Cloning of Barley *COBRA* genes and study their functions

by

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Declaration

I declare that this thesis is a record of original work and contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text.

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October 2008

Dr. Qisen Zhang

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Preface

This research was performed over 10 months as part of a Masters in Biotechnology (Plant Biotechnology). The literature review was previously assessed and the research focus has not changed. Although the research manuscript contained herein will provide the first draft of a future publication in *the Plant Journal*, due to time constraint, all data relevant to that publication has not been collected. However, additional data were collected and provided within the appendices. The research manuscript presents the cloning of five *COBRA* genes and a study of the features of *COBRA* family genes. It also reports root hair phenotype observation of *Arabidopsis AtCOBRA* mutant lines. The appendices contain all the collected ESTs sequences for a future reference by people continuing the work.

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Literature Review

1. Introduction

Cell expansion is an important process of plant morphogenesis. The plant cell wall has a great effect on cell growth. The cell wall determines cell shapes and provides mechanical protection for protoplasts. Cellulose, xyloglucan and pectin are found in the primary cell wall and are the main composition of cell wall polysaccharides. Cellulose microfibrils are the primary anisotropic materials in the cell wall and may determine the orientation of cell extension. The *COBRA* (*COB*) proteins have been identified as having secondary effect on cell elongation by affecting the microfibril organization (Roudier et al., 2005). However, the mechanism how *COBRA* genes regulate microfibril organization is still not understood.

COBRA gene families have already been identified in *Arabidopsis*, maize and rice. The members of *COBRA* gene family are 12 in *Arabidopsis*, 9 in maize and 11 in rice (Brady, et al, 2007). Genetic mapping of *COBRA* genes in rice and *Arabidopsis* has been completed. However, there is little research on the *COBRA* genes in barley.

2. Plant cell wall composition

Plant cell wall can be distinguished as primary cell wall and secondary cell wall. Primary cell wall is deposited during cell growth. Primary cell wall is a dynamic structure which is made up of cellulose, cellulose-binding hemicelluloses, and pectin. The formula of cellulose is $(C_6H_{10}O_5)_n$, it is polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units. While hemicellulose is derived from several sugars in addition to glucose, including xylose, mannose, galactose, rhamnose, and arabinose. Hemicellulose consists of shorter chains branched sugar units, usually up to 400. Polysaccharide matrix which fills the cellulose microfible interface is composited by pectin

and hemicelluloses. Glucan synthase, and xyloglucan synthase are participating in these polysaccharides' formation. Cellulose is synthesized from a 1,4-linked β-D-glucose residues, by cellulose synthases which are arranged into rosette a complex, and form cellulose microfibrils which are the frames for polysaccharide matrix. In dicotyledonous and monocotyledonous plants, the principal polysaccharide interlinking the cellulose microfibrils is usually xyloglucan and arabinoxylan. Secondary cell walls are deposited after the cessation cell growth and confer mechanical stability. Lignin is also found in secondary cell walls. In addition, plant cell walls contain hundreds of different proteins, which may participate in cell wall structure modification (Reiter, 2002).

3. Previous research on the *COBRA* gene family

3.1 The function of the *COBRA* genes

According to Hauser et al., (1995), the name *COBRA* comes from the mutations of *Arabidopsis*, which have changed the orientation of cell expansion in the root, from the longitudinal axis to the radial axis, as a result, the widening roots gain the appearance of the head of a *cobra*.

COBRA proteins are required for the oriented deposition of cellulose microfibrils during rapid anisotropic expansion. Microfibril orientations are potentially regulated by the COBRA proteins, which are possibly the plasma membrane proteins that bind with microtubules and determine the microfibril orientation (Roudier et al., 2005). The effect of *COBRA* protein on cellulose biosynthesis is secondary, since it does not directly act on cellulose synthesis. The latest model suggested that microtubules influence growth anisotropy by regulating the relative length of microfibrils, and the microtubule-dependent patterning of *COBRA* could support the formation and extension of microfibrils (Wasteneys, 2004). The model which

explains the mechanism is that cortical microtubules constrain rosette complex movement by serving either as tracks or guard rails (Giddings and Staehelin, 1991; Wasteneys, 2004). Thus, the *COBRA* genes affect the cellulose synthesis by regulating the organization of microtubules. Roudier (2005) also reported the distribution of *COBRA* during cell elongation is dependent on cortical microtubule organization. Paredez et al. (2006) have fluorescently labelled cellulose synthases (CesAs) which assemble into rosette complexes in *Arabidopsis* hypocotyls cells. These rosette complexes move in the plasma membrane along tracks defined by the co-labelled microtubules. Double labelling of *COBRA* and cortical microtubules in elongating cells has further confirmed the similar orientation of cortical microtubules and the *COBRA* proteins (Roudier et al., 2005).

The model proposed by Wasteneys(2004) explains a mechanism of how microtubules affect the cellulose arrangement and disrupt the cell elongation. Microtubules' activity at the plasma membrane influences the length of cellulose microfibrils. When the cortical microtubules are intact, the cellulose microfibrils are produced at a right angle and with an extended length, so the xyloglucan/pectic polysaccharide matrix can deposit among the cellulose microfibrils and keep the cell extending in one direction. When the microtubules are disorganized, the microfibrils are disorganized and fragmented, and the xyloglucan/pectic polysaccharide matrix will have isotropic expansion.

However, *COBRA* gene functions are still to be fully investigated. According to Emons et al (2007), the force for cellulose synthase complex movement is derived from the cellulose microfibrils production itself. Although cortical microtubules can guide the cellulose synthase complex as tracks, they are not strictly required for organized patterns of motion.

Mutations in the *COBRA* gene in *Arabidopsis*, reduce the amount of crystalline cellulose in the cell wall in the root growth zone, and *COBRA* mutants have disoriented cell expansion

(Schindelman, 2001). The barley mutant *brittle culm1*(*bc1*) was reported to have about an 80% reduction in the amount of cellulose and a decrease in breaking strength in the culms compared with those of wild-type plants (Kokubo et al., 1989, 1991). The rice *brittle culm1*(bc1) mutant was reported to have 43% and 52% decreases of forces required to break culms and leaves respectively compared with wild-type plants. The elongation ratio of the *bc1* leaves also decreased by 50% compared with wild-type (Li et al., 2003). The rice *bc1* mutant plants are also reported to have an altered cell wall composition. The amount of cellulose in *bc1* culms reduced to 70% of the wild-type, while the lignin of the *bc1* culms increased by 30%. Analysis of sequence shows that rice *BC1* encodes a *COBRA*-like protein, because it contains all the conserved features of the *COBRA* family. According to Ching (2006), maize mutant *brittle stalk–2*(*BK2*) has dramatically reduced tissue mechanical strength. The reduction of mechanical strength in the stalk tissue is correlated with a reduction in the amount of cellulose. *Brittle Stalk–2*(*BK2*) encodes a *COBRA*-like protein that is similar to the rice Bc1 protein.

3.2 Characteristics of the *COBRA* gene family

In the NCBI database, there are 12 *COBRA* genes in *Arabidopsis*, nine in Maize, and 11 in Rice. The *COBRA* gene family have common in encoding a plant-specific glycosylphosphatidylionositol (GPI)-anchored protein with a ω-attachment site at the C-terminus, a CCVS domain which is rich in Cys, an N-terminal secretion signal peptide sequence, and a hydrophobic C terminus (Roudier et al., 2002). *COBRA* protein is produced from Golgi and anchored on the extracellular side of the plasma membrane by GPI moiety, then released into the cell wall (Roudier et al., 2005).

GPI anchor is a glycolipid that can be attached to the C-terminus of a protein during posttranslational modification. It can help to anchor a protein to the cell membrane. GPI

anchor is frequently associated with polar protein sorting in animal cells (Matter and Mellman 1994), and acts as sorting signal in the Golgi to target protein specifically into apical portion of the cell membrane in both neuronal and epithelial cells of mammals (Rodriguez Boulan and Powell 1992). In animal cells, GPI proteins are targeted within the membrane, and organized into microdomains at the cell surface (Fried-richson and Kurzchalia 1998; Varma and Mayor 1998).

Immunolocalization of the *Arabidopsis* COBRA proteins shows they are localized in discrete regions along longitudinal cell surfaces in the root. These results may demonstrate the common properties of GPI-anchor motifs acting as a protein sorting signal both in plants and animals (Schindelman, 2001).

The CCVS domain is particularly rich in Cys, and contains the consensus N-glycosylation site, which is the GPI-anchoring site or ω-cleavage site. The N-terminal secretion signal peptide is required for targeting to the endoplasmic reticulum, and the hydrophobic C terminus is consistent with the cleaved peptide required for GPI linkage (Roudier, 2002).

3.3 Expression of the *COBRA* genes

Previous researches have found that *COBRA* gene expression is regulated in a tissue-specific, cell-type specific and developmental stage-specific manner. The expression of *COBRA* gene family is highly developmentally and spatially regulated (Brady et al., 2007).

In *Arabidopsis*, the *cobra* mutant is defective in cellulose synthesis and microfibrils orientation in roots (Schindelman et al., 2001; Roudier et al., 2005). Members of the *Arabidopsis COBRA* family have been demonstrated to be expressed in specific organs by using reverse transcription-PCR (Roudier et al., 2002). The majority of *COBRA* family

members are expressed at varying levels in most organs, except *AtCOB10* and *AtCOB11*, which are expressed only in inflorescence of floral tissue.

There are reports that rice *Bc1* is expressed primarily in vascular regions of the leaves and culm, and the phenotype of *bc1* results in organ brittleness rather than any obvious cell deformities. RT-PCR analysis demonstrated the *BC1* gene was expressed throughout the wild-type plant organs, including leaves, stems and roots, and regulated developmentally (Li et al., 2003).

