)(1→4)-β-D-glucan levels in barley coleoptile walls increases during elongation and then rapidly declines during maturation. [139] et al.They also reported that the relative abundance of (1→3)(1→4)-β-D-glucan increases to about 10 mol % of the wall material during the elongation phase of coleoptile and thereafter decreases to much lower levels [139]. Gibeaut *et al.* also detected xyloglucans at 8-12 % in barley coleoptile walls during all stages of development [139]. Oppositely, we report that HvXTH4 levels doesn't decrease during maturation. This brings to mind that some (1→3)(1→4)-β-D-glucan genes may have important functions during maturation. These XTH genes appear to be coordinately transcribed both during elongation and maturation.

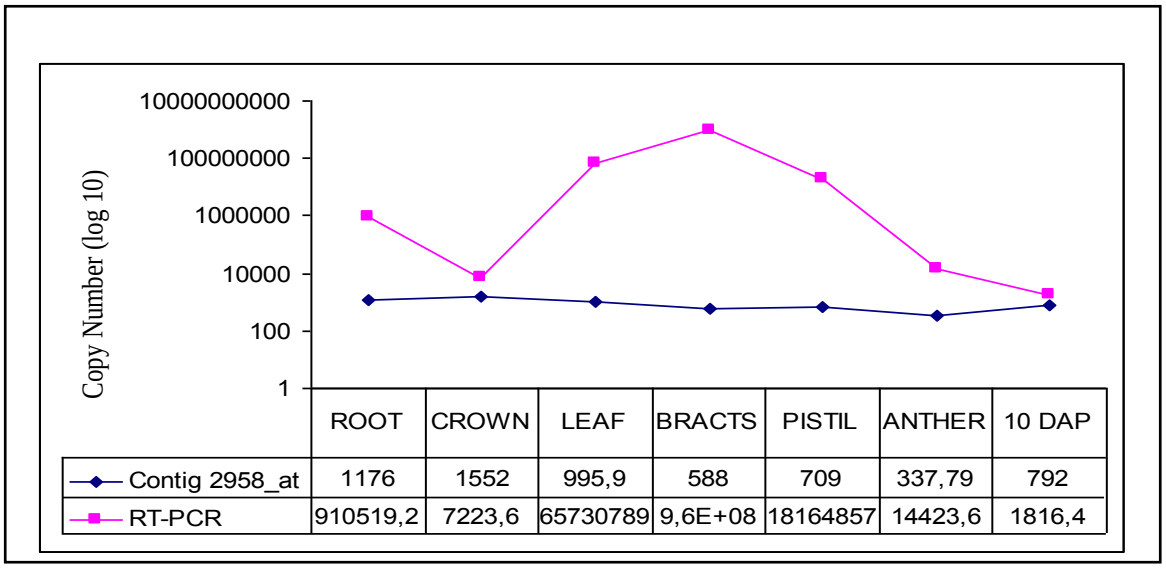


	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig2670_x_at	160	3191	10809	12161	2896	68	955
RT-PCR	51214,4	50134,8	3076013,8	3821509	406805,8	208,6	397,6

Figure 4.8. Comparison of HvXTH3 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels

XTH levels are quite high for leaf in both HvXTH2 (Appendix 3) and HvXTH3 showed in Figure 4.8 in our study. Microarray data confirmate our results. Smith *et al*. studied the regulation of XET in leaf elongation[140]. Smith *et al*. reported that non-elongation leaf tissue has XET activity which suggests that this enzyme can also have a 'housekeeping' role [140]. Smith *et al.* mentioned that different isozymes of XET might be present in the expanding barley leaf, performing different roles for elongation and maturation [140]. Smith *et al.'s* suggestion may help to explain the high expression levels for all XTH genes that were studied in this thesis.

Michael S. Schober *et al.* mentioned that the expression levels of XTH genes changes throughout segment zones for each gene [141]. Further studies could be done for these eight genes by seperating tissues into leaf base and leaf tip and also for root sections [141].



