Electronic Journal of Biotechnology ISSN: 0717-3458
http://www.ejbiotechnology.info
DOI: 10.2225/vol15-issue1-fulltext-6 *RESEARCH ARTICLE*

Cloning and expression analysis of peanut (*Arachis hypogaea* L.) CHI gene

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Received August 16, 2011 / Accepted November 27, 2011
Published online: January 15, 2012

Abstract Chalcone isomerase (CHI) is the key enzyme that catalyzes chalcone into (2S)-flavanol or (2S)-5-desoxidation flavanol. The full length cDNA (1050 bp) of *AhCHI* (*Arachis hypogaea* CHI gene) was cloned by large scale EST sequencing using a peanut immature seed cDNA library. Sequence analysis results indicated that it was a type I CHI gene (with the accession number JN660794). The ORF of *AhCHI* was 768 bp, encoding a peptide of 255 amino acids with a pI of 5.189. Sequence alignment showed that the coding region of *AhCHI* gene is highly conserved to compare with CHI genes from other plant species. Peanut cDNA microarray and semi-quantitative RT-PCR analysis indicated that *AhCHI* was highly expressed in pegs. The expression level in flower and root was higher than the expression level in stem and leaf. *AhCHI* was expressed in a high level in seeds with a purple seed coat, while its expression was low in seed with white seed coat.

Keywords: *Arachis hypogaea* L., cDNA library, chalcone isomerase, expression analysis

INTRODUCTION

Flavonoids, widely presents in plants, is a large class of secondary metabolites which derived from phenylpropanoid. Flavonoids plays an important role in keeping normal physiological activities in plants, such as the formation of colour in plants (Koes et al. 2005), UV radiation protection, resistance to pathogens (Winkel-Shirley, 2002), pollen development, plant hormones transportation (Bogs et al. 2006) and the interaction between legume roots and rhizobium. Anthocyanin is a kind of flavonoid, which is a main component of the pigment in petals and fruits. As a natural pigment, it widely used in medicine, food and chemical industry. Many enzymes including C4H (cinnamate-4-hydroxylase), CHS (chalcone synthase), CHI, DFR (dihydroflavonol-4-reducatase), and ANR (anthocyanidin reductase) were involved in anthocyanin biosynthesis pathway. CHI is the first enzyme of the flavonoid synthesis pathway catalyzing chalcone into (2S)-flavanol or (2S)-5-desoxidation flavanol.

Two major types of CHI gene, type I and type II, has been identified. Type I CHI could catalyze 6-hydrox-chalcone into (2S)-flavonoid or (2S)-5-desoxidation flavonoid. Type I CHI was found in most of plant species such as barley, rape, *Arabidopsis*, and rice (Liu et al. 1995; Druka et al. 2003). Type II CHI, which was mainly found in leguminous plants could catalyze both 6-hydrox-chalcone and 6-deoxidation-chalcone into (2S)-flavonoid or (2S)-5-desoxidation flavonoid. CHI genes from many different plants such as corn, soybean, rice, barley, lucerne, *Saussurea medusa*, fenugreek and carnation have been cloned (Forkmann and Dangelmayr, 1980; Bednar and Hadcock, 1988; Grotewold and Peterson, 1994; Mckhann and Hirsch, 1994; Druka et al. 2003; Li et al. 2006b; Qin et al. 2011).

The expression pattern of CHI gene varied in different plant species. The expression of CHI in the same species was regulated in a developmental or tissue specific manner. When the promoter of petunia CHI gene was mutated the expression level of CHI gene was decreased and resulted in the accumulation of chalcone in the pollen, which led to yellow- or green-coloured pollen (Van Tunen et al. 1991). Over expression of petunia CHI-A gene in tomatoes resulted in a drastic increase of flavonoid content in the pericarp and pulp of the transgenic tomato fruit without causing any other phenotypic defects (Muir et al. 2001). Blocking the flavonoid synthesis pathway by inactivation of CHI gene in onion resulted in high level of chalcone accumulation and reduced amount of flavonoid, and ultimately generated a yellow corn (Kim et al. 2004). Using RNA interference to directly inhibit the expression of CHI gene, the content of flavonoid decreased, the pollen and petal was faded (Nishihara et al. 2005). In addition, CHI affects the seed coat colour of Arabidopsis (Kim et al. 2007). CHI gene is important in flavonoid metabolism and it is a good candidate for fruit nutrition, flower and fruit pigmentation modification through gene engineering approach. Understanding the expression and regulation mechanism of CHI gene is also significant for plant abiotic or biotic stress resistance.

