TITLE: Genetic characterization of flowering and phytochrome genes in peanut (Arachis hypogaea L.) for early maturity

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Abstract
Background Peanut (Arachis hypogaea L.) production and cropping pattern is highly influenced by the climatic factors including temperature and rain pattern fluctuations. It is one of the most important cash crop in the rain fed areas of Pakistan and its production, under changing climatic conditions, that can be improved by developing short duration varieties. The present study was based on the molecular characterization of the maturity associated gene families in the peanut under twolight conditions.

Methods and results.Genomic analysis based on the in silico study of important gene families for early maturity associated attributes like flowering time, their pattern, duration and photoperiodism was done for a comprehensive mapping of maturity related genes. Phytochromes genes Phy A, Phy B and Phy E and flowering genes FT2a, Ft5a and COL2 were selected for in silico characterization for protein based analysis including Multiple Sequence Alignment (MSA), and Neighbor Joining (NJ) tree. MSA and NJ trees of the peanut with Arabidopsis thaliana and Glycine max showed a clear picture of the phylogenetic relationship on the basis of selected gene proteins. Expression profile of phytochrome and flowering genes revealed that photoperiod conditions i.e. short and long days, have great influence on the Phy A, Phy B and Phy E, Ft2a, FT5a and COL2 gene expression pattern. In current study, the relative expression of all studied genes was found higher in short day light condition at flower initiation stage of the plants than in the long light day condition with exception of COL2 gene protein.

Conclusions. The molecular characterization based on the in silico study of the particular genes and qPCR based gene expression profiling of the selected genes provided an evidence of the role of these genes and their comparative analysis under two photoperiodic conditions.

Keywords: Cropping pattern, Short duration, Phylogenetic relationship, Gene expression

Introduction

Changing climatic conditions have reduced plant productivity and generated food security issues. In the context of food security and alleviation of hunger, peanut exhibits promising components which make it an exceptional food to meet nutritional needs [1]. In Pakistan, this oilseed legume crop is cultivated approximately in an area of 102.9 thousand ha, with a total production of 94.5 thousand tons [2]. Although peanut is an important oilseed crop in the region, still its per acre yield is very low in Pakistan due to multiple factors including unavailability of high yielding, short-duration varieties, unprecedented climatic conditions, low rainfall, and farmers' management problems [3].

Flowering time and its plant's responses under different photoperiodic conditions have a key role which influence crop yield [4]. Genetic exploitation of the peanut genome for flowering and light responses provides an insight about the genes associated with the early maturity. Maturity is a quantitative trait and is influenced by multiple genes and environmental factors [5]. The most important physiological components associated with peanut early maturity are the flower timing and photoreceptors [6 –8]. Peanut genome has recently been sequenced, and despite the discovery of very important genes related to allergens and biosynthesis, still, the genes associated with flowering and maturity have not been properly identified and reported. Arachis hypogaea is an allotetraploid derived from crosses between A. ipaensis K30076 Å~ A. duranensis V14167 (2n = 4x = 40) [9]. Light signaling pathway and flowering play a critical role for development and early maturity of the peanut pods. Phytochromes are sensors through which plants receive information about the immediate environment, the changing environmental conditions and day length. Whereas, the characterization of molecular mechanisms of light signaling involved in plant development has not been reported extensively [10].

All the legumes have a standard complement of three main phytochromes (phyA, phyB, and phy E) which play a crucial role in the flowering pattern and maturity of the plants [11]. In Arabidopsis the CONSTANS protein integrates light and circadian photoperiod signaling [12]. In Arabidopsis and rice more than 100 genes which control flowering time have been identified and their functions and interactions has been studied [13 , 14]. The role of set of FT genes are very important to understand the mechanism of flowering control. The integration of environmental signals, for flowering and the signaling from the site of photoperiod detection in the leaves to particular flower formation sites at the shoot apex, is very well documented [15]. These gene families have been studied in detail in soya bean, pea and Medicago, Lotus [16 , 17]. Most legume species contain at least five FT-like genes in three different clades including FTa, FTb, and FTc [18]. The wellknown E series of maturity loci (E1 to E9) confer early flowering and maturity, particularly under non-inductive (LD) conditions in soya bean [19 , 20].

Genomic diversity and the evolution of peanuts serve as a genomic basis for improving peanut cultivars [21]. Since analysis of gene expression and protein accumulation patterns are very important components for authentication of particular attributes associated with early maturity [22].The molecular profiling on the basis of in silico and expression analysis for the selected gene families is required to provide an overview of the genetics of early maturity in peanut to meet the stress exerted by climatic changes. The current study is mainly based on the in silico characterization of members of Phy and FT family genes and COL2 gene for their phylogenetic relationship and protein conserved domain analysis. Furthermore this study explores the gene expression profile of the selected genes under short day and long day light conditions in peanut.