	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig 2958_at	1176	1552	995,9	588	709	337,79	792
RT-PCR	910519,2	7223,6	65730789	9,6E+08	18164857	14423,6	1816,4

Figure 4.9. Comparison of HvXTH1 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels

The results are quite different between the Q-PCR studies and the microarray for HvXTH1 as seen in Figure 4.9 and HvXTH12 (Appendix 3). For these two genes our results did not confirm the microarray data. This could be because of issues with the individual probes for these genes on the microarray where annealing temperatures were not ideal, or there may have been cross-reactivity with other gene products. Another issue may be due to varietal

differences between the barley cultivar Morex that was used in the microarray studies, and the barley cultivar Sloop that was used in our Q-PCR studies.

In summary; we have observed high levels of HvXTH4, HvXTH7, HvXTH11, HvXTH12 gene transcripts in leaf, root and coleoptiles. Transcript profiles also indicate that these 4 genes are likely to be important in the growth and development of leaf and root tissues.
From detailed analysis of transcripts, the abundance in root, leaf, bracts and coleoptile day 3 are significantly higher for all genes. Does each of these genes play a particular role or are they functionally redundant?

Nishitani *et.al*. stated that the transcription of closely related XTH genes are regulated interactively and their transcription processes are individually regulated for AtHTH17, AtHTH18, AtHTH19, AtHTH20 [142]. Nishitani *et.al*. also suggest that a specific member of this gene family, AtHTH18, plays an indispensable role in the cell elongation process of root. The results in this study may suggest that HvXTH7 has the highest activity for this 8 genes [142].

Genomic approaches of XTH gene family involved in cell wall remodeling during the devolopmental stage of barley is more efficent than it is expected. In our study, we study the expression levels of 8 XTH genes of this large family. The results of our study supports that each member of the XTH gene family has it’s own specific role in plant growth and development.

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APPENDIX A: PROBE SET OF MICROARRAY DATA COMPARED

Table A.1. Informations about the probe set of microarray data that is used to compare with target XTH genes. (plexdb database)

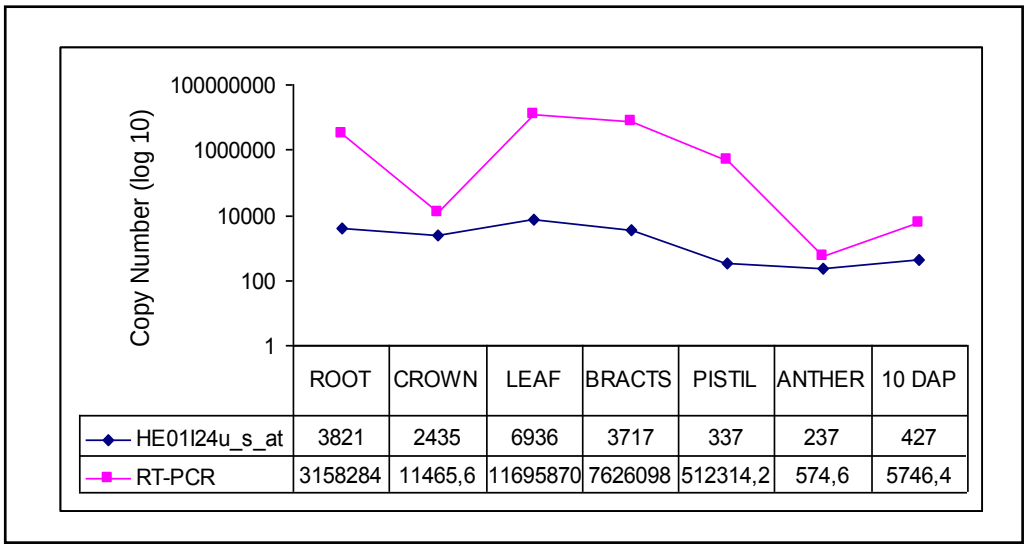
TARGET GENE	PROBE SET ID	IDENTITIES	LENGTH OF MATCH
HvXTH 1	Contig2958_at	100 %	1321
HvXTH 2	HE01I24u_s_at	94.5 %	455
HvXTH 3	Contig2670_x_at	100 %	1177
HvXTH 4	Contig2671_at	99.1 %	1237
HvXTH 7	Contig1616_at	100 %	1214
HvXTH 8	Contig7337_at	98.8 %	1286
HvXTH 11	Contig2669_at	99.4 %	1310
HvXTH 12	Contig2673_at	100 %	1191

