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Identification of low Ca^{2+} stress-induced embryo apoptosis response genes in *Arachis hypogaea* by SSH-associated library lift (SSHaLL)

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Summary

Calcium is a universal signal in the regulation of wide aspects in biology, but few are known about the function of calcium in the control of early embryo development. Ca^{2+} deficiency in soil induces early embryo abortion in peanut, producing empty pods, which is a general problem; however, the underlying mechanism remains unclear. In this study, embryo abortion was characterized to be caused by apoptosis marked with cell wall degradation. Using a method of SSH cDNA libraries associated with library lift (SSHaLL), 62 differentially expressed genes were isolated from young peanut embryos. These genes were classified to be stress responses, catabolic process, carbohydrate and lipid metabolism, embryo morphogenesis, regulation, etc. The cell retardation with cell wall degradation was caused by up-regulated cell wall hydrolases and down-regulated cellular synthases genes. *HsfA4a*, which was characterized to be important to embryo development, was significantly down-regulated under Ca^{2+}-deficient conditions from 15 days after pegging (DAP) to 30 DAP. Two *AhCYP707A4* genes, encoding abscisic acid (ABA) 8′-hydroxylases, key enzymes for ABA catabolism, were up-regulated by 21-fold under Ca^{2+}-deficient conditions upstream of *HsfA4a*, reducing the ABA level in early embryos. Over-expression of *AhCYP707A4* in *Nicotiana benthamiana* showed a phenotype of low ABA content with high numbers of aborted embryos, small pods and less seeds, which confirms that *AhCYP707A4* is a key player in regulation of Ca^{2+} deficiency-induced embryo abortion via ABA-mediated apoptosis. The results elucidated the mechanism of low Ca^{2+}-induced embryo abortion and described the method for other fields of study.

Introduction

Peanut (*Arachis hypogaea*) is an important oil and protein crop worldwide contributing to the fifth of world's oil output and 11% of the world's protein supply (Katam *et al.*, 2010). In China, peanut output takes nearly 50% of the total yield of all oil crops. Different from other plant species, peanut is a unique geocarpic plant that is marked by the underground development of its fruit, which follows pegging from its fertilized airborne flowers. Consequently, young developing fruit is entirely reliant on maternal assimilate supply and also subject to some mineral supply from the soil, especially sensitive to the availability of calcium (Ca^{2+}). Ca^{2+} deficiency in the soil of pegging zone not only greatly lowers peanut yield and quality (Gascho and Parker, 2001; Murata *et al.*, 2007; Zhang *et al.*, 2004, 2007, 2008a), but also adversely affects seed viability and germination in the following seasons (Wright *et al.*, 2009). It has been known that Ca^{2+} deficiency results in empty peanut pods during harvest season because of early embryo abortion, which is a general problem in peanut production worldwide especially in the red yellow soil regions of China, and the tropic and subtropic areas (Jain *et al.*, 2011; Zhang *et al.*, 2004, 2007, 2008a,b; Zhou, 2001). When peanut is supplied with sufficient Ca^{2+} in the soil, it will produce normal filled pods.

Preliminary molecular and physiological characterizations of events in young embryos under Ca^{2+} deficiency stress have identified several differential genes, such as MADS-box gene (PMADS08) showing up-regulated expression (Zhang *et al.*, 2008a). Several kinds of proteins controlling energy transfer (ATP synthesis), oxidoreduction, endomembrane and cytoskeleton structure systems were also present, which were expressed either abnormally up-regulated or down-regulated (Zhang *et al.*, 2007). A study evaluating a Ca^{2+}-dependent protein kinase (CDPK) isolated in mature peanut seeds validated that it showed up-regulation in response to low Ca^{2+}, which is related to the establishment and maintenance of sink strength (Jain *et al.*, 2011). Moreover, Ca^{2+} has been identified as a universal second messenger that is involved in numerous plant functions, which include cell elongation and division, membrane fluidity and permeability, ion fluxes, cellular pH, source–sink translocation of carbohydrates, N-metabolism, reproductive development and apoptosis (Hepler, 2005). Calcium, because of its nutrient and signalling specificity, acts as a mediator of both intrastimulus- and outer stimulus-response couplings in the regulation of plant

growth and development throughout their entire lifetime (Du and Poovaiah, 2005; Gleason *et al.*, 2006; Yang *et al.*, 2010), but fewer are known about its function during early embryo development. The molecular mechanism underlying peanut embryo development, especially the early embryo abortion that is induced by low Ca^{2+}, is not characterized.