Materials and methods

Data collection and selection of phytochrome and maturity related genes and their proteins
The targeted phytochromes and flowering time genes were first of all searched through literature review from the model plant Arabidopsis and soya bean (Glycine max) and FASTA sequences of Phy A, PhyB and Phy E like, FT2a and FT5a, COL2 genes and reference gene ACTIN were retrieved from the NCBI (https:// www. ncbi. nlm. nih. gov). The selected FASTA sequences were then blast in the peanut base to find the top similar sequences for further protein alignment for peanut (Arachis hypogaea). Protein sequences of Arabidopsis thaliana for aforementioned genes were obtained from the Arabidopsis Information Resource (TAIR) database (http:// www. arabi dopsis. org/) and soya bean from the phytozymes (https:// phyto zome. jgi. doe. gov/ pz/ portal. html).

Multiple sequence alignment and phylogenetic analysis To investigate the phylogenetic relationships among selected proteins which are related to maturity including Phy A, Phy B, Phy E, COL2, FT2a and FT5a, amino-acid sequences of respective gene proteins were retrieved from Arabidopsis, peanut and soya bean databases. Multiple Sequence Alignment (MSA) and Neighbor Joining (NJ) phylogenetic tree on the basis of identified amino acid sequences was constructed in Geneious software.

Conserved domain analysis
NCBI based interface i.e. CD-Search, was used to search the conserved domains of query protein sequences. RPSBLAST, was used to examine a set of pre-calculated position-specific scoring matrices (PSSMs) with a protein query. qPCR primer designing

Expression study was conducted through reverse transcriptase real time PCR using gene specific primers. Gene specific primers for qRT-PCR analysis of AhphyA, AhphyB, AhphyE-like, Col2a, FT2a and FT5a along with a reference gene actin were designed using NCBI primer website: http:// www. ncbi. nlm. nih. gov/ tools/ primer- blast/ index. cgi. Further verification of the primers for GC contents, melting temperature and self-complementarity were analyzed through sigma https:// www. sigma aldri ch. com/ confi gurat or/ servl et/ Desig nTool? prod_ type= STAND ARD) for aforementioned parameters. To verify the non-specific products, that can interfere with qPCR and not give an accurate result, both forward and reverse primers were aligned to the nucleotide sequence of the respective genes. The primer sequences are listed in Table 1.

Primer efficiency
Primers that gave no dimers and efficiencies more than 85% were selected for qPCR assay. Efficiency of each primer was determined by a dilution series of 10 DPA fiber cDNA. Efficiency of each primer was determined by a dilution series of 0.01 ng, 0.1 ng, 1 ng, 10 ng, 100 ng cDNA. The specificity of real time PCR was confirmed by a single peak in the melting temperature curve analysis. The formula used was' = 2.718^(-1/Y). It should give a value that's ideally around 2, which is 100% efficient. The charts with trend line to calculate Y was made using ct values of each primer against different concentrations.

Plant materials (sampling)
The peanut plant genotype PI 542961 01 SD was grown in growth chamber in short day (8 h light, 16 h dark) and long day (16 h light and 8 h night) light conditions in small pots.
Two different growing conditions were used to analyze the peanut plant response under different photoperiodic conditions based on the selected genes. The complete data about the genotype is available in the mentioned link https:// npgsw eb. ars- grin. gov. During light hours, at flower initiation stage the leaf samples were collected, in liquid nitrogen in Dewar

flask, using sharp blade from the plants placed in the short day and long day light conditions. The samples were then immediately stored in − 80 ÅãC freezer for mRNA extraction.

RNA extraction, cDNA synthesis and qPCR run

Total RNA was extracted from the frozen leaves using Trizol Reagent (TaKaRa, Dalian, China). mRNA was isolated and purified using DNase I to degrade DNA contamination. To quantify the RNA and check its purity, each sample (1 µL), after blank (1 µL), was tested on nanodrop and concentration was measured in ng/µL. The ratio of 280/260 concentrations was observed and values ranging near 2 were found good for better quality RNA.

The 1st strand cDNA was synthesized using Primer- ScriptTM 1st Strand cDNA Synthesis Kit (TaKaRa) and oligo (dT) primer. Gene-specific primers for qRT-PCR analysis were used. FastStart Universal SYBR Green Master Mix (Roche, USA) was used for qRT-PCR. The reaction mixture was consisted of 2 µL of 50 Å~ diluted 1st cDNA, 0.5 µL of 10 µmol of each primer and 10 µL 2 Å~ FastStart Universal SYBR Green Master Mix. An ABI 7500 real-time PCR system was used under the following program: 95 ÅãC for 10 min, then 40 cycles of 95 ÅãC for 15 s and 60 ÅãC for 1 min. Peanut actin was used as an internal control for normalization. Non-specific products were identified by melting curve analysis. The 2-delta delta CT method was used to analyze the relative expression level [22].