The colour of peanut seed coat varied from white to pink, brown, purple and black, which may reflect the content of anthocyanin in seed coat. In the present study the full length cDNA of CHI was cloned from peanut and the expression of this gene was analyzed. The results may provide useful information for understanding the molecular mechanism of peanut seed coat pigmentation.

MATERIALS AND METHODS

Plant materials

Peanut varieties Luhua14 (LH14), FT001, and FZ001 were from High-Tech Research Center in Shandong Academy of Agricultural Sciences. Zhonghua9 (ZH9) was from Oil Crops Research Institute of Chinese Academy of Agricultural Sciences. Peanuts used for this research were all grown in the farm. Root, stem, and leaf were collected from two weeks old seedlings. Flower, peg, and seed with seed coat were collected from plants grown in the same farm.

cDNA library construction and EST sequencing

The mRNA used for constructing peanut cDNA library was extracted from LH14 immature seeds with different developmental stages. 200-500 mg peanut seeds were used for total RNA extraction, following the protocol of RNAgent kit (Promega). Separation and purification of mRNA were carried out according to the protocol of PolyATtract mRNA Isolation Systems kit (Promega). The methods of cDNA library construction and EST sequencing were described in our previous paper (Bi et al. 2010).

EST sequence and protein analysis

Low quality sequences with too many unreadable nucleotides, sequences less than 300 bp, vector and primer sequences were removed manually. Cluster analysis of ESTs was done by Cap3 (with default parameters), the resulted contigs and singletons were annotated by BLASTX (with *E* value < 10^{-10}) against the NCBI database. ORF was predicted by Lasergene SeqMan II Module (DNAStar). A phylogenetic tree was constructed using MEGA 3.1 software based on deduced protein sequences of plant CHI homologs. A bootstrap analysis was carried out and the robustness of each cluster was verified in 1,000 replications. Multiple alignments were performed by ClustalW 1.83 software. The protein sequences were aligned by BoxShade program. The protein three-dimensional structure was simulated using SWISS MODEL.

Expression analysis of CHI gene

Total RNA was extracted from peanut roots, stems, leaves, flowers, pegs and seeds. The RNA quality was confirmed by measuring the ratio of optic density at 260/280 nm. The ratio of all samples was ranged from 1.8 to 2.0. The ratio of sharp 28S and 18S rRNA bands was approximately 2:1 in 1% non denaturing agarose gel. First-strand cDNA wassynthesized using PrimeScript 1st Strand cDNA Synthesis kit (TaKaRa) using 5 μg total RNA as template. Forward primer (5'-CCGACAAGACCTTCTTCCT-3') was located on the upstream of *AhCHI* (*Arachis hypogaea* CHI gene)

first exon and the reverse primer (5'-GAGATCCGTTGGGTAATAGTG-3') was designed on the downstream of *AhCHI* second exon. The target fragment was amplified using 2 x PCR Master Mix (TaKaRa) by the following program: 94ºC for 5 min, and then 30 cycles at 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 50 sec, with a final extension at 72ºC for 5 min. Actin was used as a loading control.

RESULTS

Cloning of *AhCHI* full length cDNA

More than 17000 peanut ESTs were sequenced from the peanut cDNA library. Among these sequences one singleton showed high similarity (82%) to alfalfa CHI gene (BT052270). It was considered as *AhCHI* (with the accession number JN660794). The full length of *AhCHI* cDNA was 1050 bp with an ORF of 768 bp, encoding a peptide of 255 amino acids with pI of 5.189. The overall structure of CHI resembles an upside-down bouquet that adopted an open-faced β-sandwich. A large β-sheet and a layer of α-helices comprise the core structure. Three short β-sheets were located on the opposite side of the large β-sheet. The three-dimensional fold and enzymatic activity of CHI were unique to plant kingdom (Jez et al. 2000). The predicted structure of *AhCHI* protein was consistent with the model described above (Figure 1).