We conducted a screening test for important genes induced by low Ca^{2+} to gain insight into the molecular events that affect early embryo development under Ca^{2+}-deficient conditions. Various strategies have been used to identify differentially expressed genes, as follows: differential display of expression genes (Qin *et al.*, 2011; Stein and Liang, 2002), representational difference analysis (RDA) (Panaud *et al.*, 2002), subtractive hybridization (SH), suppression subtractive hybridization (SSH) (Diatchenko *et al.*, 1996; Soler *et al.*, 2011; Zhou *et al.*, 2011), serial analysis of gene expression (SAGE) (Malig *et al.*, 2006; Velculescu *et al.*, 1995), DNA microarray and transcriptome sequencing (Burgess, 2001; Chen *et al.*, 2014; Puissant *et al.*, 2015; Wang *et al.*, 2010a,b). The polymerase chain reaction (PCR)-based methods were sensitive and powerful in identifying differentially expressed genes, but these methods are time-consuming and prone to errors (Li *et al.*, 2005; Zhang *et al.*, 2008b). Microarray analysis (Crawford *et al.*, 2002; Leelatanawit *et al.*, 2011) and SAGE (Malig *et al.*, 2006) allow high-throughput and comprehensive analysis of differential genes; transcriptome sequencing can isolate differentially expressed genes directly (Labaj *et al.*, 2011; Motameny *et al.*, 2010). However, these methods are expensive and difficult to perform on a regular basis (Hogh and Nielsen, 2008). Furthermore, many of these methods are either not sensitive enough to identify very rare transcripts or provide too many differential genes for identification. SSH can highly and sensitively identify both the abundant and the rare expressed transcripts with low false positives by dramatically enriching the target sequence (Deokar *et al.*, 2011; Diatchenko *et al.*, 1996; Soler *et al.*, 2011). Currently, the screening of SSH cDNA library mainly relies on the cDNA dot-blot macroarray derived from PCR, but this strategy is time-consuming and expensive (Masuda *et al.*, 2007; Sahu and Shaw, 2009; Zou *et al.*, 2010). In the present study, we described the differential analysis of SSH library associated with macroarray library lift (SSHaLL) to identify Ca^{2+} deficiency-induced genes that may play important roles in peanut early embryo development. This method integrates SSH techniques to evenly enrich differential genes into much smaller volume of SSH cDNA libraries and the library colony lift to quickly display and screen differential expression genes simply and high effectively.

Initially, low Ca^{2+}-induced empty pods were characterized as embryo apoptosis with cell wall degradation. We constructed forward and reverse SSH cDNA libraries comprising genes from peanut embryos that were subjected to deficient and sufficient Ca^{2+} treatments. Then, we efficiently screened differentially expressed genes from the SSH cDNA library of the Ca^{2+} deficiency-treated embryos using SSHaLL. Subsequently, genes were identified by microarray, quantitative real-time reverse transcription (qRT)-PCR and bioinformatics. Cytochrome P450 (*CYP707A*) and *HsfA4a* were characterized. These two genes were shown to exert important functions in early embryo development and are involved in apoptosis. We also confirmed the presence of *AhCYP707A* by its over-expression in transgenic *Nicotiana benthamiana*. We found that this protein is a key player in the control of apoptosis-induced early embryo abortion via catabolism of ABA under low Ca^{2+} stress. Therefore, we provided an excellent foundation for the potential cloning of differentially expressed genes of interest from unknown sources and plants with a complicated genome, such as peanut.

Results

Ca^{2+} deficiency-induced embryo cell apoptosis leads to empty pods in peanut

To obtain ideal embryos for important differential gene discovery, we grew peanut plants under Ca^{2+}-deficient and Ca^{2+}-sufficient soil conditions. Such conditions effectively mediated the abortion or normal development of embryos (Figure 1; Experimental Procedures). The deficient Ca^{2+} level in soil inhibited the developmental progression of peanut fruits much less, but had visibly deleterious effects on embryo development. We observed that Ca^{2+} deficiency increased the incidence of peg or pod rot and led to nearly complete early embryo abortion resulting in empty pods. Most peanut embryos showed a tendency to be aborted 15 days after pegging (DAP), and some embryos showed sterility before 15 DAP or after 30 DAP (Figure 1A–C). Using periodic acid–Schiff (PAS) method to study the polysaccharides, which are main components of early embryo cells, we found that embryo cells grown under Ca^{2+}-sufficient conditions had normal cell walls with ordered cell structure from 9 DAP to 21 DAP (Figure 1D–F). By contrast, embryo cells grown under Ca^{2+}-deficient conditions showed normal cell structure only at earlier stage and tended to dissolve their cell walls with diminished starch grains and disordered structure at 21 DAP (Figure 1G–I). Obviously, low Ca^{2+} conditions resulted in embryo apoptosis marked with cell wall degradation, which resulted in embryo abortion and empty pod production.

SSHaLL is a simple method for differential gene analysis

To identify differentially expressed genes involved in early embryo abortion in response to low Ca^{2+} stress, we used mixed RNAs of embryos grown under Ca^{2+}-deficient and Ca^{2+}-sufficient conditions at 6, 9 and 15 DAP. The differentially expressed genes were determined by SSHaLL (Figure 2). SSHaLL method is based on screening SSH libraries by macroarray library lift using probes of subtractive cDNAs or double-strand cDNAs derived from reverse transcription of tester or driver mRNA. We prepared high-quality RNA by CTAB-LiCl methods and mRNA using *Oligotex™-dT30<Super>* mRNA Purification Kit (Takara, Dalian, China) for SSH libraries construction (Figure 3a,b). The differential cDNA library was constructed, after which a pooled cDNA library was prepared for screening with subtractive cDNA probes amplified from subtracted cDNAs of Ca^{2+} deficiency (tester) and sufficiency (driver) stresses. To develop a safer method for library screening, DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim, Germany) was primarily used, but the results were abortive. We subsequently developed a simple, cost-effective and efficient method associated with DIG chemiluminescence detection, and we obtained a clear background (Figure 4a,b). The use of SSH library reduced the screening work and focused on already enriched rare differential genes. Using subtractive cDNA as probes further decreased the cross-screening work, and the use of double-strand cDNA probes from reverse transcripts maintained original differential expression profile for effective hybridization. Only clones that were dramatically up- or down-regulated on visual inspection were selected (Figure 4).