Results

Phylogenetic analysis

The Phytochrome, FT family and COL genes are not reported as studied genes in the peanut data base. Henceforth, analysis of these genes to find out their linkage distance and evolutionary pattern is of significant importance. The homologs of selected proteins are divided into three major clades. Gene for each protein is distributed in a different clad. Arachis hypogaea Actin and Phy E proteins are more close to the Arabidopsis Actin and Phy E protein whereas the other four gene proteins of Arachis are closely linked with the Glycine max protein in three different clades (Fig. 1).

Conserved domain analysis

The conserved domain analysis of the selected gene proteins is shown in Fig. 2. Conserved domain analysis of Phy A, Phy B and Phy E proteins showed domains of PAS_2, PAS, HisKA, and HATPase containing proteins. In case of FT2a and FT5a proteins, conserved domain, Phosphatidyl Ethanolamine-Binding Proteins (PEBPs), is involved in regulation of flowering plant stem architecture. In case of COL2 proteins, three conserved domains were identified including CCT motif, found in a number of plant proteins, and two B-box-type 1 zinc finger in B-box (BBX) family, which is involved in seedling photomorphogenesis, photoperiodic regulation of flowering, shade avoidance, and responses to biotic and abiotic stresses in plants. In Actin protein, nucleotide-binding domain of the sugar kinase/HSP70/actin superfamily was identified as conserved regions. These proteins of related superfamily are involved in the closure of catalytic site cleft.

Gene expression analysis

Primer efficiency Efficiency of each primer was determined by a dilution series of 0.01 ng, 0.1 ng, 1 ng, 10 ng, 100 ng cDNA. The charts with trend line to calculate Y was made using ct values of each primer against different concentrations (Fig. 3a–c). All the selected gene primers showed their values in specific range (value around 2) and were further used to analyze the gene expressions. Gene expression analysis of six selected genes at flower

initiation stage qPCR based expression analysis of six genes, to anlyse their responses under short day and long day light growing conditions, was carried out to investigate the protein accumulation in plants for phytochrome' response and flowering time which are associated with the early maturity in peanut. Relative transcripts abundance of selected genes was examined in peanut at time of initiation of flowering in long days and short days light on the basis of CT values of selected gens with the reference gene Actin (Fig. 4). The graph bars with standard error shows relative abundance of each gene in short day and long day conditions and the expression of all studied genes was found relatively higher in plants grown under short duration except COL2 gene.

The heatmap analysis

This heat-map shows qPCR expression analysis using average linkage clustering method by Euclidean distance measurement algorithm (Fig. 5). Proteins FT5a and FT2a showed values below mean average on short day length and above mean average on long day length that is why they are placed together in a separate clade (Fig. 5). COL2 protein did not show any similarity in trend with other proteins and is placed in a separate clade in both short day and long day conditions. Its value is below mean average in Short Day Light (SDL) and above mean average in Long day Light (LDL) conditions. Phy A Phy B and Phy E proteins are placed together in single cluster for both SDL and LDL conditions on the basis of their expression profile. These proteins have positive Z score in short day light conditions and negative Z score in the long day light conditions. Similarly, the other gene family members FT2a, FT5a show a distinct trend on the basis of their expression and are grouped in a separate clade. These gene proteins show a negative Z score in short day light conditions and positive Z score in the long day light conditions. COL2 proteins was found in a separate clad for both long day and short day conditions with negative Z score in short day light conditions and positive Z score in the long day light conditions

Discussion

The current study was based on the identification, selection and expression analysis of maturity related and light sensitive genes in peanut. The gene homologs associated with maturity and light response were selected mainly from the Soya bean (Glycine max) and Arabidopsis. The phytochrome genes including Phy A, Phy B and Phy E and flowering genes including Ft2a, Ft5a and COL2 were selected to observe their expressions in two different light conditions (short day and long day). For the functional protein analysis, the predicted structures with protein conserved domains were observed as previous studies of Zhang et al. [22] for Phytochromes and Gao et al. [23] for patatin-like protein (PLP) family genes. Soya bean phytochrome genes were reported to be associated with the elongation of branches and transition of vegetative to reproductive phase and senescence during growth and development of plants [24]. Phy A was reported to express its protein for proper function at low fluorescence while Phy B and Phy E interplay in high irradiance. However, flowering is greatly influenced by light stimuli received by these photoreceptors or phytochromes, hence regulate photoperiod. Constans (CO) and flowering locus T (FT) genes were reported to activate the flower initiation [25].

On the basis of these evidences genes linked with maturity (flowering) and light sensitivity were selected and their primers were designed for expression profiling.