Maize *Bk2* is highly expressed during the first two weeks from germination, but the expression is lower once the brittle phenotype at the four-week stage is visible. *bk2* exhibits a brittle phenotype and decreased cellulose content in leaves and mature stem. (Sindhu et al, 2007).

3.4 *COBRA* gene and phytochelatin synthase gene

Phytochelatins (PCs) are family of peptides which are enzymatically generated from glutathione (GSH, γ-Glu-Cys-Gly). They are necessary to protect cells from divalent heavy metals by binding and rendering them insoluble and nontoxic in the cytoplasm (Ortiz et al. 1992). Their 2-11 $(\gamma\text{-Glu-Cys})_n$ repeats act as high affinity metal chelators, and facilitate the vacuolar sequestration of heavy metals, such as Cd^{2+}(Zenk, 1996). PCs were identified in the fission yeast *Saccharomyces pombe* and termed cadystins (Kondo, N et al., 1984). They have also been found in some fungi, marine diatoms and all plant species investigated (Cobbett, 1999).

PCs are rapidly induced in cells and tissues exposed to a range of heavy metal ions, including the cations Cd, Ni, Cu, Zn, Ag, Hg and Pb, and the anions arsenate and selenite. A model

suggests that catalytic activity of PC synthases is conserved in N-terminal domains which have cysteine residues that interact with metal ions. The C-terminal domain consisting of cysteine residues is not absolutely required for catalysis or activation, but it has some role in activity, which possibly binds and brings metal ions in contact with the activation site (Cobbett, 1999).

A partial *COBRA* cDNA was reported to complement a *Saccharomyces pombe* mutant defective in phytochelatin synthesis (Leuchter et al. 1998). The partial *COBRA* cDNA was able to confer resistance to cadmium in *S. pombe*, but did not contain the first 94 amino acids of COBRA. These missing amino acids contain the putative N-terminal signal sequence as well as the domain in which the *COBRA*-3 mutation is found. It is possible that COBRA binds divalent metals and has functional relevance with phytochelatin synthesis activity (Schindelma, 2001).

A large-scale proteomics project identified a staggering 122 proteins from a tubulin affinity chromatography column (Chuong et al., 2004). The diversity of microtubule-binding proteins suggests that microtubules play numerous roles beyond coordinating cell division and morphogenesis (Wasteneys, 2004). According to Wasteneys, signalling molecules, bound microtubules either directly or indirectly through protein complexes, may be released to the cytoplasm and activate when microtubules are depolymerised. Many environmental triggers, including exposure to heavy metals, are associated with reorganization of microtubule. These give further linkages that heavy metals are associated with COBRA proteins and microtubule organization.

4. Conclusion

The *COBRA* genes have been reported to have secondary effects on cellulose elongation by affecting the microtubule organization. *COBRA* gene family have been identified to have the four common properties: a plant-specific glycosylphosphatidylionositol (GPI)-anchored ω-attachment site at the C-terminus, a CCVS domain rich in Cys, an N-terminal secretion signal peptide sequence, and a hydrophobic C terminus. Analysis of the *COBRA* gene family shows *COBRA* gene members are expressed in multiple organs and regulated in a cell-type and developmental stage-specific manner. In addition, phytochelatin synthesis activities and heavy metal binding are believed to be related with microtubules organization and the *COBRA* genes.

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Research Manuscript

Molecular Cloning and Expression Analysis of Barley *COBRA* family Genes

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Summary

The *COBRA* family is an ancient gene family that exists in dicots and monocots. They be divided into two subfamilies based on phylogenetic analysis and intron/exon structure. We report here the cloning of five barley *COBRA* genes via a PCR-based strategy. All five have the common features of the *COBRA* gene family: a glycosylphosphatidylinositol (GPI) anchor, a CCVS amino-acid domain and a signal peptide domain. Q-PCR experiments were conducted to investigate the *COBRA* gene expression levels in different barley tissues. Q-PCR analysis showed that all five *COBRA* genes were expressed in all tissues, but at different levels. In order to investigate *COBRA* gene functions, we examined the root hair phenotype of six *Arabidopsis* mutant lines and found one line, Salk_099933C, with deficient root hair growth. Transformation of this line with a barley *COBRA* gene has also been conducted.

Keywords: cloning, *COBRA* gene family, cell wall, cellulose, microtubule, root hair, transformation.

Introduction

Cell expansion is an important process of plant morphogenesis. The plant cell wall has a great effect on cell growth, determines cell shape and provides mechanical protection for protoplasts. Cellulose, xyloglucan and pectin are found in the primary cell wall and are the main components of cell wall polysaccharides. Cellulose microfibrils are the primary anisotropic materials in the cell wall and may determine the orientation of cell extension. As cellulose is a major component of the plant cell wall and the most abundant carbon resource on the earth, and can be used to generate bioethanol, it is regarded as a potential renewable source of energy.

Cellulose is synthesized by the cellulose synthase complex (CSC), also known as the rosette complex. The model of the rosette complex is that: six CesA polypeptides form a rosette subunit, and six rosette subunits interact to form a rosette complex. Each CesA polypeptide synthesizes a cellulose chain and the 36 cellulose chains form a cellulose microfibril (Doblin, et al., 2002).

The *COBRA* family genes are believed to have effects on cellulose synthesis. According to Hauser et al., (1995), the name *COBRA* comes from the mutations of *Arabidopsis*, which have changed the orientation of cell expansion in the root, from the longitudinal axis to the radial axis. As a result, the widening roots gain the appearance of the head of a *cobra*. The model proposed by Wasteneys (2004) provided a mechanism by which microtubules affect the cellulose arrangement; this model can help to interpret the phenotype of the *COBRA* mutant. Cortical microtubule activity at the plasma membrane influences the length of cellulose microfibrils. When the cortical microtubules are intact, the cellulose microfibrils are produced at correct angle and with an extended length, so the xyloglucan/pectic polysaccharide matrix can be deposited among the cellulose microfibrils and keep the cell extending in one

direction. When the microtubules are disorganized, the cellulose microfibrils are disorganized and fragmented, and the xyloglucan/pectic polysaccharide matrix will have isotropic expansion (Wasteneys，2004). COBRA proteins are required for the oriented deposition of cellulose microfibrils during rapid anisotropic expansion. Microfibril orientations are potentially regulated by the COBRA proteins, which are possibly the plasma membrane proteins that bind with microtubules and determine the microfibrils orientation (Roudier et al., 2005). The effect of COBRA proteins on cellulose biosynthesis is secondary, since they do not directly act on cellulose synthesis. Microtubules influence growth anisotropy by regulating the relative length of microfibrils, and the microtubule-dependent patterning of *COBRA* protein could support the formation and extension of microfibrils (Wasteneys, 2004). Cortical microtubules constrain rosette complex movement by serving either as tracks or guard rails (Giddings and Staehelin, 1991; Wasteneys, 2004). Roudier et al., (2005) also reported that the distribution of *COBRA* protein during cell elongation is dependent on cortical microtubule organization. Paredez et al. (2006) have fluorescently labelled cellulose synthases (CesAs) which assemble into rosette complexes in *Arabidopsis* hypocotyl cells. These rosette complexes move in the plasma membrane along tracks defined by the co-labelled microtubules. Double labelling of *COBRA* protein and cortical microtubules in elongating cells has further confirmed the similar orientation of cortical microtubules and the COBRA bands (Roudier et al., 2005). However, *COBRA* gene functions are still to be fully investigated. According to Emons et al., (2007), the force for cellulose synthase complex movement is derived from the cellulose microfibril production itself, and although cortical microtubules can guide the cellulose synthase complex as tracks, they are not strictly required for organized patterns of motion (Emons et al., 2007).

All *COBRA* gene family members encode a plant-specific glycosylphosphatidylionositol (GPI) anchored protein with a ω-attachment site at the C-terminus, a CCVS domain which is rich in Cys, an N-terminal secretion signal peptide sequence, and a hydrophobic C terminus

(Roudier et al., 2002). All members of the *COBRA* gene family contain a central phytochelatine synthase (PCS) domain (Hochholdinger et al., 2008). COBRA proteins are produced from Golgi and anchored on the extracellular side of the plasma membrane by a GPI moiety, then released into the cell wall (Roudier et al., 2005). *COBRA* family genes have been found in several plant species. In the NCBI database, there are 12 *COBRA* genes in *Arabidopsis*, called *COBRA* or *COBRA*-like genes; nine in maize, called *brittle stalk* genes; and 11 in rice, called *brittle culm* genes. A recent article reported that the maize roothairless3 gene encodes a *COBRA*-like protein which affects root hair elongation and grain yield (Hochholdinger et al., 2008). However, there is no report about the *COBRA* gene family in barley.

In this study we cloned five *COBRA* genes from barley. In order to investigate the function of COBRA proteins, we ordered several *Arabidopsis COBRA* mutant lines from The Arabidopsis Biological Resource Center (ABRC) and observed their root hair phenotype. We identified one line with deficiency of root hair elongation. Transformation of the *HvCOBRA4* gene into this line has also had been conducted.

Results

Cloning Barley *HvCOBRA* cDNA

Two hundred and seventeen ESTs were obtained by a Blast search of the NCBI and TIGR databases, using rice, maize and *Arabidopsis COBRA* gene sequences (Appendix D). These ESTs were assembled into 13 contigs using ContigExpress (Invitrogen®). Five contigs were selected for cloning as they have relatively more ESTs assembled than the other contigs. Their sequences were shown in Appendix A. The cloning was conducted using the PCR method. PCR primers are designed according to the predicted sequences; the primers and expected product sizes are listed in Table 1. The cDNAs of three *HvCOBRA* genes have been amplified and sequenced. They are full length and contain putative start codons. Another two *HvCOBRA* cDNAs fragments have been partially cloned and sequenced. These sequence are predicted to be nearly full length.