Identification of differentially expressed genes by SSHaLL

Construction of high-quality differential libraries

The construction of high-quality SSH libraries is the basis for successful differential gene screening. Double-strand cDNAs were synthesized using qualified mRNA induced by Ca^{2+} deficiency and sufficiency with M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China) and were thoroughly digested using *Rsa* I enzyme (Figure 3c). After ligation of adaptor, subtracted hybridization, and nested PCR, the cDNA size of tester appeared as a smear band ranging from 200 to 1000 bp. To estimate the subtraction efficiency, subtracted tester, driver cDNA and unsubtracted ones as templates were amplified separately by the first and second PCR. Meanwhile, both the subtracted and the unsubtracted skeletal muscle cDNA, together with a PCR control-subtracted cDNA (kit supplied), were used as control templates to evaluate PCR performance. Numerous specific bands existed in the second PCR products, and the banding patterns obviously differed among the products of the subtracted and unsubtracted cDNA templates, both in Ca^{2+}-deficient cDNA (tester) and Ca^{2+}-sufficient cDNA (driver) (Figure 3d, lanes 1–4). Distinct Hae III-digested φX174 DNA bands were observed in the second PCR product of the subtracted skeletal muscle cDNA, which was similar to that of the kit-supplied PCR control-subtracted cDNA (Figure 3d, lane 5–7). We constructed forward and reverse SSH

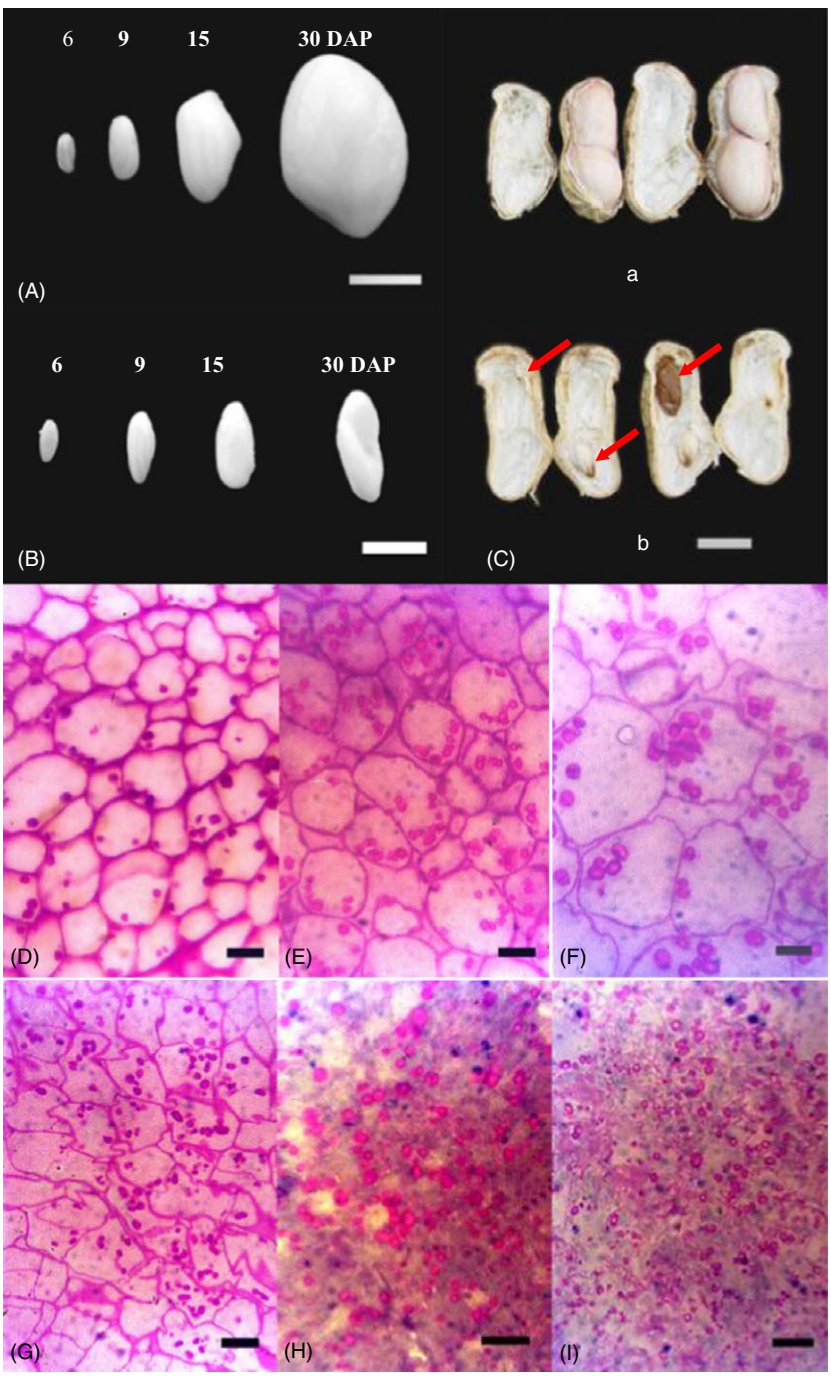

Figure 1 Morphological changes of developing embryos from peanut plants growing in the soil with Ca^{2+} sufficiency and deficiency. A. The representative embryos developed in the soil with sufficiency Ca^{2+} for 6, 9, 15 and 30 days after pegging (DAP). B. The representative embryos developed normally in deficiency Ca^{2+} before 15 DAP, but showed abortion afterwards. C. Mature pods of peanut grew under sufficient (a) and deficient (b) Ca^{2+} in the soil bearing with full pods and empty pods (with abortive embryos) respectively. Arrows indicate abortive embryos. D–I, embryo cell morphology in peanut. D–F, embryo cells of 9, 15, 21 DAP in sufficiency Ca^{2+}, cells with normal cell walls; and G–I, cells of 9, 15, 21 DAP of deficiency Ca^{2+} treatments, cell walls were hydrolyzed and completely dissolved after 21 DAP and cell tended to apoptosis. Bars indicated in A, B and C are 5, 5 and 10 mm; and Bars in D–I are 5 μm.