Expression profile of phytochrome and flowering genes revealed that photoperiod conditions i.e. short and long days, have great influence on the Phy A, Phy B and PhyE, COL2, Ft2a and FT5a gene expression pattern. In current study the relative expression of all studied genes was higher in short days condition at flower initiation stage of the plants than in the long days condition with exception of COL2 gene protein. However, COL2 gene showed more expression in the plants grown under long day conditions. Constans (CO) proteins are central for the circadian rhythms and their expression is

highly controlled by the light and dark period (photoperiod) in plants. It is highly influenced by the phytochrome gene family and triggers expression of FT genes [12 , 26]. In long day plants CO promotes the FT expression while in short day plants it acts as repressor for FT expression [27]. The phytochromes genes were classified in same clade in both short day and long day conditions which shows a close linkage of these genes in evolutionary pattern in peanut. Similarly, both FT 2a and FT 5a were grouped in same clade based on of expression profiling showing the same evolutionary linkage. Earlier reports of Zhang et al.
[22] on expression analysis Phy A, Phy B and Phy E of peanut leaves showed significant difference. Similarly, the pattern of expression of three phytochromes in the leaves
were found in the same manner as in the recent study. The natural variation in FT gene family involved with the flowering time and maturity was reported by Jiang et al. [28].
Flower time FT2a and FT5a are reported with different functions in controlling post-flowering stem growth [29]. It is observed from the current studies that the selected
genes has a correlation between their expression and photoperiodism (short day and long day light conditions). These genes including FT, Phy and Col has established a different pattern of expression under short day and long day light conditions. Henceforth, the genetics of the plants including flowering pattern and response of light receptors
and external factors, including light conditions for growth, have a major role in maturity of the crop under changing climatic conditions [30].

Conclusion

This research work has showcased very interesting aspects in peanut genome associated with early maturity genes and their expression. Genetic characterization of the peanut
genome for genes associated with flowering and light responses provided a correlation with the early maturity. The in silico data and gene expression profile provided an
insight in the peanut genome for flowering FT and photoreceptor Phy and COL genes. Therefore, evidences provided in current data can further be used for evaluation of peanut
genotypes for earliness study, for improvement of the peanut crop under changing climatic conditions, particularly in the rainfed areas of Pakistan.

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Figure Legends

Fig. 1 A Neighbor Joining phylogenetic analysis in peanut (Arachis hypogaea) Arabidopsis thaliana and soya bean (Glycine max) on the basis of the Phy A, Phy B, Phy E, COL2, FT2a and FT5a. The phylogenetic tree was constructed using Geneious. The homologs from three plant species were identified by homology searches in Gene Bank database using Arabidopsis proteins as entry. The clades were divided into three major groups, branches of which were showing varied protein clusters

Fig. 2 Protein conserved domains of a PhyA, PhyB, Phy E (PAS, BaeS and HATPase superfamily) b FT2a, Ft5a (PEBP superfamily) c COL2 (Bbox_SF, CCT superfamily), and d Actin (with NBD_sugar-kinase_HSP70_actin superfamily) using NCBI based interface i.e. CD-search

Fig. 3 The graph showed primer efficiency values calculated from the formula for a Phy A, Y = 1.775342377 with primer efficiency was 88.77% b Phy B, Y = 1.957214 with primer efficiency was

97.86% c Phy E, Y = 1.962538755 with primer efficiency was 98.13%
d COL2, Y = 1.979787047 with primer efficiency was 98.99% e FT2a, Y = 1.99860407 with primer efficiency 99.93% and f FT5a, Y = 1.784813992 with primer efficiency was 97.86%

Fig. 4 Quantitative reverse transcriptase analysis of maturity related genes in two different light conditions (Short days: SD and Long days: LD) at flowering initiation stage in peanut (Arachis hypogea). Genes include a Phy A, b Phy B, c Phy E, d COL2, e FT2a and f FT5a. Error bar represents Å} S.E. of three experimental replicates

Fig. 5 Heat map of six early maturity related genes in in short day light and long day light. 1. Phy A; 2. Phy B; 3. Phy E; 4. FT 5a; 5. FT 2A; 6. COL2.
Heat map depicting clusters of differentially expressed five genes in two different light conditions. The value of the z-score tells you how many standard deviations you are away from the mean. If a z-score is equal to 0, it is on the mean