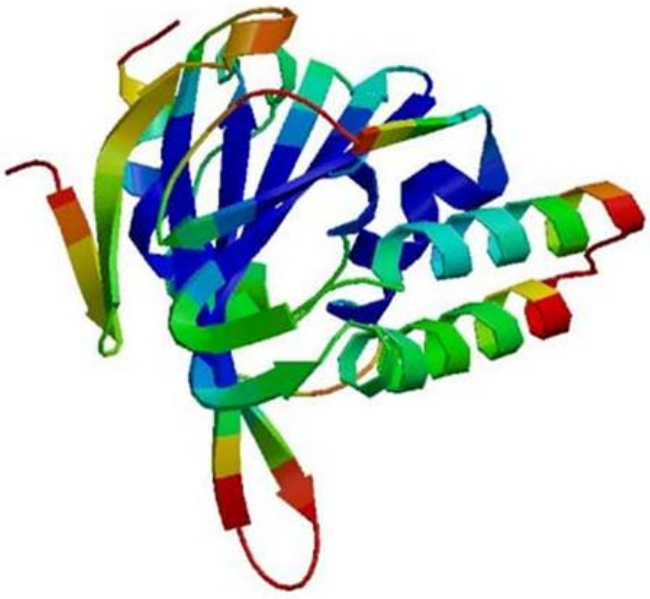

Fig. 1 The predicted three-dimensional structure of *AhCHI* protein by SWISS-MODEL.

Protein sequences comparison of CHI from 13 different plant species showed that these proteins were highly conserved especially within the 200 aa chalcone domain, the substrate binding domain and the catalytic site. The sequences of N- and C-terminal varied significantly both in length and the composition of amino acids, which could be associated with cellular localization and species-specific function. The genomic structures of type I and type II CHI were quite different. Most of the Type I CHI gene contained four exons and three introns, while type II CHI contained three exons and two introns. In addition, type II CHI was only found from leguminous plants, while type I CHI was found in almost all plant species. The decisive amino acid residues for substrate binding were distinct in these two types of CHI (Figure 2). *AhCHI* identified in this study was considered as type I CHI based on the comparison of amino acid sequences. The cloning and characterization of peanut type II CHI is in progress.

Phylogenetic analysis of *AhCHI* and CHIs from other plants

Eighteen CHI proteins from different plant species were analyzed and the Phylogeny-neighbour joining tree was generated using MEGA software through phylogeny-bootstrap Test. The result showed that *AhCHI* protein was most closely related to CHIs of soybean and alfalfa. *AhCHI* also showed high similarity to CHIs of *Lotus japonicas*, *Gossypium hirsutum* and *Populus trichocarpa*. Type I and type II CHIs were grouped in different clusters. Type II CHIs were all from legumes with a short genetic distance. The sequence of type I CHI from snapdragon varied greatly from type I CHI from other plants. CHI of monocots such as corn and rice were clustered together in one class. CHIs from plants within the same family showed high sequence similarity, for examples, tobacco and petunia, soybean, *Lotus japonnicus* and alfalfa (Figure 3).