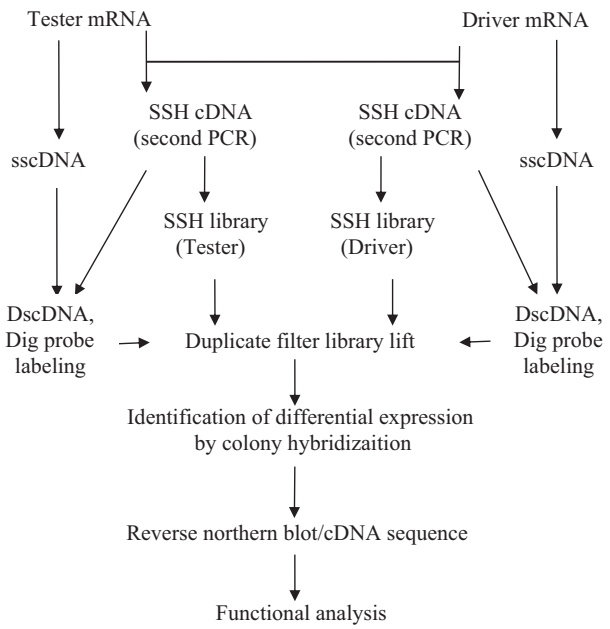

Figure 2 Schematic outlines for the SSHaLL protocol. This scheme contains four steps: (i) Construction SSH library, (ii) Differential hybridization analysis of cDNA clones, and (iii) Clone analysis by sequencing and reverse northern blot analysis. Finally, RT-PCR/Real time RT-PCR/northern blot analysis was used to confirm the differential expression cDNA clones.

libraries with 65 300 and 52 300 primary clones, respectively, using second PCR products of subtracted cDNA from embryos developed under Ca^{2+}-deficient and Ca^{2+}-sufficient conditions. The quality of SSH libraries was evaluated using both PCR amplification and enzyme digestion of the randomly chosen clones. Results showed that all clones had a single insertion with a fragment length of approximately 200–800 bp (Figure 3e,f). The subtraction of tester and driver cDNAs was successfully conducted, and the high-quality libraries were constructed.

Screening and identification of differentially expressed genes

Differentially expressed genes were screened using macroarray library lift technique. For effective screening, Ca^{2+}-deficiency SSH cDNA library was used to create subpools, and clones were grown on nylon films. Approximately 2000 clones were present on each nylon film. We initially performed colony lift for differentially expressed genes several times using DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland), but this method produced a black background around colonies or resulted in the accumulation of the probe around colonies, which affected subsequent comparison and screening (data not shown). Subsequently, we changed the kit manual using the following: 0.15 M sodium phosphate 2% (w/L) SDS solution for prehybridization; new hybridization solution (containing 1% BSA, 1 mM EDTA, 7% SDS, 0.5 M sodium phosphate, pH 7.0) with probe for hybridization; Tris-NaCl-Tween (TNT) for blocking; and TNT with milk for antibody association (as described in the Experimental Procedures). Immunodetection was performed according to the Roche protocol. This method produced a very clear background with excellent results (Figure 4a,b). By SSHaLL, more than 20 000 clones were primarily screened, and 62 clones (including 52 up-regulated and 10 down-regulated genes) that showed more than fivefold higher or lower intensity on X-ray film were selected, sequenced and subjected to BLASTX (Table 1 and Data S1). Eighteen up-regulated and 10 down-regulated genes selected by SSHaLL were reidentified by microarray analysis quantitatively, and results were consistent with the up-/down-regulated expression from SSHaLL (Figure 5c). This finding showed that SSHaLL is an effective method for screening differentially expressed genes.

Functional annotation and evaluation of differentially expressed genes

Annotation of differentially expression genes

The sequences of the screened Ca^{2+} deficiency stress-induced differential genes provided important clues to their possible functions and potential insights into the mechanisms by which Ca^{2+} deficiency stress caused embryo abortion (Table 1 and Data S1–S3). In particular, the genes identified by SSHaLL were classified into the functions of response to stimuli or stresses, multicellular organismal development, carbohydrate and lipid metabolic processes, catabolic process, anatomical structure morphogenesis, DNA/RNA metabolic process, regulation, translation, and generation of precursor metabolite and energy (Figure 5a,b). The functional retardation of the genes selectively expressed under Ca^{2+} deficiency stress is shown in Table 1. We observed a spectrum of genes with up-regulated expressions. Such genes were implicated in cell survival and defences, such as cytochrome P450s, aluminium-activated malate transporter 9-like, desiccation-related protein pcc13-62-like, AP2/ERF domain transcription factor, rRNA intron-encoded homing endonuclease and precatalytic spliceosome. Some proteins associated with hydrolase activity, cell wall-associated hydrolase, cellulase protein, phosphopyruvate hydratase activity and glycolysis in response to deficient Ca^{2+} were increasingly expressed, thereby indicating the tendency of progressive retardation among Ca^{2+}-stressed embryos (Figure 5c; Tables S1–S3; Figures S1–S3). Furthermore, we observed that two cellulose synthase-like protein h1, late embryogenesis-abundant group 6, a heat-shock transcription factor A-4a-like (abbreviated *AhHsfA4a*) and three unknown genes were down-regulated (Table 1). Many of these genes should be associated with embryo abortion characterized by dissolved cell wall.