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Figures

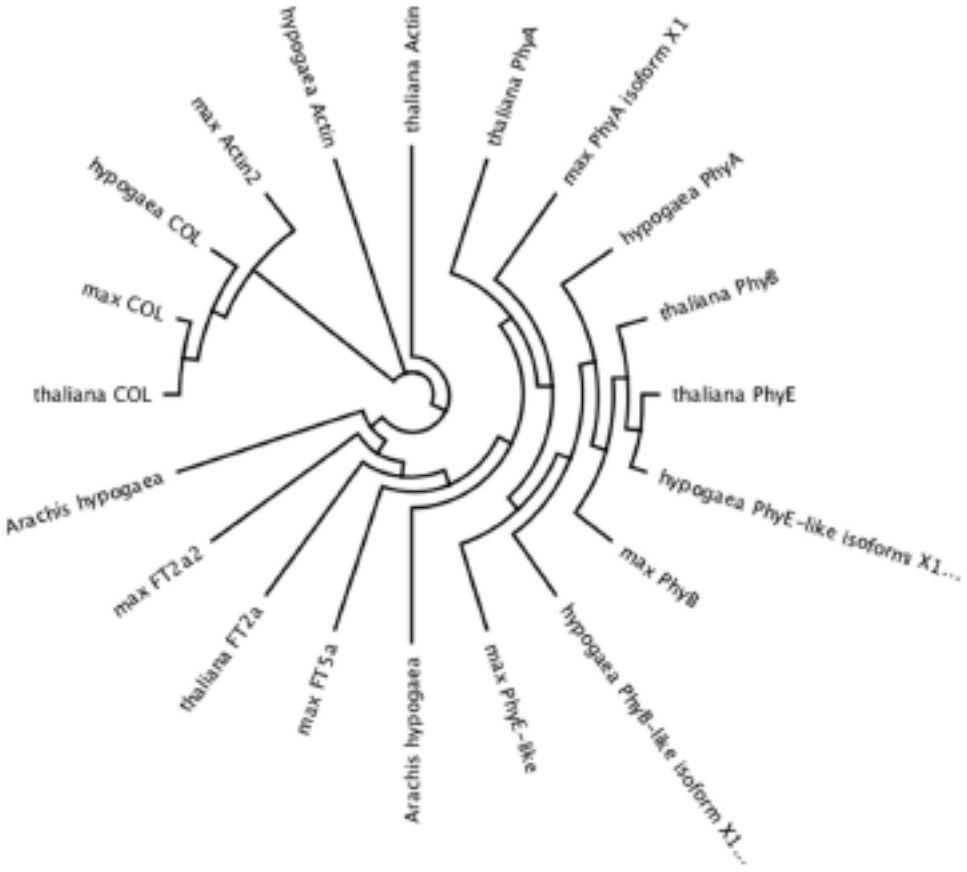


Figure 1

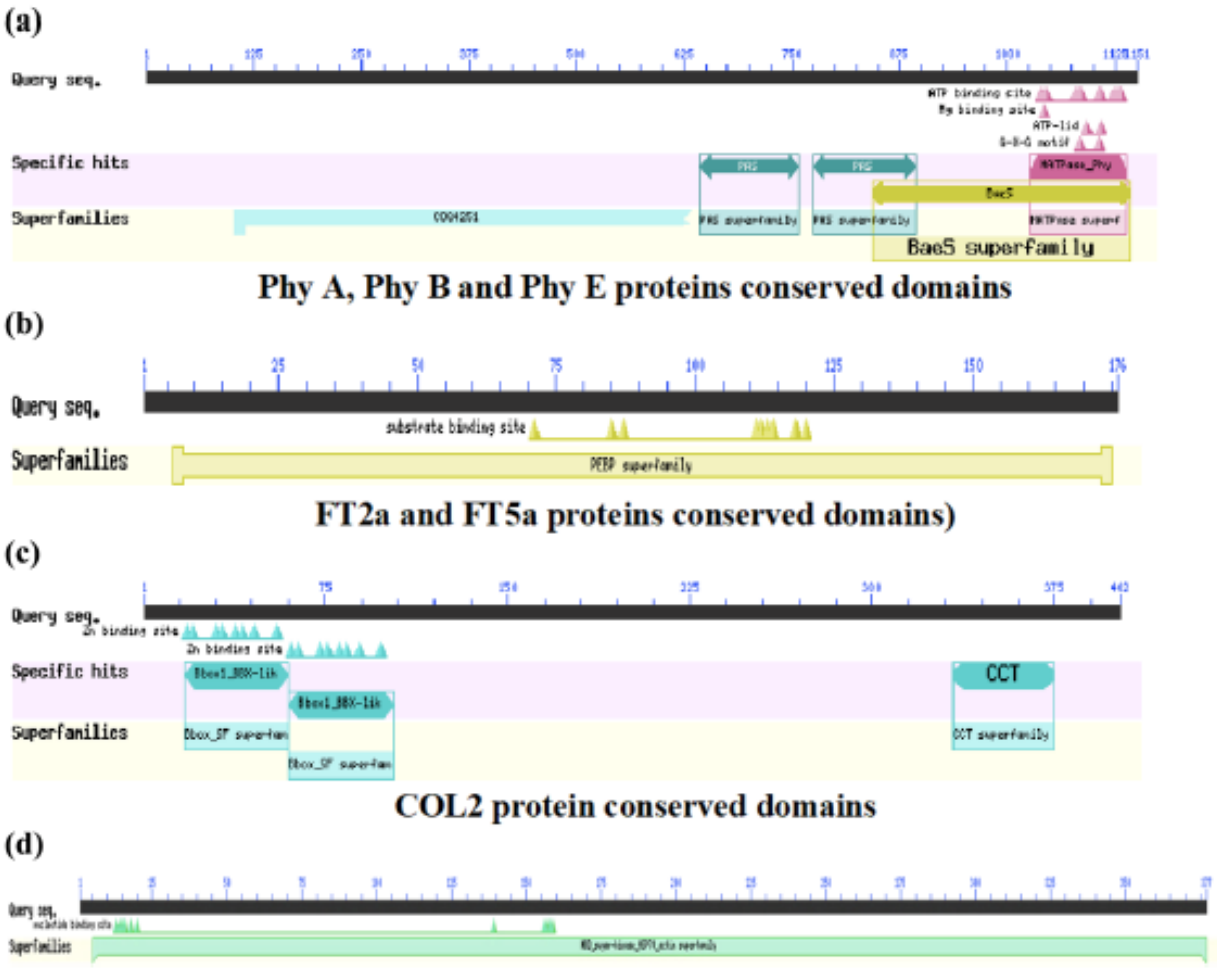

(a)
Query seq.
Specific hits
Superfamilies
Bas5 superfamily
Phy A, Phy B and Phy E proteins conserved domains
(b)
Query seq.
substrate binding site
Superfamilies
PEBP superfamily
FT2a and FT5a proteins conserved domains)
(c)
Query seq.
Zn binding site
Specific hits
CCT
Superfamilies
COL2 protein conserved domains
(d)
Query seq.
Superfamilies