Properties of *HvCOBRA* genes

Table 2 and Table 3 show the identity among the nucleotide and amino-acid sequences of these five COBRA genes and proteins respectively. One putative phytochelatin synthase nucleotide sequence (accession number: CAD42639) was included, since this sequence has 100% identity with the *HvCOBRA2*. As the central phytochelatin synthase domain is a common feature of the *COBRA* gene family, it is possible that this putative phytochelatin synthase sequence is actually a partial sequence of the *HvCOBRA2*. According to Li et al., (2003), the *COBRA* gene family can be divided into two subfamilies, which is according to the exon/intron organization of each subfamily member. One subfamily has multiple introns/exons, the other subfamily has a single exon (Li et al., 2003). Table 3 shows HvCOBRA1, HvCOBRA3, HvCOBRA4 and HvCOBRA5 have over 59% identity with each other, while HvCOBRA2 has less than 30% identity with other sequences. HvCOBRA2 is classified to the second group of *COBRA* subfamily. Phylogenetic analysis also divided the

five *HvCOBRA* genes into 2 groups (Figure 1). *HvCOBRA*2 is alone in the second group with *AtCOBRA7*, *AtCOBRA8*, *AtCOBRA9*, *AtCOBRA10* and *AtCOBRA11*, while the rest of barley *COBRA* genes were clustered with *AtCOBRA1-6*. Alignment of amino acid sequences for both barley and *Arabidopsis* genes also shows two distinct groups of *COBRA* genes (Figure 2).

COBRA gene features

Figure 3 and Figure 4 show the alignment of subfamily COBRA proteins. It shows that the barley COBRA contains the common features of the COBRA proteins: all the HvCOBRA and AtCOBRA proteins have a signal peptide region at N-terminal, the CCVS domain, and a GPI cleavage site (ω-site).

Expression profile of _HvCOBRA_ genes

The expression pattern of *HvCOBRA* genes in different tissues was analysed by Quantitative Real-Time PCR (Figure 5) with gene specific primers (Table 4). The experiments were kindly performed by Dr. Neil Shirley (The University of Adelaide). The data show that, *HvCOBRA*1 mRNA levels are high in leaf base and root tip, which is the highly growing area during elongation. *HvCOBRA2* mRNA was generally present in all tissues. *HvCOBRA4* had high mRNA levels in leaf tip and leaf base, and was also expressed in other tissues. *HvCOBRA5* mRNA was found at high levels in peduncle, leaf base and stem, but have relatively low levels in other tissues. *HvCOBRA*6 mRNA was at low levels in all the tissues. Barley leaves were sectioned into five different zones and analysed for *COBRA* gene expression by QPCR (Figure 6). *HvCOBRA*4 and *HvCOBRA*5 mRNA levels were found to be low in most of the leaf blade but increased in expression in the leaf base zone (Figure 6). Analysis of expression pattern of *HvCOBRA* genes in developing grains showed that *HvCOBRA*2 mRNA level was

higher than other *HvCOBRA* genes at all time point (Figure 7). There was an increase in HvCOBRA mRNA levels after 20 DAP.

Identifying phenotypes of *Arabidopsis COBRA* mutant lines

In order to study the function of barley *COBRA* genes, we wanted to transform barley *COBRA* genes into *Arabidopsis* T-DNA insertion lines with *COBRA* knockouts. Six *COBRA* gene mutant homozygous lines were ordered from the *Arabidopsis* Biological Resource Center (ABRC), the germplasm codes and their information are listed in Table 5. Observation of seed germination in petri dish under a microscope showed that only Salk_099933C mutant has different phenotype from the wide type *Arabidopsis* **(***Columbia***).** Salk_099933C mutant generally had less root hair growth than wild type *Arabidopsis* (Figures 8 and 9). Salk_099933C mutant contains a defection in *AtCOBRA*-like9 gene function. This line was used for transformation of *HvCOBRA* genes.

Transformation of *HvCOBRA* gene

*HvCOBRA*4 was cloned into a transformation vector (pTool2 from Invitrogen) containing the 35S promoter. The construct was transformed to the Salk_099933C mutant line by *Agrobacteria* through flower dipping. The transformants were selected after sowing the seeds on a medium containing selection reagent PPT (DL-Phosphinothricin, Duchefa Biochemie, Netherlands). Several seedlings grew in the selection medium, where it was possible to observe any changes in root phenotypes. Further observation of the root hair phenotype have not been conducted due to time constraints in the Masters degree program.

Discussion

COBRA genes are found in many higher plants and proposed to affect cell wall cellulose biosynthesis. Here, we have identified at least 11 barley contigs from barley EST databases.

Five of them have been cloned and they all show common features of *COBRA* genes from other plant species. Their functionalities were studied by transforming *Arabidopsis* mutant plants. We have obtained several transformants, which will be grown for seeds. After homozygous plants are obtained, the root hair growth will be examined in these transformants.

Barley cellulose is synthesized by cellulose synthases on plasma membrane. The cellulose in primary cell wall is synthesized by HVCesA1, HvCesA2 and HvCesA6, while the cellulose in secondary wall is thought to be synthesized by HvCesA4, HvCesA7 and HvCesA8 (Burton et al. 2004). The catalytic subunits of the HvCesAs form an enzyme complex (Brown and Saxena, 2000), which may be visible under a transmission electron microscopy. *COBRA*s are suggested to control cellulose microfibril orientation through the interaction with microtubules. Thus, there is a very complicated mechanism for the control of cell wall biosynthesis. Our data have suggested that there are about 11 *COBRA* genes in barley. It may be assumed that some of the *COBRA* genes function with the HvCesA genes responsible for primary wall biosynthesis (HvCesA1, HvCesA2 and HvCesA6) and some of the *COBRA* genes may be associated with secondary wall biosynthesis and work together with CesA4, CesA7 and CesA8. Our QPCR data showed that *HvCOBRA*1 is highly expressed in leaf base and root tip (Figures 5 and 6). It was assumed that this gene may be associated with primary wall synthesis. This assumption is supported by QPCR data obtained by Dr. Neil Shirley (ACPFG, personal communication). Dr. Shirley also showed that HvCOBRA5 is associated with barley secondary wall biosynthesis by correlation analysis of gene expression. Over-expression or knockout of barley *COBRA* genes and examination of cell wall architecture in barley will help to confirm this assumption in future.

Some of the *COBRA* genes may be tissue specific. In the six *Arabidopsis* T-DNA insertion lines we started, we found that only the defect of *AtCOBRA*9 (Salk_099933C mutant line)

affected root hair growth. We need to transfer all 11 barley *COBRA* genes into this *Arabidopsis* mutant to see which *HvCOBRA* gene will complement the function of *AtCOBRA*9. *COBRA* genes can be divided into two groups (Li et al., 2003), *AtCOBRA*9 is in the second group (Figure 1). *HvCOBRA*2 is the only barley *COBRA* gene in the second group. It would be meaningful to transform *HvCOBRA*2 into Salk_099933C mutant to see whether it will make root hair growth. However, due to time constrain, we only transformed the HvCOBRA4.

Experimental procedures

Bioinformatics

Sequences of 12 *Arabidopsis COBRA* or *COBRA*-like genes, nine maize *brittle stalk* genes and 11 rice *brittle culm* genes were collected from the NCBI (http://www.ncbi.org) database. All these sequence were used to search against the barley EST (expressed sequence target) clones which similar to *COBRA* family genes. The databases used were NCBI (http://www.ncbi.org) and TIGR (http://Plantta.tigr.org). The sequences of barley EST similarity to *COBRA* family genes are presented in Appendix D. All the ESTs were assembled by ContigExpress (Invitrogen, Australia) to constitute the potential genes. Eleven contigs were generated. Five of the sequences were selected for cloning since they have relatively higher EST numbers. The five predicted *HvCOBRA* gene sequences are included in Appendix A.

RNA extraction

Total RNA was isolated from wild type barley plants. The frozen tissues were ground to powder under liquid nitrogen using a sterilised mortar and pestle. Ground tissue was mixed with Trizol-like reagent (38% phenol (pH 4.3), 11.8% guanidine thiocyanate, 7.6% ammonium thiocyanate, 3.3% 3 M sodium acetate (pH 5) and 5% glycerol) in a 1.5 ml tube, shaken for 5 min at room temperature, and centrifuged for 5 min at 11,000 rpm at 4℃.