Chip analysis of expression patterns of the identified genes

To further characterize the SSHaLL-selected genes, temporal expression levels of 28 higher induced genes with 6, 9 and 15 DAP under deficient vs. sufficient Ca^{2+} stresses were measured by microarray hybridization using the double-strand cDNAs made from total RNA from each sample (Data S2–S4). Eight expression patterns, including four up-regulated and four down-regulated, were grouped based on the temporal profile of gene expressions (Figure 6a–d, e–h). Among 18 up-regulated genes, seven genes (group A in Figure 6a) expressed peaked at 6 DAP, and then decreased up-regulated expression at 9 DAP but increased again at 15 DAP. Two gene expression (group B in Figure 6b) also peaked at 6 DAP and declined up-regulation with time. Group A and group B genes probably represent early-response genes. Four genes (group C, Figure 6c) were found to increase up-regulated expression consistently from 6 to 15 DAP, and five gene (group D, Figure 6d) elevated expression between 6 and 9 DAP and decreased up-regulated expression at 15 DAP under deficient Ca^{2+}. Among the 10 down-regulated genes, there were also four corresponding expression patterns, and the group F contained six

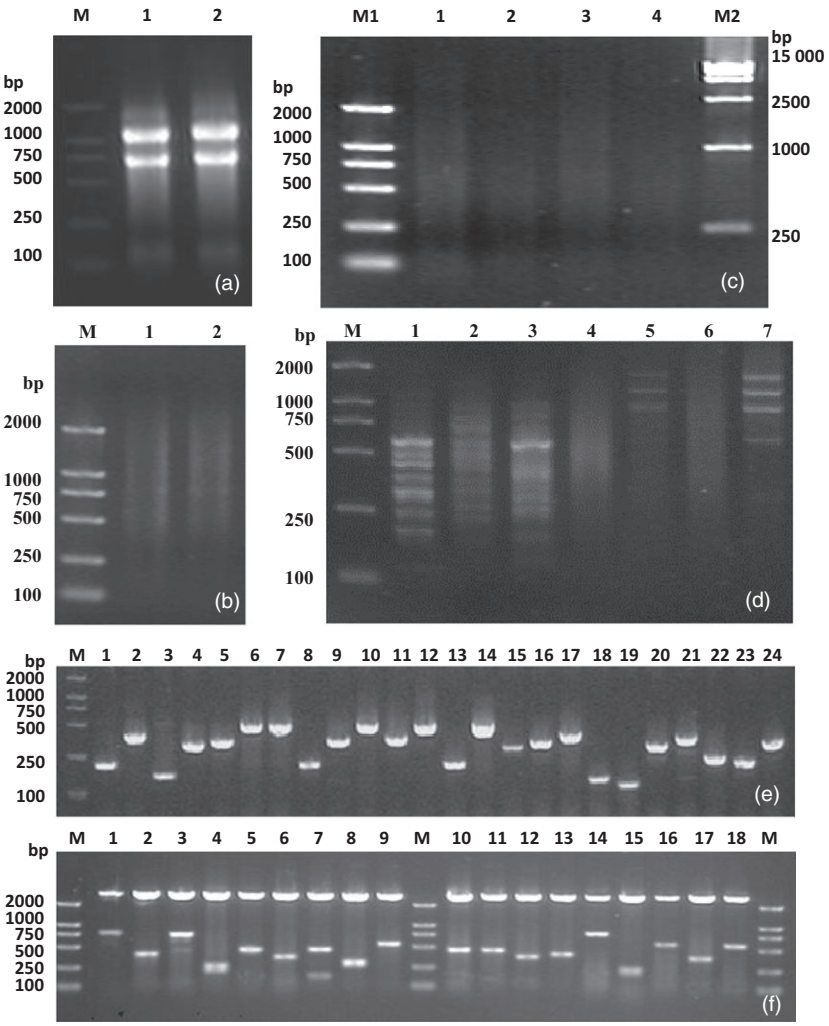

Figure 3 Progress of SSH library construction. (a): Lanes 1 and 2 are total RNA from peanut early embryos induced by deficiency and sufficiency Ca^{2+} treatments, respectively; Lane M, Maker DL2000. (b): Lanes 1 and 2 are mRNA of early embryos induced by deficiency and sufficiency Ca^{2+}, respectively; Lane M, Maker DL2000. (c): Double strains cDNA of early embryos before and after *Rsa I* digestion. Lane 1, double strains cDNA induced by deficiency Ca^{2+} before *Rsa I* digestion; Lane 2, double strains cDNA induced by deficiency Ca^{2+} after *Rsa I* digestion; Lane 3, double strains cDNA induced by sufficiency Ca^{2+} before *Rsa I* digestion; Lane 4, double strains cDNA induced by sufficiency Ca^{2+} after *Rsa I* digestion; Lane M1, DL2000; Lane M2, DL15000. (d): Electrophoresis of the second PCR products. Lane 1, subtracted PCR products of peanut early embryos induced by deficiency Ca^{2+}; Lane 2, unsubtracted PCR products of early embryos induced by deficiency Ca^{2+}; Lane 3, subtracted PCR products of early embryos induced by sufficiency Ca^{2+}; Lane 4, unsubtracted PCR products of early embryos induced by sufficiency Ca^{2+}; Lane 5, control PCR products of subtracted skeletal muscle cDNA; Lane 6, control PCR products of unsubtracted skeletal muscle cDNA; Lane 7, positive control of subtracted PCR products; Lane M, Marker DL2000. (e): Colony PCR identification of SSH library of peanut early embryo induced by deficiency Ca^{2+}. (f) Enzyme digestion Identification of SSH library of early embryo induced by deficiency Ca^{2+}; Lane M, Marker DL2000.