Figure 2

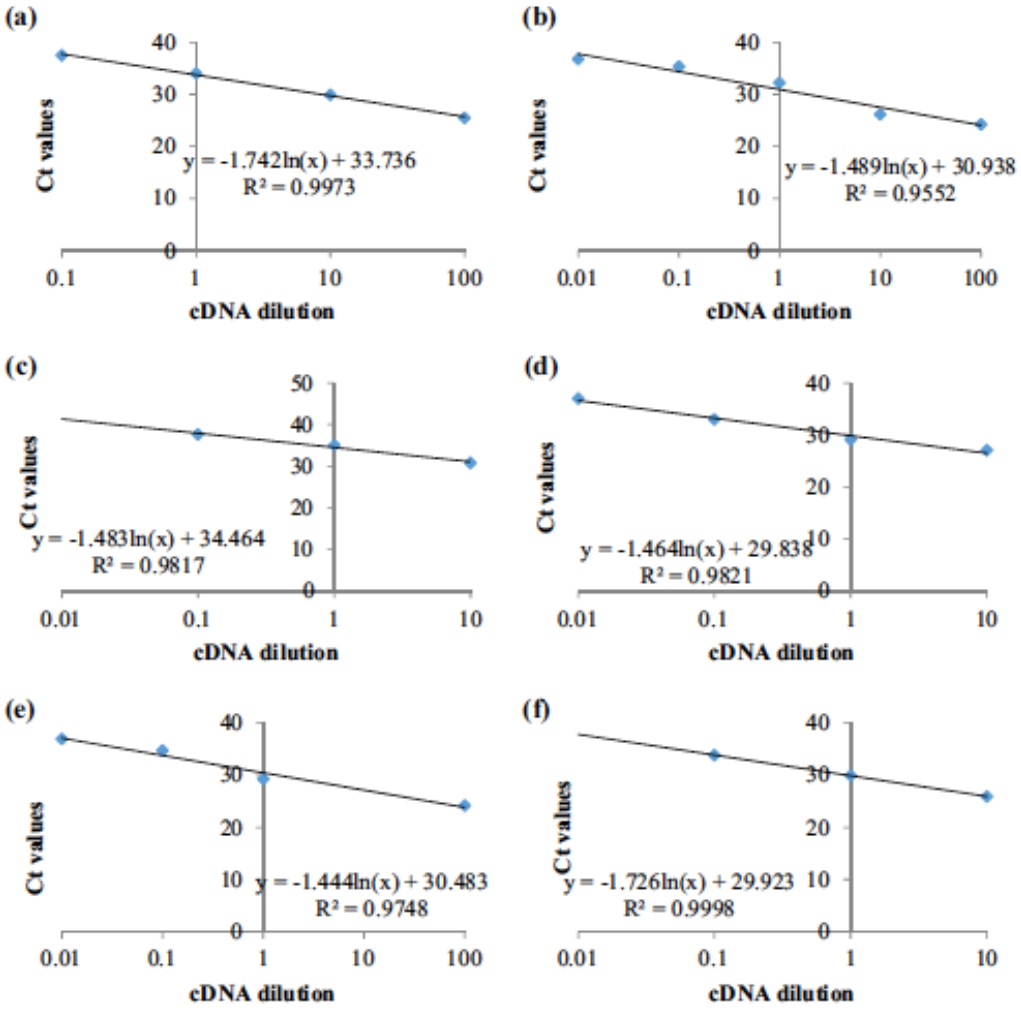
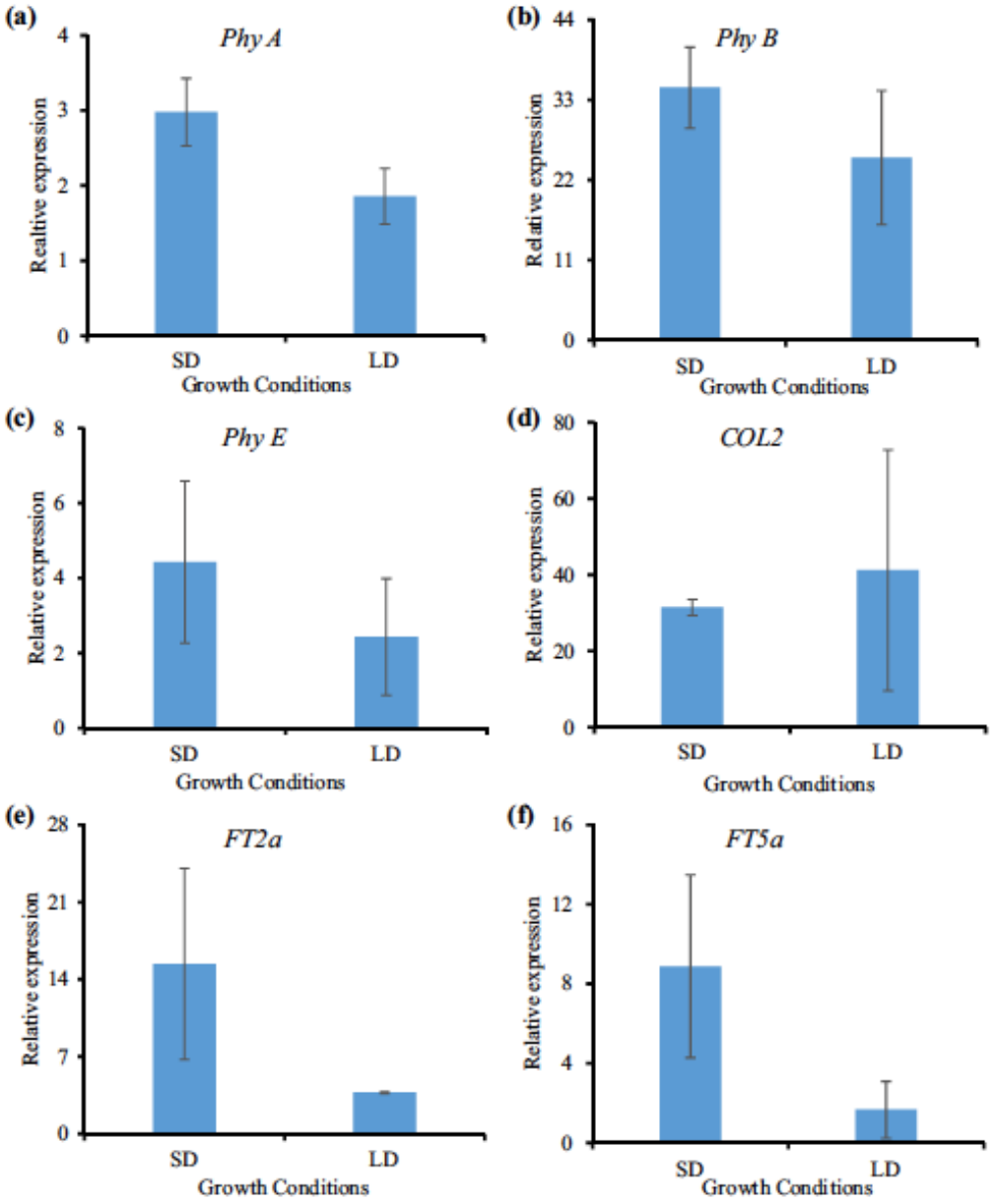

(a)
Ct values
y = -1.742ln(x) + 33.736
R² = 0.9973
cDNA dilution
(b)
Ct values
y = -1.489ln(x) + 30.938
R² = 0.9552
cDNA dilution
(c)
Ct values
y = -1.483ln(x) + 34.464
R² = 0.9817
cDNA dilution
(d)
Ct values
y = -1.464ln(x) + 29.838
R² = 0.9821
cDNA dilution
(e)
Ct values
y = -1.444ln(x) + 30.483
R² = 0.9748
cDNA dilution
(f)
Ct values
y = -1.726ln(x) + 29.923
R² = 0.9998
cDNA dilution