APPENDIX B: EXPRESSION LEVELS

Table B.1. Expression levels of target genes in each tissue studied. (plexdb database)

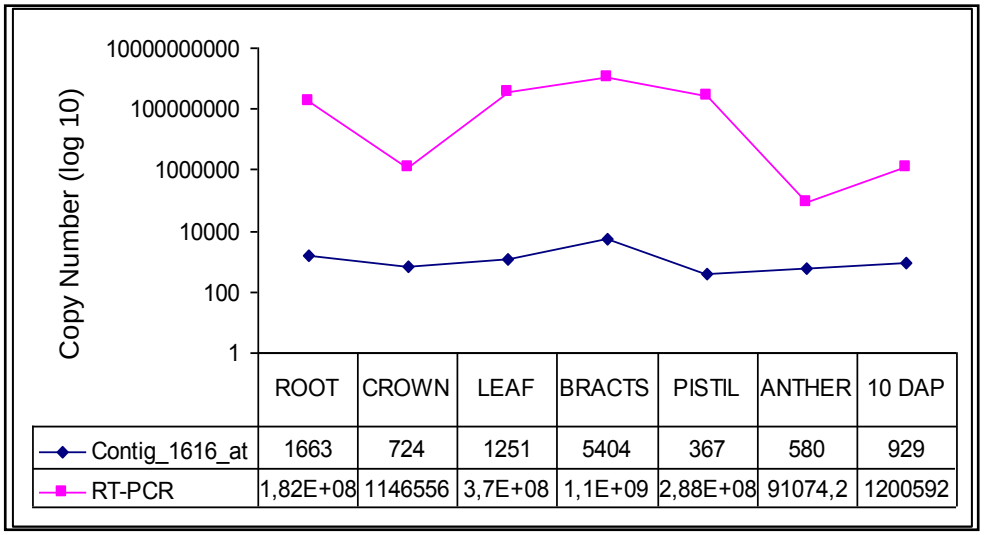
TARGET GENE	PROBE SET ID	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10DAP
HvXTH 1	Contig 2958_at	1176	1552	995,9	588	709	337	792
FHvXTH 2	HE01I24u_s_at	3821	2435	6936	3717	337	237	427
HvXTH 3	Contig2670_x_at	160	3191	10809	12161	2896	68	955
HvXTH 4	Contig2671_at	1192	4096	2418	855	1910	639	315
HvXTH 7	Contig1616_at	1663	724	1251	5404	367	580	929
HvXTH 8	Contig7337_at	284	163	388	294	137	377	58
HvXTH 11	Contig2669_at	54	357	1734	4096	2521	130	144
HvXTH 12	Contig2673_at	6,27	891,44	4096	6208	4608	128	207