genes whose down-expressions peaked at 6 DAP under deficiency Ca^{2+} and consistently decreased the down-regulation with time (Figure 6f), which represent early down-regulated response genes. Importantly, three cell wall degradation-related genes were up-regulated, one (SSHQ-11) up-regulated near 10-fold at 6 DAP, then the expression declined to base line (Figure 6a) and the other two (SSHQ-16 and SSHQ-19) increased up-regulation consistently after pegging under deficient vs. sufficient Ca^{2+} (Figure 6c), thereby showing a up-regulated expression level declined slower after pegging (Figure 6i). Moreover, two cellular synthesis genes (SSHQ-55 and SSHQ-56) showed down-regulation especially at early stage (Figures 6f,j). The results well accounted for embryonic cell wall degradation under deficient Ca^{2+} stress. Strikingly, the CYP707A (SSHQ-6 in group A) and the HSF (SSHQ-60) showed the greatest up-regulation at 6 DAP or down-expression after 15 DAP.

Characteristics of Ca^{2+} deficiency-induced cytochrome P450 expression

Three cytochrome P450s, SSHQ-1, SSHQ-6 (P450.1) and SSHQ-10 (P450.2), were selected by up-regulation using SSHaLL; SSHQ-1 was associated with a very long-chain lipid synthesis, whereas the latter two were identified to play key roles in abscisic acid (ABA) catabolism through ABA 8′-hydroxylase activity (Table 1; Figures S4–S6, S10). P450.1 and P450.2 were both similar to CYP707A4 (XP_002885304), but their transcripts accumulated by >21-fold and >3.7-fold, respectively, in comparison with the embryos grown under Ca^{2+}-sufficient conditions (Figure 5c). We studied *P450* expression patterns by microarray and found the two *P450s* were up-regulated significant higher at an early stage of 6 DAP under deficient Ca^{2+} than sufficient Ca^{2+} conditions (Figures 6a and 7a). This indicated that low Ca^{2+} stress affected *P450* expression starting from the initial stage of embryo development. We further studied the *P450* transcripts of embryos using qRT-PCR under sufficient and deficient Ca^{2+} conditions, and noted that *P450s* were relatively highly expressed from 10 to 30 DAP, but were lowly expressed after 30 DAP under Ca^{2+}-sufficient conditions. Similarly, the transcripts were dramatically up-regulated under deficient Ca^{2+} stress (Figure 7b,c).

To know the nature of *AhCYP707A4* in peanut, we spatially compared the expressions of the *P450.1* and *P450.2* under normal Ca^{2+} level, which showed that both *P450s* were expressed with similar features. They were expressed at very low level in embryos, root and pegs, but at a much higher level in other tissues. The *P450.1* had higher expression level than *P450.2* in tissues other than that of the testa (Figure 7d). The results indicate peanut embryo normally has lowest level of *CYP707A4*.

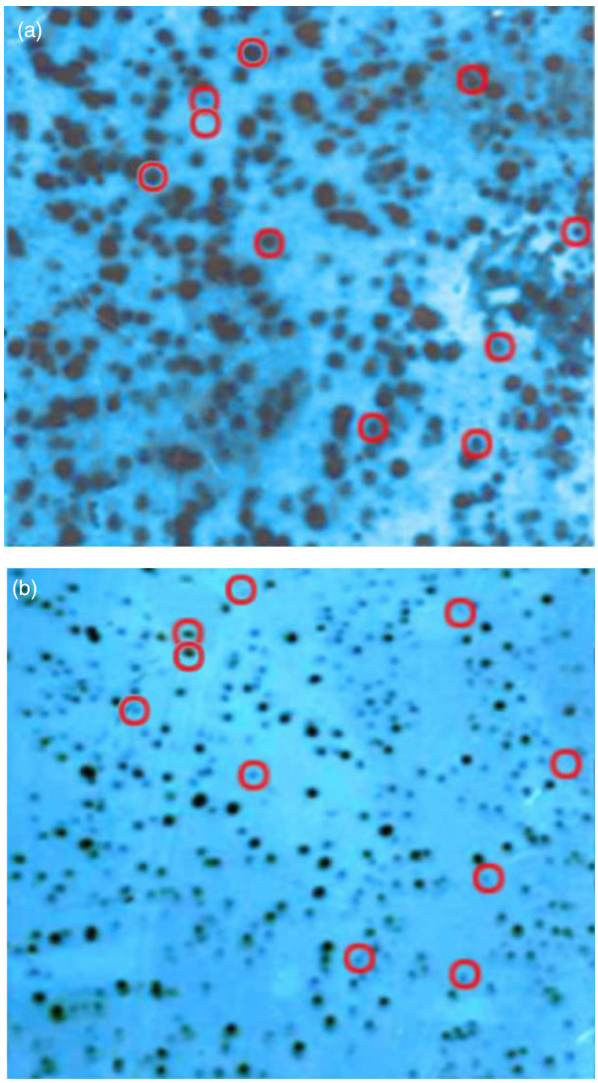

Figure 4 Result of library screening by colony hybridization. (a): Hybridization of Ca^{2+} deficiency induced embryo SSH cDNA library with probes of deficiency Ca^{2+} induced SSH cDNAs. (b): Hybridization of Ca^{2+} deficiency embryo SSH cDNA library with probes of sufficiency Ca^{2+} induced SSH cDNAs. The red circles indicate the differential expression genes.