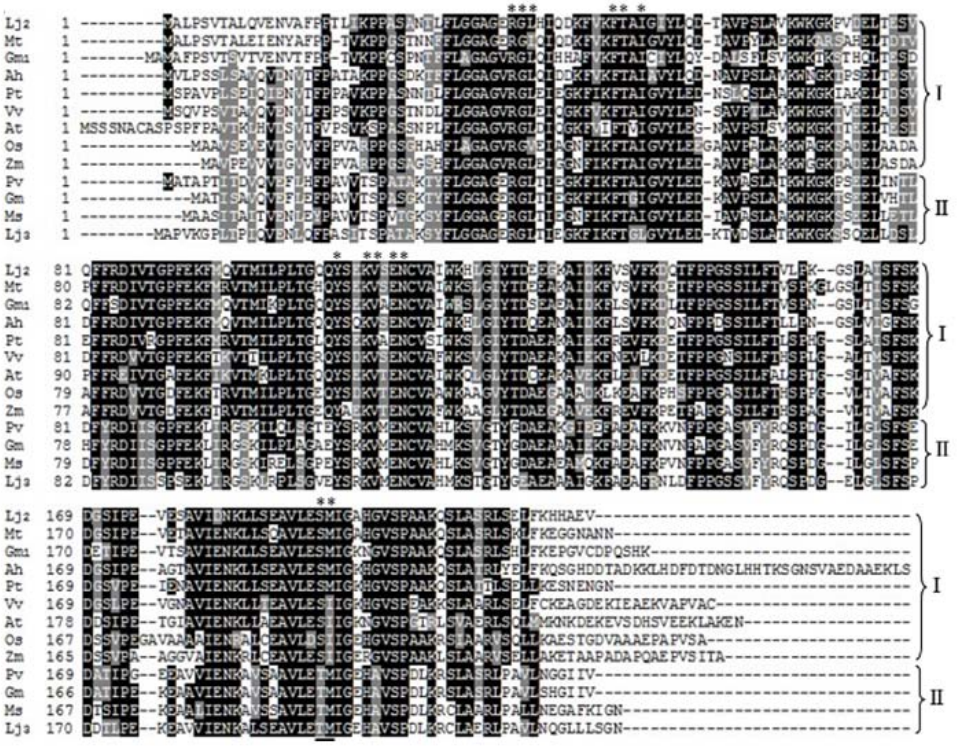

Fig. 2 Amino acid sequence alignment of CHI proteins from different plant species. Residues highlighted in black and gray represent identical and similar amino acids, respectively. The residues that compose the active site are indicated with asterisks. Underlined residues were proposed to affect substrate preference. Abbreviations and accession numbers, Os: *Oryza sativa*, AF474922; Zm: *Zea mays*, BT040681; Lj: *Lotus japonicus*, Lj2 (AB054802), Lj3 (AB073787); Mt: *Medicago truncatula*, BT052270; Gm: Glycine max, Gm (AF276302) Gm1 (BT089600); Pt: *Populus trichocarpa*, XM_002315222; At: *Arabidopsis thaliana*, NM_115370; Vv: *Vitis vinifera*, X75963; Pv: *Phaseolus vulgaris*, X16470; Ms: *Medicago sativa* M91079.

Expression analysis of *AhCHI*

We analyzed the expression level of *AhCHI* in different tissues of peanut using cDNA microarrays. The results indicated that the expression level of *AhCHI* was significantly higher in pegs than that in any other tissues. The expression level in pegs was about 2 folds as the expression levels in roots, flowers and seeds. The expression level of *AhCHI* was relatively low in stems and leaves to compare expression levels in other tissues (Figure 4). To confirm the cDNA microarray results, *AhCHI* specific primers were designed for semi-quantitative analysis of *AhCHI* expression. RT-PCR results indicated that a strong amplification of *AhCHI* was detected in pegs, while the amplification of CHI in stem was very weak (Figure 5a). The expression pattern revealed by RT-PCR was consistent with the microarray results.