Supernatant was transferred to a new 1.5 ml tube and 200 µl of chloroform was added. Shake for 15 min at room temperature and centrifuged for 15 min at 11,000 rpm. The aqueous phase was transferred into a fresh tube and 500 µl isopropanol added. The tube was left at room temperature for 10 min, and centrifuged at 11,000 × g for 10 mins at 4°C. The supernatant was removed and the pellet was washed with 1 ml of 75% ethanol. The sample was mixed by vortexing and centrifuged at 7,400 × g for 5 min at 4°C. Ethanol was removed and tubes were left open in fume hood to dry the RNA pellet. The RNA pellet was resuspended in 30-50 µl of RNase-free water. The concentration of the RNA was determined by spectroscopy of 1:100 dilutions of the RNA samples and absorbance measurements were taken at 260 nm.

cDNA synthesis

RNA was treated with DNase to remove residual DNA prior to cDNA synthesis by using the TURBO DNA-free™ kit (Ambion, USA). The 10x DNaseI Buffer (0.2 µl) and 1µl of DNaseI were added and incubated for 30 min at 37℃. DNase Inactivation Reagent was added and the reaction was incubated for 2 min at room temperature prior to centrifugation at 10,000 g for 90 sec. The supernatant was retained.

cDNA synthesis was performed using SuperScript™ III Reverse Transcriptase (Invitrogen). Reactions were performed in a 0.2 ml PCR tube. Total RNA (2 µL) was combined with 1 µL of 50 µM oligo$(dT)_{18}$ primer and 1µL of 10mM dNTPs and added with water to a total volume of 8 µL, incubated at 65℃ for 5 min in a water bath. Immediately placed the tube on ice and added 10µL of the reverse transcription master mix which contained 4 µL of 5x first strand buffer, 1 µL of 0.1 M DTT, 1 µL RNaseOUT and 50 units of SuperScript™ III Reverse Transcriptase. The cDNA synthesis reactions were performed at 52℃ for 1 hr and the reaction was terminated by incubation for 15 min at 70℃. Water was added to the cDNA reactions to give a final volume of 50 µL. The cDNA was stored at -20℃.

PCR

PCR primers were designed to amplify the barley *COBRA* fragments. The primers used to amplify each fragment and the expected fragment size are shown in Table 1. Polymerase chain reactions of typically 25 μL were prepared in 0.2 ml PCR tubes on ice. These reactions contained 1 μL of template cDNA, 1 μL of 10 mM of each of forward and reverse primers, 2.5 μL of 10x PCR buffer (100 mM Tris-HCL buffer containing 500 mM potassium chloride, pH 9.0, 10% Trition X-100)), 1.5 μL of magnesium chloride (25 mM), 1 μL dNTPs (5 mM), 1U of Taq DNA polymerase, and added sterile MQ water up to 25 μL. PCR reactions were performed in a DNA Engine Tetrad 2 Thermal Cycler with cycling as follow: 94℃ for 1 minute, 25 to 40 cycles of 94℃ for 30 sec, 30 sec at an annealing temperature between 48℃ and 62℃ according to the primer's Tm, and extension at 72℃ for 30 sec, followed by a final extension of 72℃ for 1 min.

Making constructs for *Arabidopsis* transformation

Full length *HvCOBRA*4 cDNA fragment was amplified by PCR as described above. Recombination reactions were performed using the purified DNA fragments, and the pCR8 (Invitrogen) entry vector to create an entry construct. The pCR8 vector is Gateway enabled and reaction was performed according to the manufacturer instruction (Invitrogen P/L, Australia). Briefly, the recombination reactions were performed in a 5 μL volume reaction containing 1 μL of purified DNA fragment, 1 μL of pCR8 vector reaction mix (10 ng μL^{-1}), 1 μL of salt solution and water up to 5 μL. Reaction mixture was incubated at room temperature for 1 hr. The reaction products were transformed into One Shot® Mach1^{TM} –T1^{R} Chemically Competent *E. coli* (Invitrogen®, Australia) via Heat Shock method. Products and competent cell were combined in a 1.5 ml tube and placed on ice for 20 min, and incubated at 42℃ for 1 min, placed on ice for 2 min, added LB media immediately and incubated in 37℃ with shaking for 40 min, then platted to LB plate containing spectromysin at 100 μg/ml. Colonies

which containing construct insert were selected for plasmid preparation after PCR screening and restriction enzyme digestion. The pCR8 vector containing the gene of interest (entry construct) was cloned into pTool2 vector through LR reaction. The reaction solution contained 1 μL pTool2 vector and 1μL entry clone plasmid, 2 μL reaction buffers, 2 μL LR reaction enzyme mix, and TE buffer to a total of 10 μL. Incubated overnight at 25℃, then added 1μL of proteinase K and incubated at 37℃ for 10 min to stop the reaction. After LR reaction, transfered the reaction mix into a tube, added E. coli competent cells and did transformation through heat shock method as before. Colonies containing gene of interest were selected by PCR for plasmid preparation. The plasmids were sequenced to confirm the right orientation of the gene and correct reading frames in the destination construct. This construct will be used for transformation into *Agrobacterium* and then to *Arabidopsis*.

Plasmid DNA Mini-preparations and Sequence Analysis

Colonies were picked from overnight plates and transferred to 10 ml tubes containing 3 ml of LB + Spectromycin (100μg/ml) and placed in a 37℃ shaking incubator for 16 hr. Plasmid DNA was prepared by alkaline lysis and resuspended in 40 μL MQ water. Plasmid DNA sequencing reactions were performed using the BigDye® v3.1 Cycle Sequencing Kit. Reactions were set up in 12 μL volumes containing 3μL of BigDye Sequencing Buffer, 1μL BigDye enzyme, 1μL of Gateway forward or reverse primer, 1-2 μL of DNA template and add water to a total of 12μL. Cycling parameters were as follows: 96℃ for 30 sec, 25 cycles of 96℃ for 10 sec, 50℃ for 5 sec and 60℃ for 4 min. After cycling, sequencing reactions were precipitated using freshly prepared wash solution (75% ethanol and 0.2μM $MgSO_4$) and incubating at room temperature for 15 min. The samples were pelleted by centrifuging at 13,000 rpm for 15 min and the supernatant was discarded. The remaining pellet was dried in dark before being submitted for sequencing to AGRF (Australian Gene Research Facility, SA, Australia).

Transformation of *Agrobacteria* by Electroporation

Agrobacteria were transformed with the destination construct which contained the gene of interest by electroporation using a Gene-Pulser apparatus. (Both the entry construct, pCR8/*HvCOBRA4* and the destination construct, pTool2/pCR8/*HvCOBRA4* are displayed in Appendix B and Appendix C respectively). A programmed setting was used for *Agrobacteria* transformation (cuvette size: 1 mm, Voltage: 1250V, Capacitance 25 μF, Resistance: 200 Ω). Cuvettes (1 mm) were placed on ice for 1 hr before use. Purified plasmid (1 μL) was added to 40 μL of electrocompetent Agrobacteria cells within 5 minutes of their removals from the -80 ℃ freezer. This mixture was transferred to the cuvette and pulsed. Transformed cells were immediately resuspended in 300 μL of YM media, transferred to a 1.5 ml eppendorf tube and placed in a 25℃ water bath for 3 hours. The cells were plated onto YM Agar plates containing Carbenicillin (100 μL ml^{-1}) Rifampicin (100 μL ml^{-1}) and Kanamycin (100 μL ml^{-1}) and incubated at 25 ℃ for a few days. The colonies will be used for transformation of *Arabidopsis*.

Transformation of *Arabidopsis* by *Agrobacteria* floral dip method

A single colony was selected and inoculated *Agrobacterium* culture medium (4 ml YM medium containing 0.4% Yeast Extract, 10% Mannitol, 0.1% NaCl, 0.2% $MgSO_4.7H_2O$, 0.5% $K_2HPO_4.3H_2O$). The culture was incubated at 28°C for 2 days. Once cultures looked cloudy, it was transfered into 50 ml YM with selection antibiotics and was grown for two days at 28°C. Inoculate 500 ml YM with selection antibiotics (25mg/L Rifampicin, 25 mg/L Kanamycin, 50 mg/L Carbenicillin), with the 50 ml starter culture and grow at 28°C until cloudy. Poured cultures into 4 x 250 ml centrifuge tubes and spin down for 15 min at 4000 rpm at 4°C. Made 1000 ml 5% sucrose solution. Resuspended *Agrobacterium* pellet in sucrose solution, pour into plastic bottle, and add 500 μl Silwet L-77. Pour *Agrobacterium*

into a lettuce crisper. Inverted plants with fresh flowers into the *Agrobacterium* solution and gently swirled in the solution for 30 seconds. The plants will continue grow and set seeds. The seeds will be selected for transformants in a medium containing antibiotics.

Seed germination and transformant selection

The seeds sowed in a medium containing selection antibiotics (10mg/mL PPT DL-PHOSPHINOTHRICIN, Duchefa Biochemie, Netherlands). The seedlings containing gene of interest will survive while the seedlings without the transformation construct will dye.

Microscopy observation for root phenotypes

Six *Arabidopsis* Salk line seeds and widetype seeds were grow on petri dish with MS Agarose media, 800 ml media containing 1.7g Murashige and Skoog Basal Salt Mixture (MS, Sigma), 7.2 g sucrose and 9 g Agarose. Root growths were observed directly by a Leica MZFLIII microscope (Leica, Germany). The images were taken by a Leica DC 300 digital camera (Leica, Germany). The images were taken for the roots of seedling at 1 day to 14 days old after being grown in short day time (8 hours) green house cabinet.