We treated the peanut plants with various stresses and performed microarray assay to further characterize these *P450s*. We found that both the *P450.1* and *P450.2* in leaves were up-regulated in response to ABA, low temperature, and drought, as well as *Ralstonia solanacearum* inoculation to some extents, but were down-regulated by ethylene treatment. However, only the deficiency of Ca^{2+} increased *P450.1* and *P450.2* expression by large scale (Figure 8a). These demonstrated that *CYP707As* responded to many stresses especially under low Ca^{2+}-induced embryos.

Furthermore, ABA contents of peanut embryos grown under low and sufficient Ca^{2+} conditions were compared by HPLC to test whether up-regulation of CYP707A4 will affect ABA (Figure 8b,c). Under normal (sufficient Ca^{2+}) conditions, ABA level was increased from 10 to 30 DAP, but decreased to the lower content after 30 DAP, indicating a curve with a single peak at 30 DAP. This result demonstrated a balance between ABA content and *CYP707A* expressions. However, under deficient Ca^{2+} condition, ABA content in seeds was significantly reduced, particularly in the early stages confirming that the up-regulation of *CYP707A4* reduced ABA level in the early embryos under deficient Ca^{2+}.

Characterization of Ca^{2+} deficiency-induced *Hsfs* genes in peanut

The *AhHsfA4a* gene exhibited wide aspects of cellular functions and many biological pathways, which respond to a wide of stresses and stimuli, and was involved in system development, nucleic acid metabolic process, macromolecule metabolic process, reproductive developmental process and postembryonic morphogenesis (Figures S7–S9; Tables S7–S9). This gene was selected as the down-regulated gene through SSHaLL and was identified through microarray analysis with significant down-regulation over 10-fold under Ca^{2+} deficiency (Figure 5c). It showed significantly reduced expression pattern from 15 DAP (group G in Figure 6g). Thus, this gene may play an important role in regulating the Ca^{2+} deficiency-induced embryo abortion in peanut. To identify whether the reduction of *AhHsfA4a* expression induced by Ca^{2+} deficiency implicated in early embryo abortion, we examined the transcript levels of *AhHsfA4a* by real-time RT-PCR using embryos aged 10, 20, 30, 40, 50 and 60 DAP grown under normal Ca^{2+} condition and embryos aged 6, 9, 15 and 30 DAP grown under Ca^{2+} deficiency/sufficiency (Figure 9). Under sufficient Ca^{2+} condition, *AhHsfA4a* expression was at a lower level in embryos aged earlier than 20 DAP. *AhHsfA4a* expression dramatically increased in 30 DAP embryos before decreasing back to trace level in embryos older than 30 DAP. Ca^{2+} deficiency stress increased the *AhHsfA4a* expression in young embryos (6–15 DAP) by a small scale, but expression decreased significantly at 30 DAP by nearly fivefold when *HsfA4a* expression level drastically increased under sufficient Ca^{2+} in contrast (Figure 9b,c). This result agreed with the stage of embryo abortion marked with contraction of embryos under Ca^{2+} deficiency stress. Thus, given that embryos require *AhHsfA4a* for rapid growth, decreasing *AhHsfA4a* expression level induced by low Ca^{2+} concentration prevents the occurrence of many cellular and biochemical processes, resulting in cells apoptosis and, consequently, embryo abortion. However, *AhHsfA4a* seems to play functions downstream of *P450.1* and *P450.2*.

Over-expression of *AhCYP707A4 in N. benthamiana* produced a high degree of embryo abortion

To confirm the finding that ABA should play an important role in low Ca^{2+}-induced embryo abortion, the fusion gene of *35S::AhCYP707A4* was introduced into *N. benthamiana* by *Agrobacterium tumefaciens*-mediated transformation. Transgenic plants were generated, and T_1 and T_2 kanamycin-resistant lines were recovered. Genomic PCR and RT-PCR analyses were carried out to confirm the presence of the transgenes starting from the T_0 generation (data not shown). Three T_2 homozygous lines were selected after identification. ABA metabolism in these lines was further characterized. The plants over-expressing *AhCYP707A* were grown along with wild-type (WT) plants. The plants over-expressing *AhCYP707A* grew slowly and showed easier wilting than the WT under the same water condition (Figure 10a). Compared with the WT, corollas in plants over-expressing *AhCYP707A* developed slowly and were shorter; the fruits were smaller and produced numerous smaller

Table 1 BLASTX results of differentially expressed genes screened from Ca^{2+}-deficiency SSH library