APPENDIX C: Q-PCR MICROARRAY COMPARISON GRAPHS OF EXPRESSION LEVELS



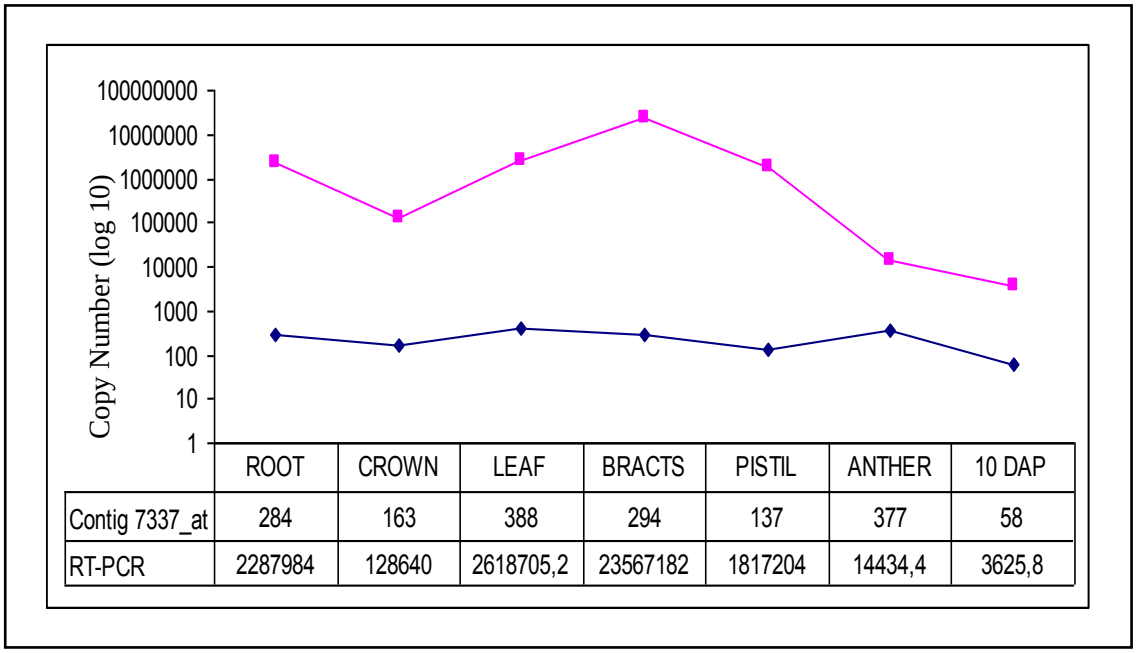
	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
HE01I24u_s_at	3821	2435	6936	3717	337	237	427
RT-PCR	3158284	11465,6	11695870	7626098	512314,2	574,6	5746,4

Figure C.1. Comparison of HvXTH2 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels.



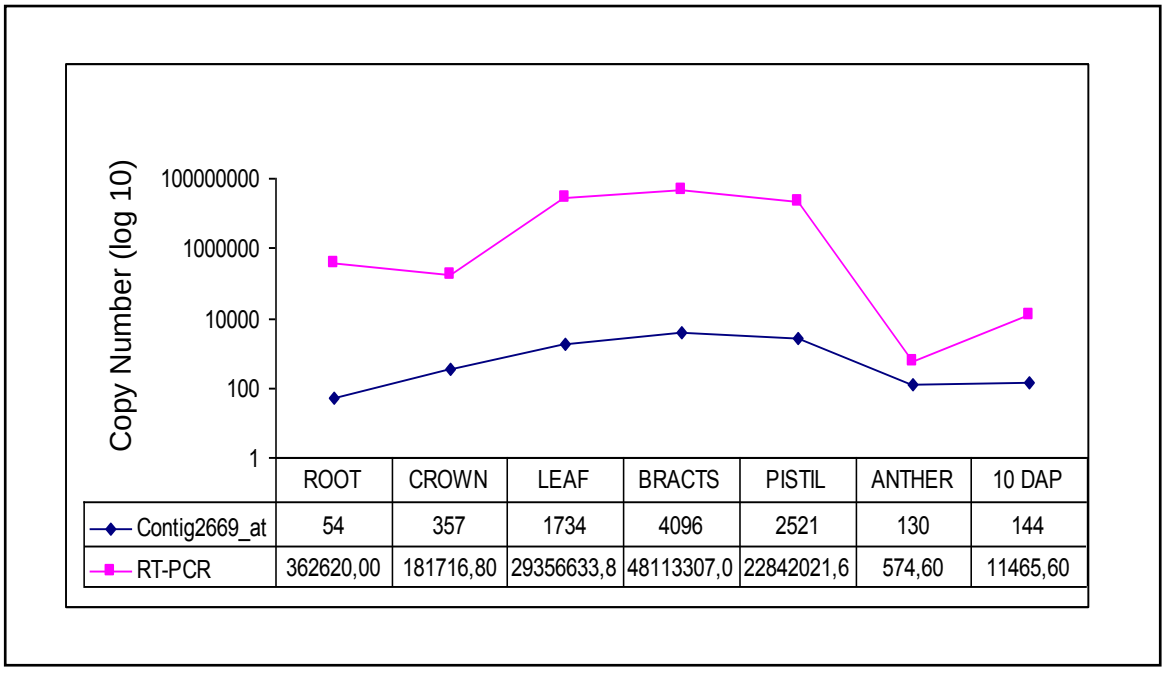
	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig_1616_at	1663	724	1251	5404	367	580	929
RT-PCR	1,82E+08	1146556	3,7E+08	1,1E+09	2,88E+08	91074,2	1200592