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Table list

Tables

Table 1. Primers used for cloning the genes of interest

Barley Gene Contig Name	Forward Primer Name/sequence	Reverse Primer	Fragment size (bp)
*HvCOBRA*1	COB1F1: ACTGAGGCGCGGAAGGAAGC	Oligo dT	
*HvCOBRA*1	COB1F2: CGCACATGCTTTAAGTCC	COB1R2: ACAATGCTAGATTATCTCAACC	1946
*HvCOBRA*2	COB2F001: CGGTCATCGACGCCAACTC	Oligo dT	
*HvCOBRA*2	COB2F002: TCCAGGCATACCCCACCAG	COB2R3: GCACTGCTTAGATGGGTCCTG	1252
*HvCOBRA*3	HvCOB3F1: AGCTGGTTCTTGAGACTCGA	HvCOB3R1: TTCAATTTTTTTGGGGTTCA	1602

*HvCOBRA*3	HvCOB3F2: TACATCGCTCCTGCCCTC	HvCOB3R2: ATCCTCAAACCTGAATCTGC	1452
*HvCOBRA*4	HvCOB4F1: GTGTCTCGTCGCAGTCCA	HvCOB4R1: GACCAAAAGCATCCTCTCCA	1737
*HvCOBRA*4	HvCOB4F2: TGCCTTCTGTCCGCCTC	HvCOB4R2: GCACACCAGAAGCTCGC	1483
*HvCOBRA*5	HvCOB5F1: GGTCAGGTCGATCCATCAC	Oligo dT	
*HvCOBRA*5	HvCOB5F2: TCCGCAGCTCTATGCTTG	HvCOB5R2: CTTGACATGACAACCACACACA	1532

Table 2. Nucleotide sequence identity comparison

	*HvCOBRA*1	*HvCOBRA*2	*HvCOBRA*3	*HvCOBRA*4	*HvCOBRA*5	Putative Phytochelatin Synthase
*HvCOBRA*1	100%					
*HvCOBRA*2	43.6%	100%				
*HvCOBRA*3	58.8%	40.1%	100%			
*HvCOBRA*4	72.4%	42.7%	61.9%	100%		
*HvCOBRA*5	54%	45.8%	58.7%	57.8%	100%	
Putative Phytochelatin Sythase	43.4%	96.7%	43.6%	41.3%	42.2%	100%

Table 3. Amino-acid sequence identity comparison

	*HvCOBRA*1	*HvCOBRA*2	*HvCOBRA*3	*HvCOBRA*4	*HvCOBRA*5	Putative Phytochelatin Synthase
*HvCOBRA*1	100%					
*HvCOBRA*2	27.5%	100%				
*HvCOBRA*3	65.3%	27%	100%			
*HvCOBRA*4	84.9%	27.6%	64.8%	100%		
*HvCOBRA*5	63.2%	28.4%	59.1%	63.7%	100%	
Putative Phytochelatin Sythase	28%	100%	28.3%	28%	29.8%	100%

Table 4. Gene specific primers for Q-PCR analysis

Name	plant	cultivar	forward primer	reverse primer
*Hvcobra*1	Hv	sloop	TTAAAAGGGGCTGGAAATGTT	TTAAATGTCTGCGTCTGCTAT
*Hvcobra*2	Hv	sloop	GCCCTTGCTTTGGTCTTGCT	CATCTCTCTTGCGGTTCATCTT
*Hvcobra*4	Hv	sloop	AACGCGAGCCCTCTGATGAAG	CAACTGTGGAACCGAATGAAC
*Hvcobra*5	Hv	sloop	CGCCGGATTCCTACCCTTACC	GCATCCGCACGGACCACTCTG
*Hvcobra*6	Hv	sloop	CGTTGCCTTCTTGGTTCTCC	AGATCCAAATGTCACATTGCCA

Table 5. Information on the six *Arabidopsis* mutants used in this study

Germplasm code	Gene	Mutation type	Genotype
CS8541	COB	T-DNA	homozygous
CS8542	COB	Ethyl methanesulfonate	homozygous
CS8543	COB	X-ray	homozygous
SALK_020771C	COB9	T-DNA	homozygous
SALK_099933C	*Cobra*-like9 Phytochelatin-like	T-DNA	homozygous
SALK_106466C	COB9	T-DNA	homozygous

Figure legends

1. **Figure 1.** The phylogenetic tree of the 11 *Arabidopsis COBRA*-like proteins and 5 barley COBRA proteins. *AtCOBRA*1, *AtCOBRA*2, *AtCOBRA*3, *AtCOBRA*4, *AtCOBRA*5, *AtCOBRA*6, *HvCOBRA*1, *HvCOBRA*3, *HvCOBRA*4 and *HvCOBRA*5 are divided into one group, HvCOBRA2, AtCOBRA7, AtCOBRA8, AtCOBRA9, AtCOBRA10 and AtCOBRA11 are belong to the other group.

2. **Figure 2.** Alignment of Arabidopsis and barley COBRA proteins. The alignment shows that AtCOBRA and HvCOBRA can be divided into two subfamilies. HvCOBRA2, AtCOBRA7, AtCOBRA8, AtCOBRA9, AtCOBRA10 and AtCOBRA11 belong to one group, the rest belong to the other group. Identical and similar amino acid are shaded in black and gray.

3. **Figure 3.** Protein sequence alignment of *AtCOBRA*7, *AtCOBRA*8, *AtCOBRA*9, *AtCOBRA*10, *AtCOBRA*11 and *HvCOBRA*2 (Subgroup 2). The predicted signal peptide is underlined, with cleavage site marked by an arrowhead. A consensus amino-acid sequence CCVS is boxed. The ω-amino acid of the predicted GPI cleavage site is also boxed.

4. **Figure 4**. Protein sequence alignment of *AtCOBRA*1, *AtCOBRA*2, *AtCOBRA*3, *AtCOBRA*4, *AtCOBRA*5, *AtCOBRA*6, *HvCOBRA*1, *HvCOBRA*3, *HvCOBRA*4 and *HvCOBRA*5 (Subgroup 1). The predicted signal peptide is underlined, with cleavage site marked by an arrowhead. A consensus amino-acid sequence CCVS is boxed. The ω-amino acid of the predicted GPI cleavage site is also boxed.

5. **Figure 5**. The mRNA levels of *HvCOBRAs* in different barley tissues

6. **Figure 6**. The mRNA levels of *HvCOBRAs* in different sections of barley leaves

7. **Figure 7.** The mRNA levels of *HvCOBRAs* in developing barley endosperm

8. **Figure 8**. Microscopic examination showed different root hair growth between Salk_099933c mutant line (a and b) and wild type plants (c and d) 3 days after germination. (Scale: 400X)

9. **Figure 9.** Microscopic examination showed different root hair growth between Salk_099933c mutant line (a and b) and wild type plants (c and d) 7 days after germination. (Scale: a and c 100X; b and d 400X)

Figures

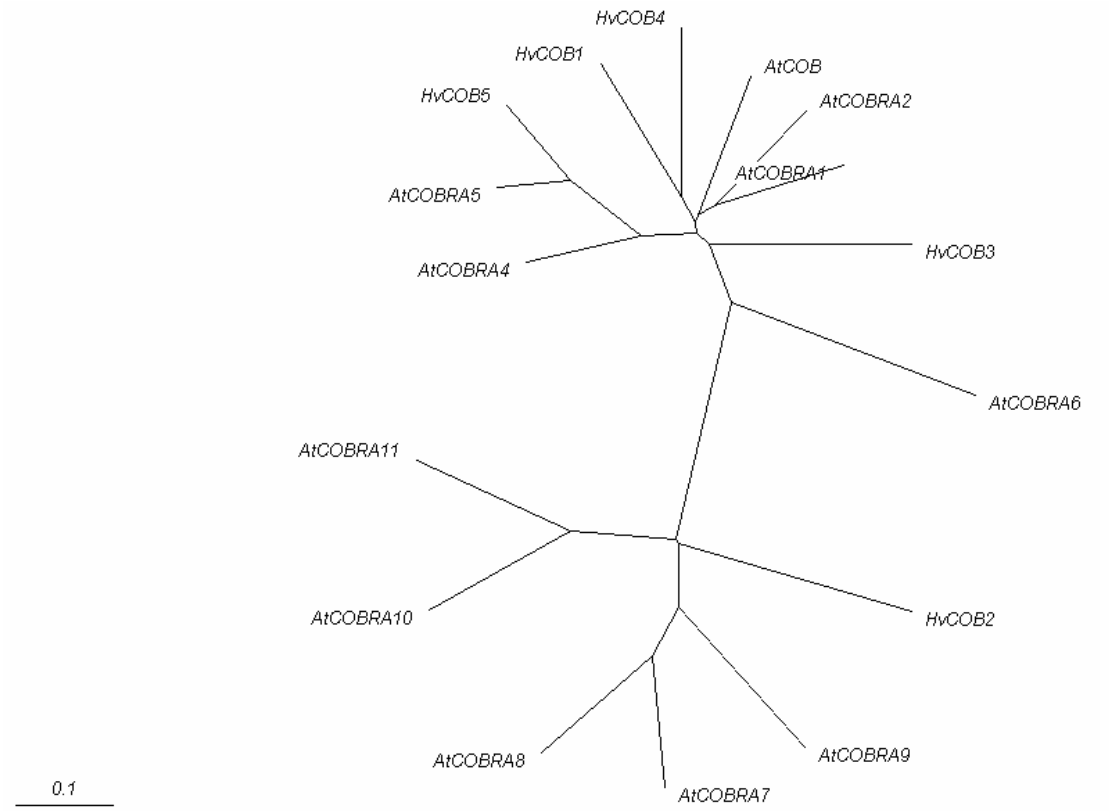

Figure 1. The phylogenetic tree of the 11 *Arabidopsis COBRA*-like proteins and 5 barley COBRA proteins. *AtCOBRA*1, *AtCOBRA*2, *AtCOBRA*3, *AtCOBRA*4, *AtCOBRA*5, *AtCOBRA*6, *HvCOBRA*1, *HvCOBRA*3, *HvCOBRA*4 and *HvCOBRA*5 are divided into one group, HvCOBRA2, AtCOBRA7, AtCOBRA8, AtCOBRA9, AtCOBRA10 and AtCOBRA11 are belong to the other group.