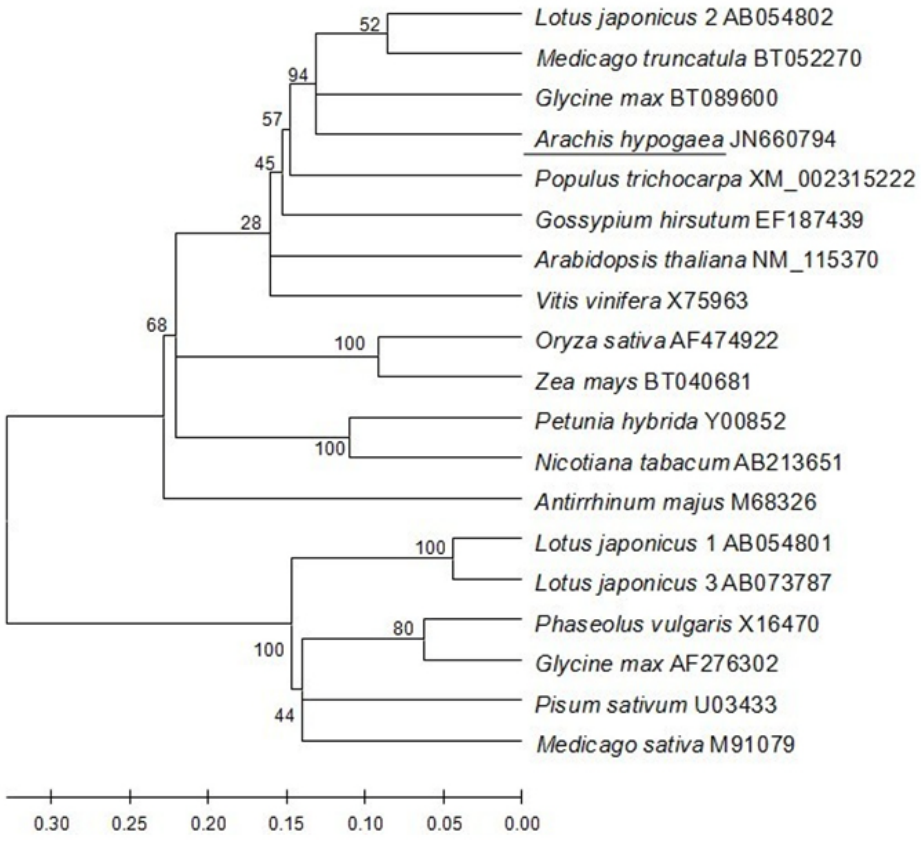

Fig. 3 Phylogenetic tree of *AhCHI* and CHI from other plant species. The ruler represents expected number of substitutions per site, 0.1 means 10% changes were observed between two sequences.

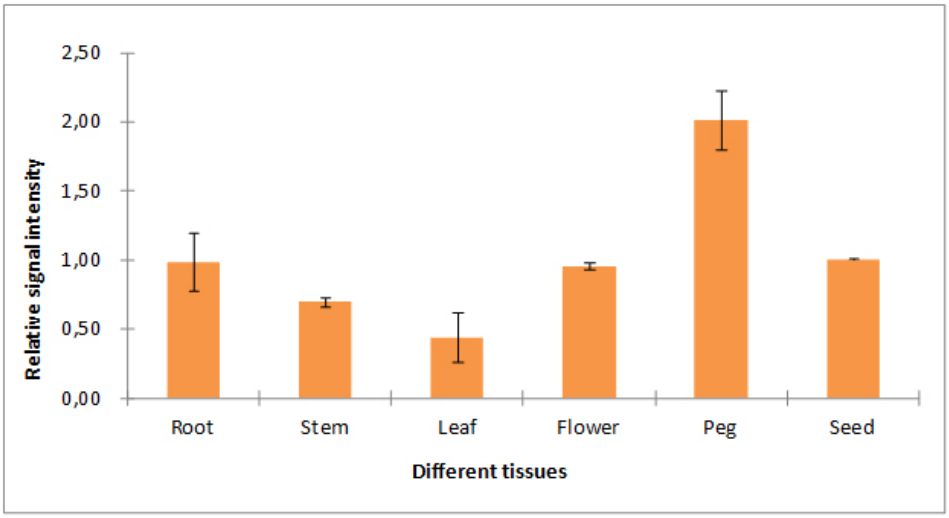

Fig. 4 The expression analysis of *AhCHI* in different tissues using cDNA microarray.

The peanut cultivars Luhua14 (LH14), FT001, FZ001 and Zhonghua9 (ZH9) seeds (with seed coat) were selected to analyse the expression of *AhCHI*. The seed coat colour of these cultivars were pink, dark red, white and black (Figure 5b), respectively, while their seeds were all white. RT-PCR showed that the expression level of *AhCHI* were significantly different between these cultivars. The expression of *AhCHI* was barely detected in the white seed cultivar FZ001. A low level expression of *AhCHI* was observed in FT001, while the expression levels of *AhCHI* in LH14 and ZH9 were very high (Figure 5c).