Figure C.2. Comparison of HvXTH7 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels



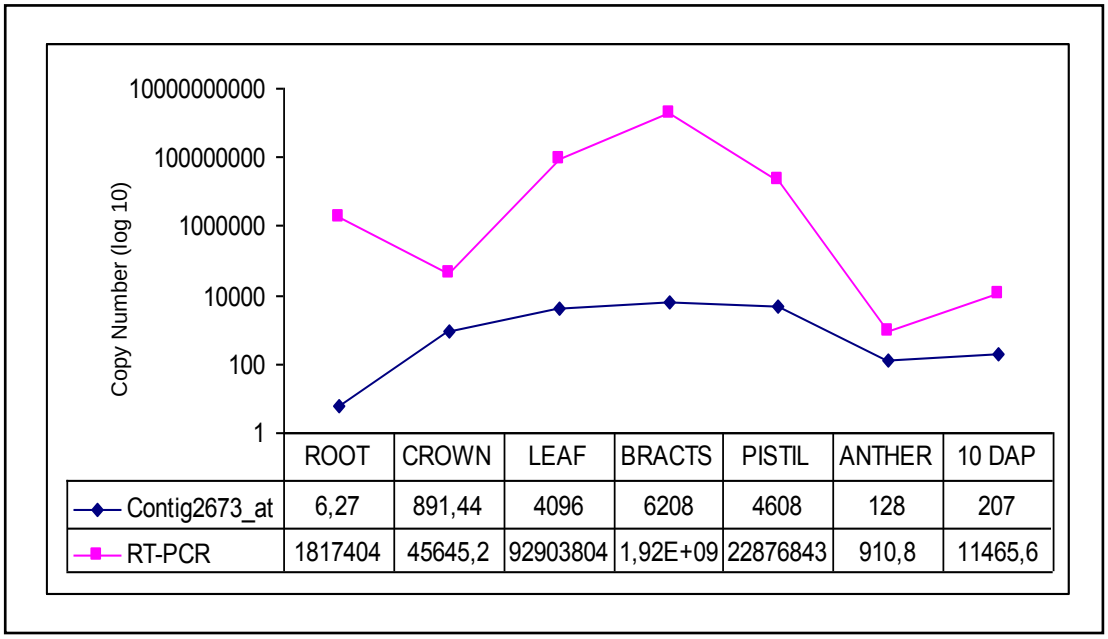
	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig 7337_at	284	163	388	294	137	377	58
RT-PCR	2287984	128640	2618705,2	23567182	1817204	14434,4	3625,8

Figure C.3. Comparison of HvXTH8 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels



	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig2669_at	54	357	1734	4096	2521	130	144
RT-PCR	362620,00	181716,80	29356633,8	48113307,0	22842021,6	574,60	11465,60

Figure C.4. Comparison of HvXTH11 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels



	ROOT	CROWN	LEAF	BRACTS	PISTIL	ANTHER	10 DAP
Contig2673_at	6,27	891,44	4096	6208	4608	128	207
RT-PCR	1817404	45645,2	92903804	1,92E+09	22876843	910,8	11465,6

Figure C.5. Comparison of HvXTH12 expression levels in both Q-PCR and microarray studies. Values are both generated and normalized in different ways between the two experiment types leading to the overall difference in levels