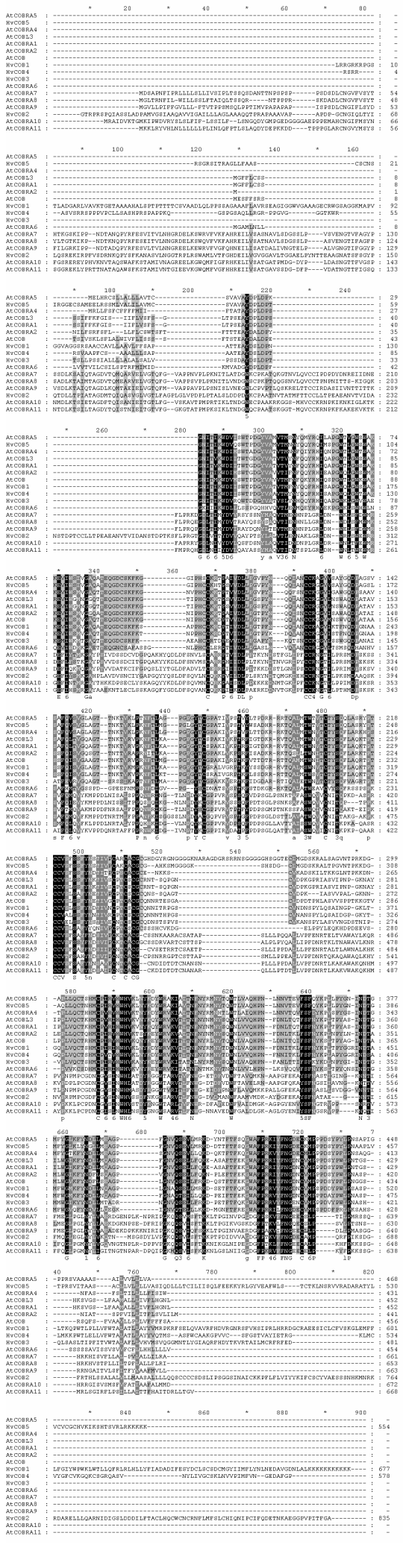

Figure 2. Alignment of Arabidopsis and barley COBRA proteins. The alignment shows that AtCOBRA and HvCOBRA can be divided into two subfamilies. HvCOBRA2, AtCOBRA7, AtCOBRA8, AtCOBRA9, AtCOBRA10 and AtCOBRA11 belong to one group, the rest belong to the other group. Identical and similar amino acid are shaded in black and gray.

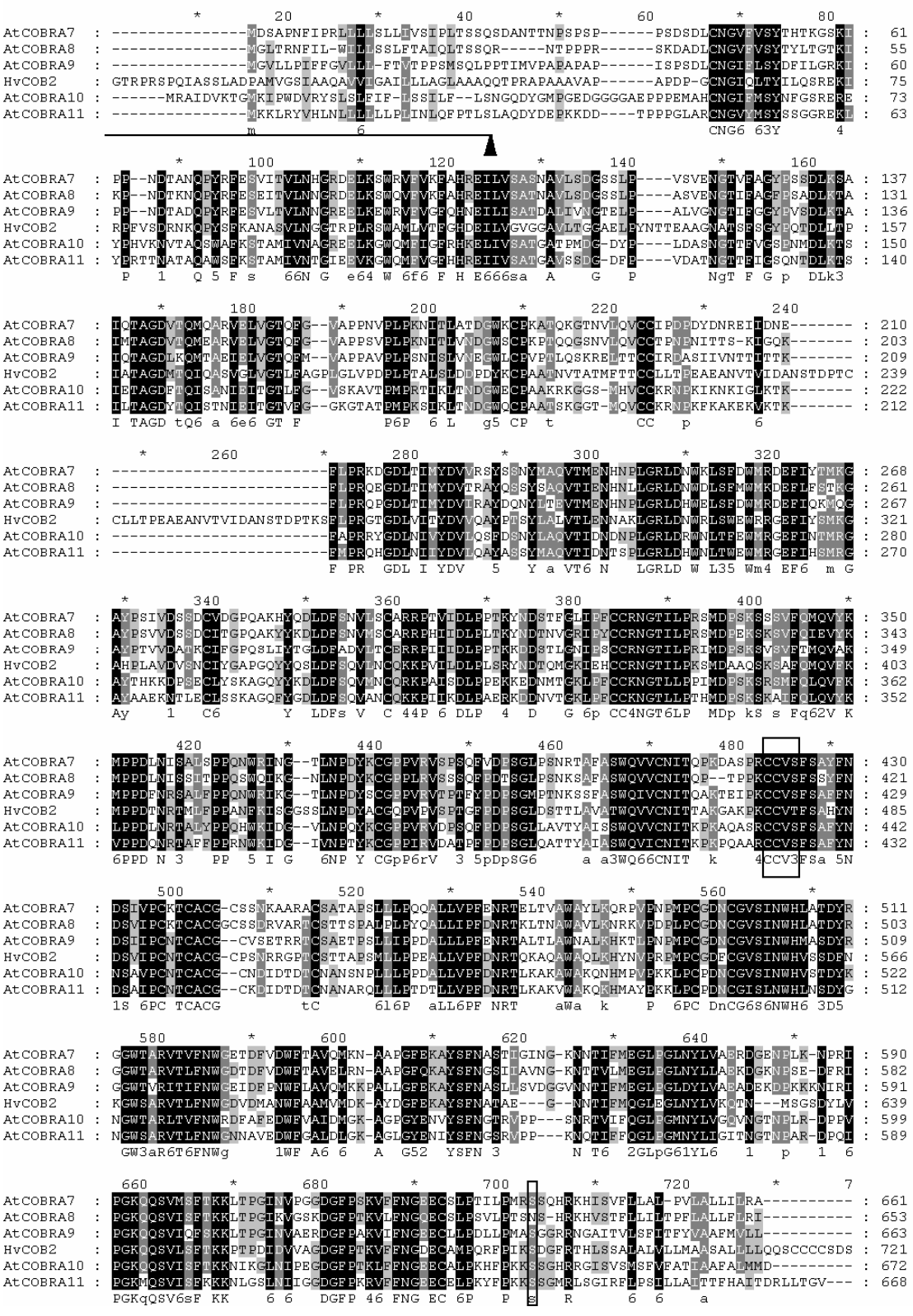

Figure 3. Protein sequence alignment of *AtCOBRA*7, *AtCOBRA*8, *AtCOBRA*9, *AtCOBRA*10, *AtCOBRA*11 and *HvCOBRA*2 (Subgroup 2). The predicted signal peptide is underlined, with cleavage site marked by an arrowhead. A consensus amino-acid sequence CCVS is boxed. The ω-amino acid of the predicted GPI cleavage site is also boxed.

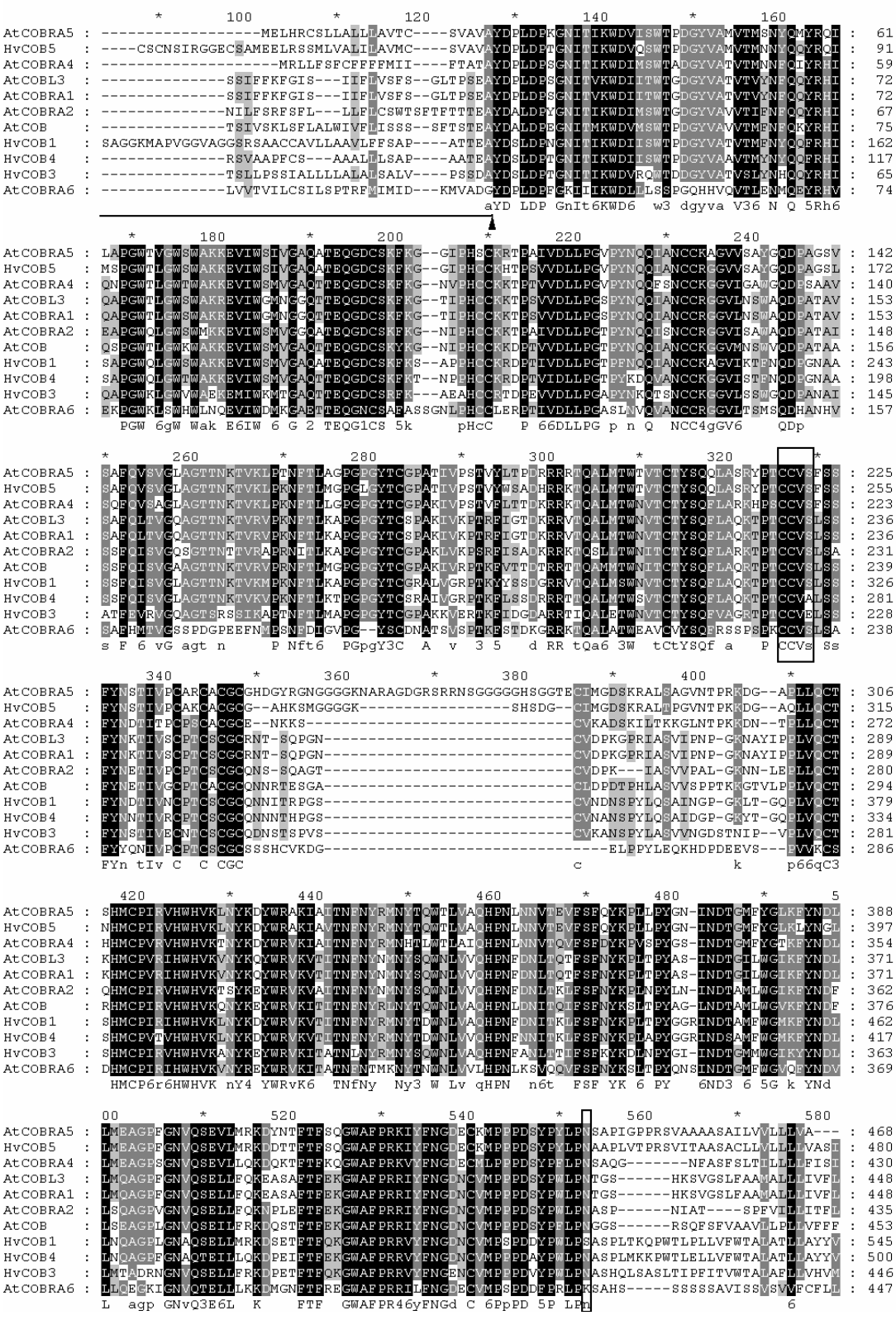

Figure 4. Protein sequence alignment of *AtCOBRA*1, *AtCOBRA*2, *AtCOBRA*3, *AtCOBRA*4, *AtCOBRA*5, *AtCOBRA*6, *HvCOBRA*1, *HvCOBRA*3, *HvCOBRA*4 and *HvCOBRA*5 (Subgroup 1). The predicted signal peptide is underlined, with cleavage site marked by an arrowhead. A consensus amino-acid sequence CCVS is boxed. The ω-amino acid of the predicted GPI cleavage site is also boxed.