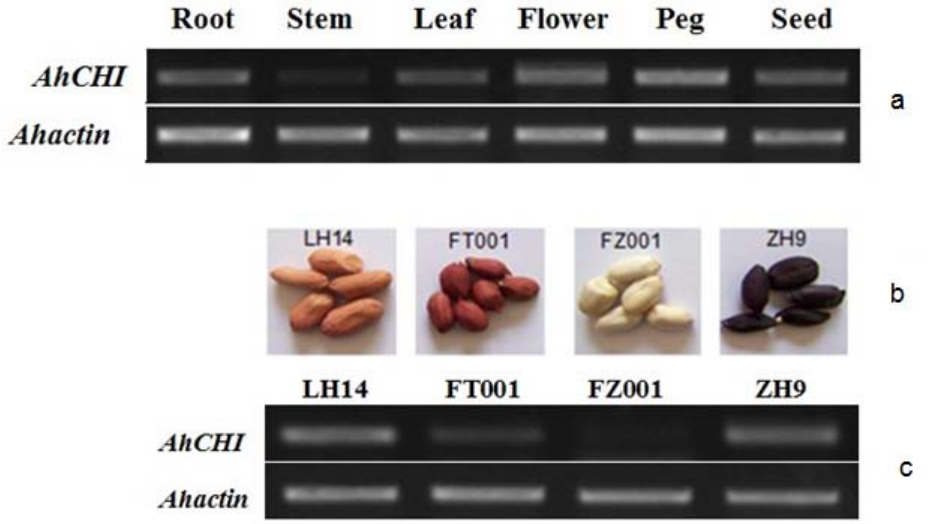

Fig. 5 RT-PCR analysis of *AhCHI* gene expression. (a) The expression level of CHI in different tissues of LH14; (b) Seed coat colour of different peanut cultivars; (c) The expression of *AhCHI* in seed of different cultivars.

DISCUSSION

In this study, we detected the expression level of *AhCHI* in different tissues and cultivars. *AhCHI* expressed highly in pegs where the accumulation of anthocyanin was high, indicating the tissue specific expression of *AhCHI*; *AhCHI* expressed much higher in seeds with black, dark red, pink seed coat than in seeds with white seed coat. This result indicated the positive correlation between *AhCHI* expression and anthocyanin accumulation in different tissues or organs.

As a key enzyme in flavonoid biosynthesis pathway, CHI has been studied extensively in terms of modifying the flavonoid biosynthesis pathway. Over expression of CHI gene from Scutellaria and *Glycyrrhiza uralensis* led to increased production of flavonoids in hairy roots (Zhang et al. 2009; Park et al. 2011b). Reducing the expression level of CHI in carnation, *Callistephus chinensis* and cyclamen caused over accumulation of chalcone and led to yellow flowers (Kuhn et al. 1978; Forkmann and Dangelmayr, 1980; Miyajima et al. 1991). Transferred the sense CHI of *Saussurea medusa* into tobacco, the transgenic plants accumulated five times more flavonoid than the control. The major flavonoid accumulated in the transgenic tobacco was routine. While expression of the CHI antisense sequence in tobacco caused significant reduction of flavonoid in transgenic plants (Li et al. 2006a). Anthocyanin synthesis is a complex process which requires the participation of many key enzymes (Wei et al. 2011) and different types of transcription factors (Ohno et al. 2011; Park et al. 2011a). The expression of the genes encoding enzymes or the transcription factors were highly regulated by environmental signals, such as light and temperature. Light and UV-A could induce the expression of CHI in turnip roots and caused anthocyanin accumulation (Zhou et al. 2007); CHI-A expressed in the petunia seedlings grown under the ultraviolet radiation. During the development of corolla, corm and anther the expression of CHI was regulated by light and was induced by ultraviolet radiation (Van Tunen et al. 1989). Over expression of Antirrhinum MYB and bHLH family transcription factors in tomato led to significant increased anthocyanin content in transgenic tomato and generated purple colour tomato fruits (Butelli et al. 2008).

Due to the nutritional and physiological roles of flavonoids, it is important to understand flavonoid pathway in crop plants including the oil plant peanut. Cloning and characterization of genes encoding key enzymes or transcription factors is the first step to understand the regulatory mechanisms controlling flavonoid biosynthesis in peanut. It will be helpful in discovering new strategy for efficient genetic modification of peanut to alter flavonoid content for nutritional purpose as well as stress tolerance.

Financial support: Foundation for Excellent Young Scientists of Shandong Province (2006BS06008), National Natural Science Foundation of China (30871324, 31000720), Shandong Province Natural Science Foundation (ZR2010CZ002, ZR2011CZ002,Y2008D44, ZR2010CQ008), Shandong Province "Taishan Scholar" foundation.

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How to reference this article:

ZHANG, Y.; XIA, H.; YUAN, M.; ZHAO, C.; LI, A. and WANG, X. (2012). Cloning and expression analysis of peanut (*Arachis hypogaea* L.) CHI gene. *Electronic Journal of Biotechnology*, vol. 15, no. 1. http://dx.doi.org/10.2225/vol15-issue1-fulltext-6