Figure 3


(a)
Phy A
Realtive expression
4
3
2
1
0
SD
LD
Growth Conditions
(b)
Phy B
Relative expression
44
33
22
11
0
SD
LD
Growth Conditions
(c)
Phy E
Relative expression
8
6
4
2
0
SD
LD
Growth Conditions
(d)
COL2
Relative expression
80
60
40
20
0
SD
LD
Growth Conditions
(e)
FT2a
Relative expression
28
21
14
7
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Growth Conditions
(f)
FT5a
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SD
LD
Growth Conditions

Figure 4

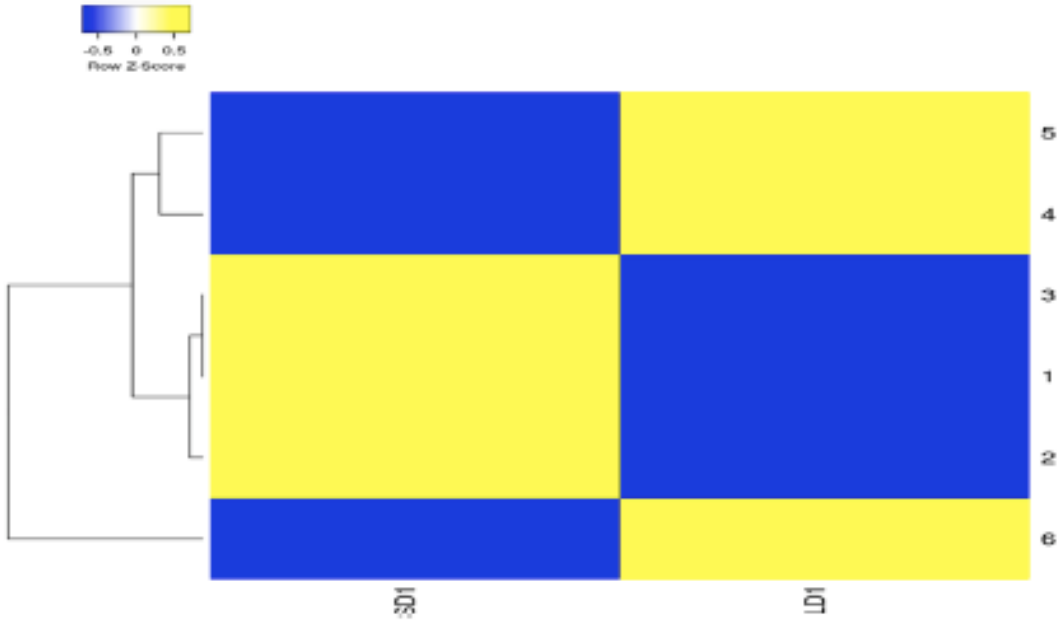

Figure 5

Table 1

Table 1 Selected primers related to phytochromes, flowering genes and reference gene *actin* of peanut (*Arachis hypogaea*) for qPCR analysis

Sr. no	Primers		Sequence (5–> 3')	Template strand	Length	Start	Stop	Tm	GC%	Self comp	Self 3' comp	Primer dimer	Secondary structure	Product length
1	Phy A	Forward	ACACATCCTGGTAGT GGGGT	Plus	20	3169	3188	60.18	55	5	1	No	Weak	142
		Reverse	TTCGTGACA ACAACC GACCT	Minus	20	3310	3291	59.82	50	3	0	No	Weak	
2	Phy B	Forward	GCTATGCGCCTTCTC CAGAT	Plus	20	3125	3144	59.97	55	4	2	No	None	127
		Reverse	GGAGGAACACCTTCA CCAGG	Minus	20	3251	3232	59.96	60	3	3	No	Weak	
3	Phy E-like	Forward	TTCAGGACGGCCATG AGTTT	Plus	20	3026	3045	59.6	50	6	0	No	very weak	115
		Reverse	TGTGTAGTCCATTGA GTCCCT	Minus	21	3140	3120	58.1	47.62	4	1	No	None	
4	Col 2a	Forward	AGATAGTGTGGTGCC GGTTC	Plus	20	753	772	59.75	55	4	2	No	None	132
		Reverse	TCTGGCACTACTCCA ACTTCC	Minus	21	884	864	59.38	52.38	4	0	No	Weak	
5	FT2a	Forward	TATTCCCGCCACA AC TGGAC	Plus	20	277	296	60.04	55	3	1	No	Weak	78
		Reverse	TGAATCCCAGATGTT GGTCGT	Minus	21	354	334	59.37	47.62	4	0	No	Weak	
6	FT5a	Forward	GGCTACCACAAACAC CACCT	Plus	20	405	424	60.18	55	2	0	No	none	136
		Reverse	ACGCCATTCAGGGGG AAAAA	Minus	20	540	521	60.18	50	3	0	No	Very Weak	
7	Actin	Forward	ATCTCTGCTTTGGCT CCCAG	Plus	20	955	974	59.74	55	3	1	No	Weak	134
		Reverse	TCAGCCTTGGCAATC CACAT	Minus	20	1088	1069	59.96	50	5	2	No	Weak	