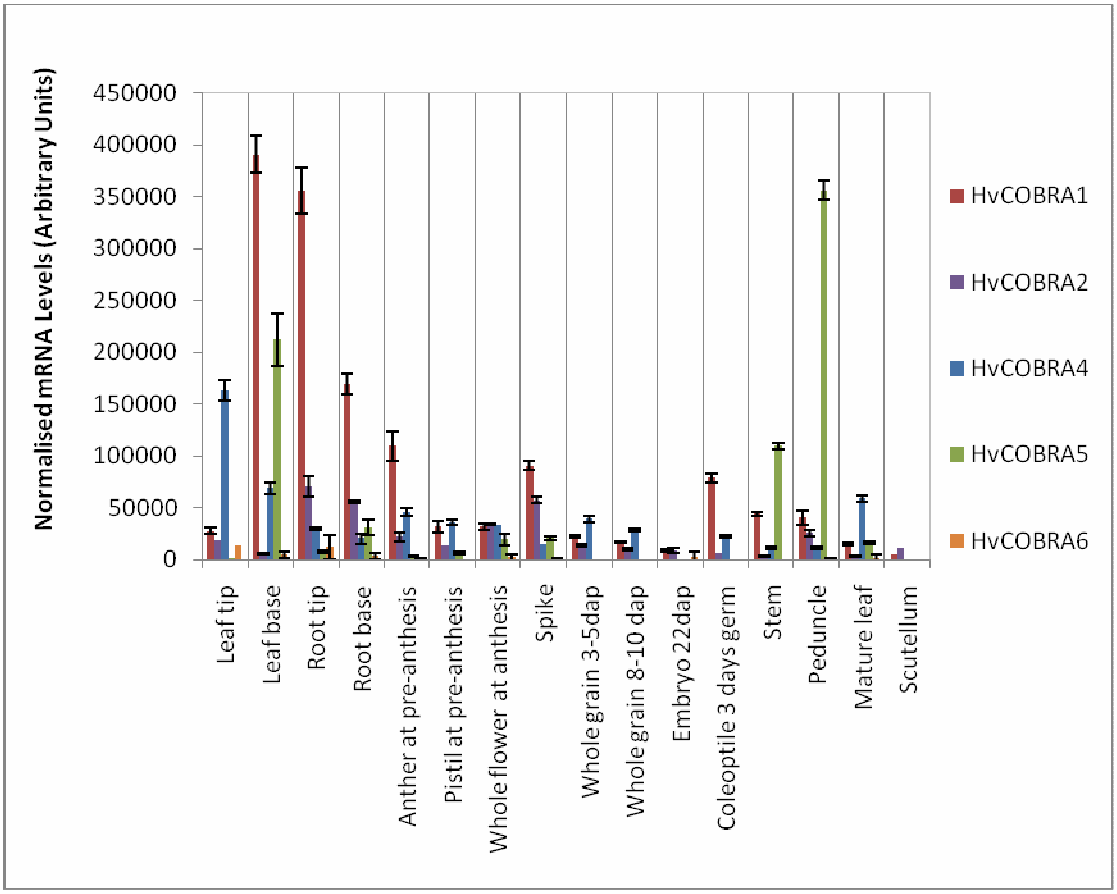

Figure 5. The mRNA levels of *HvCOBRAs* in different barley tissues

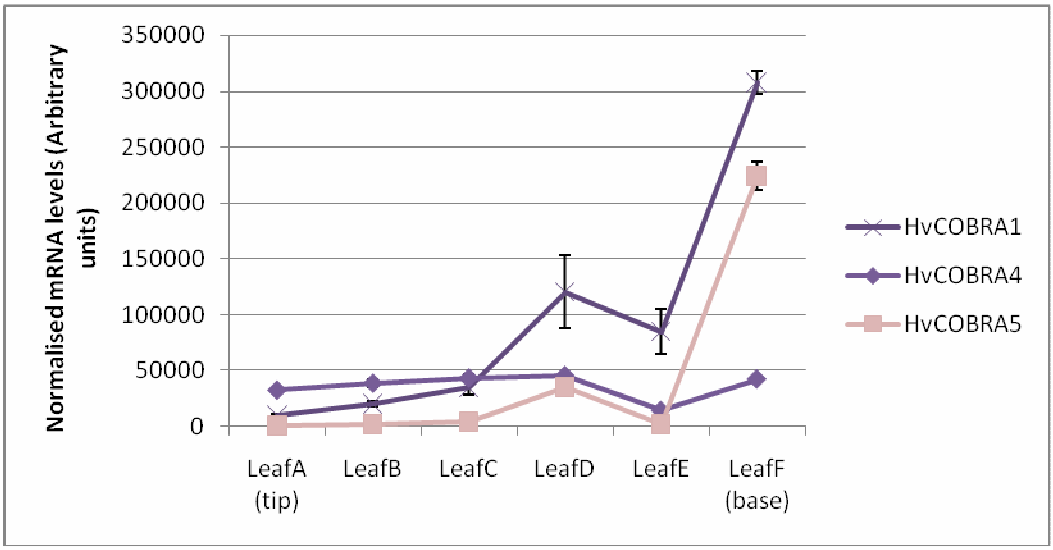

Figure 6. The mRNA levels of *HvCOBRAs* in different sections of barley leaves

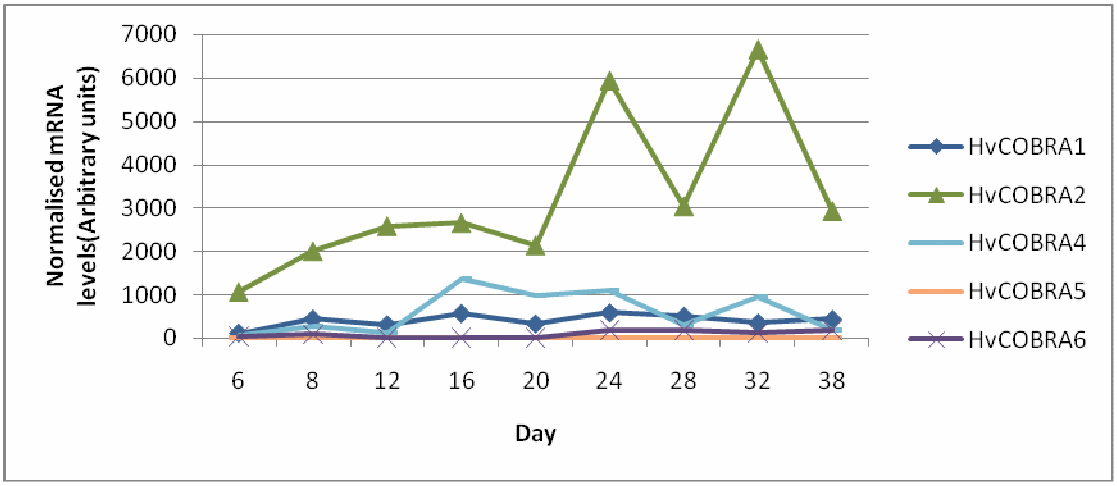

Figure 7. The mRNA levels of *HvCOBRAs* in developing barley endosperm

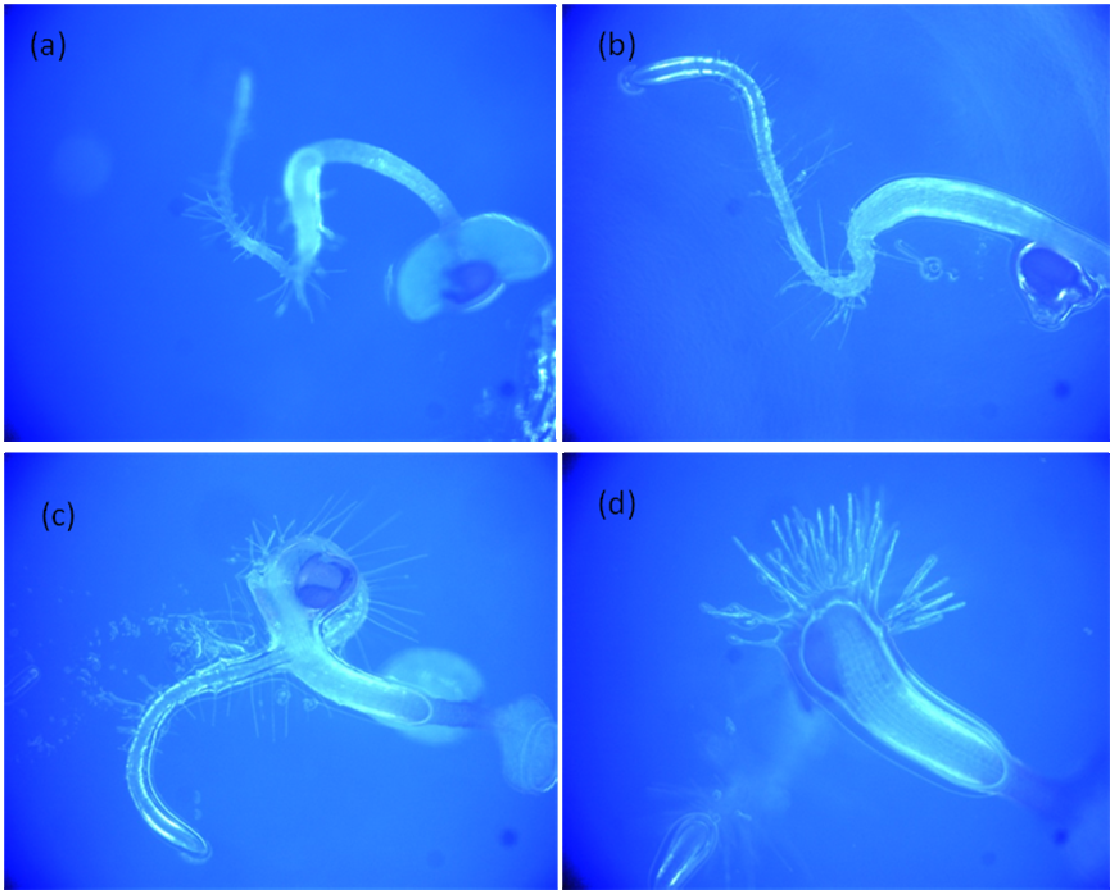

Figure 8. Microscopic examination showed different root hair growth between Salk_099933c mutant line (a and b) and wild type plants (c and d) 3 days after germination. (Scale: 400X)

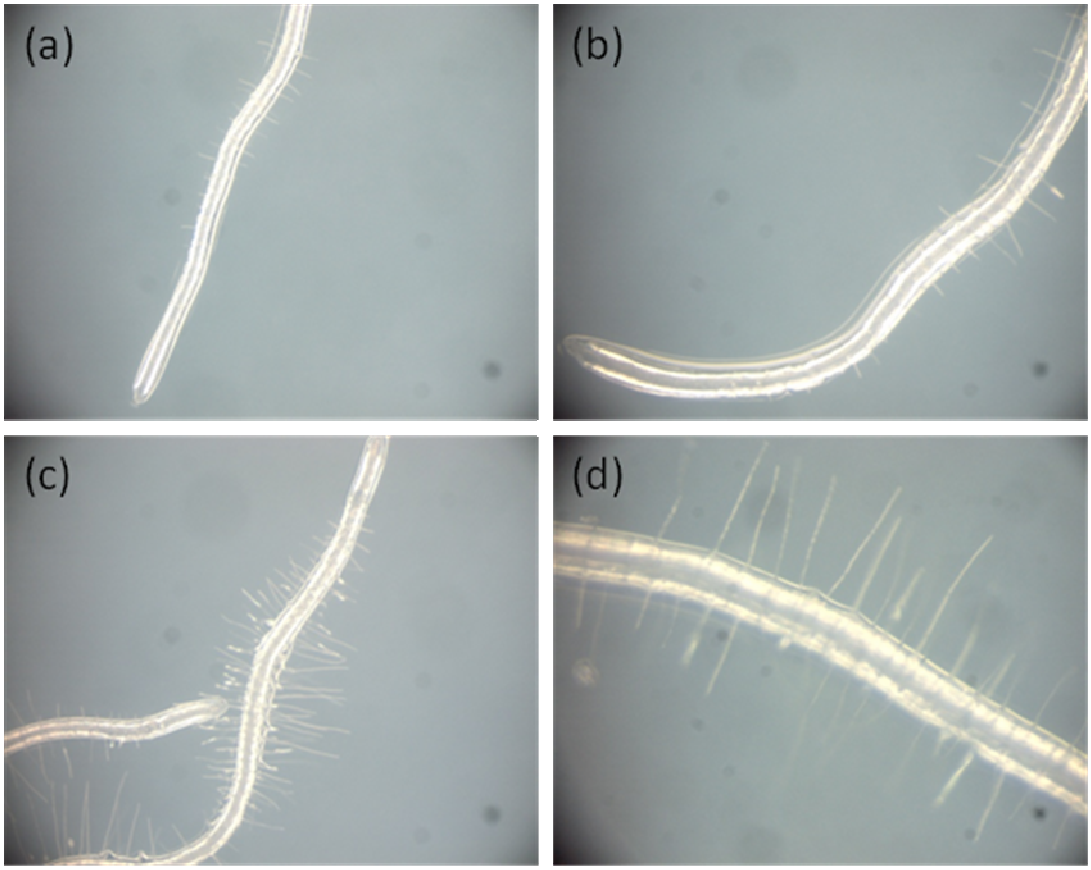

Figure 9. Microscopic examination showed different root hair growth between Salk_099933c mutant line (a and b) and wild type plants (c and d) 7 days after germination. (Scale: a and c 100X; b and d 400X)

Appendix list

Appendix:

Appendix A. Sequence details of *HvCOBRA* genes. The regions highlighted in yellow are sequenced. Start codons are highlight in green, stop codons are highlight in red. Primers designed for cloning the cDNAs are in red characters.

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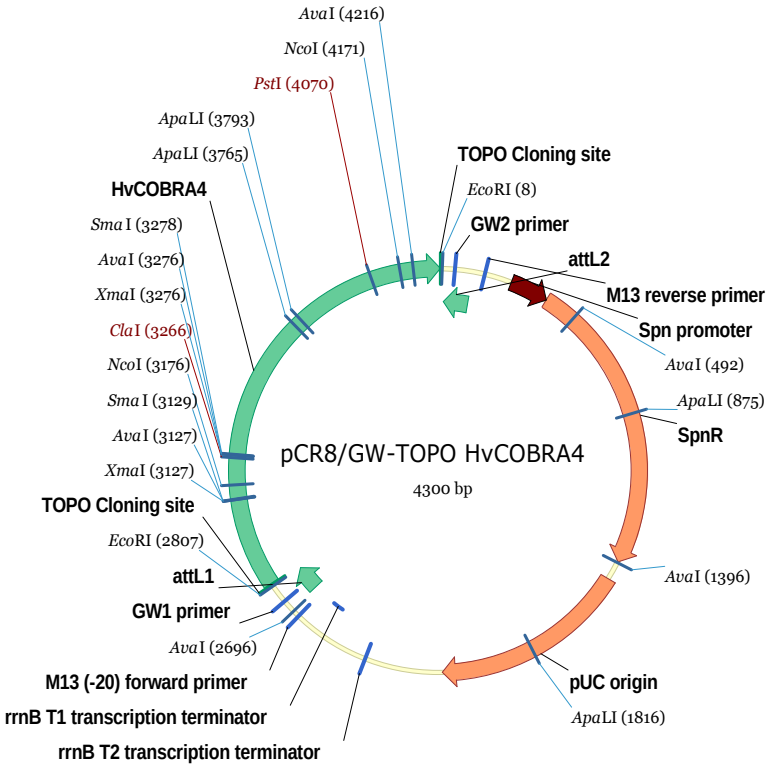
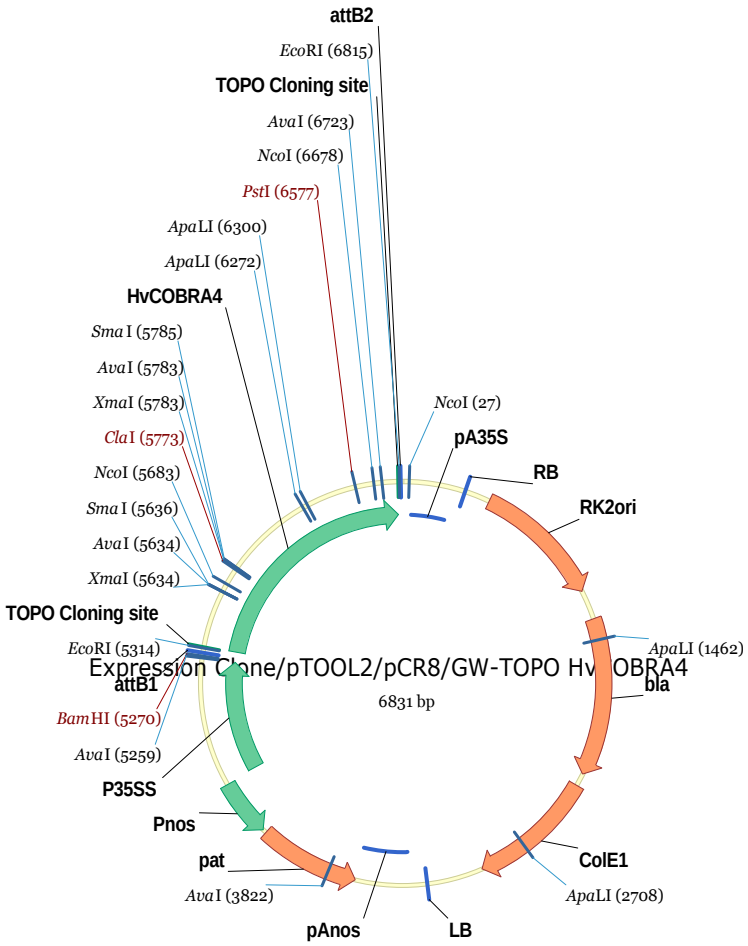
Appendix B. Construct of pCR8/*HvCOBRA4*



Appendix C. Construct of pTool2/pCR8/*HvCOBRA4*



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