Sample no.	Sequences producing significant alignment	Length (bp)	Access number	E-Value	Identities (%)	Note
SSHQ-1	Cytochrome p450	404	XP_003607255	1.54E-29	83.90	U
SSHQ-2	Inner membrane protein yecn	502	ZP_12479558	7.84E-106	99.70	U
SSHQ-3	Aldehyde dehydrogenase	364	XP_003525266	3.24E-29	89.65	U
SSHQ-4	Chloroplast small heat-shock protein	459	XP_003523325	3.50E-26	78.45	U
SSHQ-5	Pathogenesis-related protein class partial	213	ACA79908	9.99E-38	89.40	U
SSHQ-6	Cytochrome p450	228	AFV61750	1.12E-16	76.15	U
SSHQ-7	Dehydration-responsive element binding protein 2	474	NP_001235779	6.77E-30	90.05	U
SSHQ-8	Momilactone A synthase	293	ACJ84543	1.96E-23	72.05	U
SSHQ-9	Kunitz-like protease partial	827	XP_003620188	4.32E-50	63.30	U
SSHQ-10	Cytochrome p450	329	XP_002330327	2.41E-26	87.80	U
SSHQ-11	Cell wall-associated hydrolase	500	ZP_16527378	6.27E-45	76.05	U
SSHQ-12	Epimerase family protein slr1223-like	715	AFK47694	1.09E-122	85.80	U
SSHQ-13	NA	178				U
SSHQ-14	NA	486				U
SSHQ-15	Kunitz-like protease partial	818	NP_001238098	5.03E-33	70.65	U
SSHQ-16	Cellulase (glycosyl hydrolase family 5) protein	226	NP_189244	5.42E-09	73.55	U
SSHQ-17	NA	244				U
SSHQ-18	NA	302				U
SSHQ-19	Hydrolysing o-glycosyl	185	XP_003552802	3.21E-26	80.15	U
SSHQ-20	Aluminium-activated malate transporter 9-like	487	XP_003525258	1.11E-99	90.45	U
SSHQ-21	hmg1/2-like	460	ACF74312	1.25E-18	92.10	U
SSHQ-22	14-3-3-like protein	297	NP_001238389	1.12E-22	90.10	U
SSHQ-23	PREDICTED: uncharacterized protein LOC100785168	184	XP_003531794	9.95E-09	66.50	U
SSHQ-24	T1	534	ACM24794	2.06E-26	79.00	U
SSHQ-25	rrna intron-encoded homing endonuclease	389	XP_003541241	9.66E-38	91.60	U
SSHQ-26	rrna intron-encoded homing endonuclease	388	EGF98539	7.80E-30	89.20	U
SSHQ-27	40s ribosomal protein s25-2	406	XP_003537862	1.03E-41	97.35	U
SSHQ-28	Retrotransposon protein	538	AAO23078	1.18E-38	58.20	U
SSHQ-29	60s ribosomal protein l13a	568	XP_003547798	2.59E-114	95.20	U
SSHQ-30	NA	258	XP_003522929			U
SSHQ-31	PREDICTED: uncharacterized protein LOC100800976	253	XP_003552802	5.71E-04	85.00	U
SSHQ-32	NA	255				U
SSHQ-33	Desiccation-related protein pcc13-62-like	346	EMJ24187	5.15E-20	73.40	D
SSHQ-34	Enolase	557	CAA41116	2.48E-119	94.45	U
SSHQ-35	PREDICTED: uncharacterized protein LOC100785168	275	XP_003531794	2.69E-22	68.00	U
SSHQ-36	Enolase	557	CAA41116	2.48E-119	94.45	U
SSHQ-37	Lectin	397	3ZVX_A	1.07E-53	75.30	U
SSHQ-38	Enolase	557	CAA41116	2.48E-119	94.45	U
SSHQ-39	Aluminium-activated malate transporter 9-like	488	XP_003525258	3.27E-100	90.50	U
SSHQ-40	Hydrolysing o-glycosyl	545	XP_003539156	2.44E-69	70.90	U
SSHQ-41	NA	258				U
SSHQ-42	NA	244				U
SSHQ-43	NA	121				U
SSHQ-44	NA	346				U
SSHQ-45	NA	433				U
SSHQ-46	NA	434				U
SSHQ-47	NA	494				U
SSHQ-48	RNA-binding protein 42-like	451	XP_001952469	3.02E-43	85.00	U
SSHQ-49	NA	250				U
SSHQ-50	NA	251				U
SSHQ-51	NA	499				U
SSHQ-52	NA	539				U
SSHQ-53	PREDICTED: uncharacterized protein LOC100790544	468	XP_003552697	2.01E-19	67.90	U
SSHQ-54	Late embryogenesis-abundant group 6	779	ADQ91847	4.07E-45	82.55	D
SSHQ-55	Cellulose synthase-like protein h1	338	XP_003539497	2.01E-16	79.80	D
SSHQ-56	Arachin ahy-4	722	ABI17154	1.57E-39	94.10	D
SSHQ-57	Cellulose synthase-like protein h1	876	XP_003539497	1.04E-80	69.90	D

Table 1 Continued

Sample no.	Sequences producing significant alignment	Length (bp)	Access number	E-Value	Identities (%)	Note
SSHQ-58	NA	456				D
SSHQ-59	Embryonic abundant protein usp92	821	ADQ91849	0	69.80	D
SSHQ-60	Heat stress transcription factor a-4a-like	355	XP_003531269	1.65E-43	72.65	D
SSHQ-61	NA	483				D
SSHQ-62	NA	244				D

Note: Letter 'U or D', up-/down-regulation in library screening, respectively.

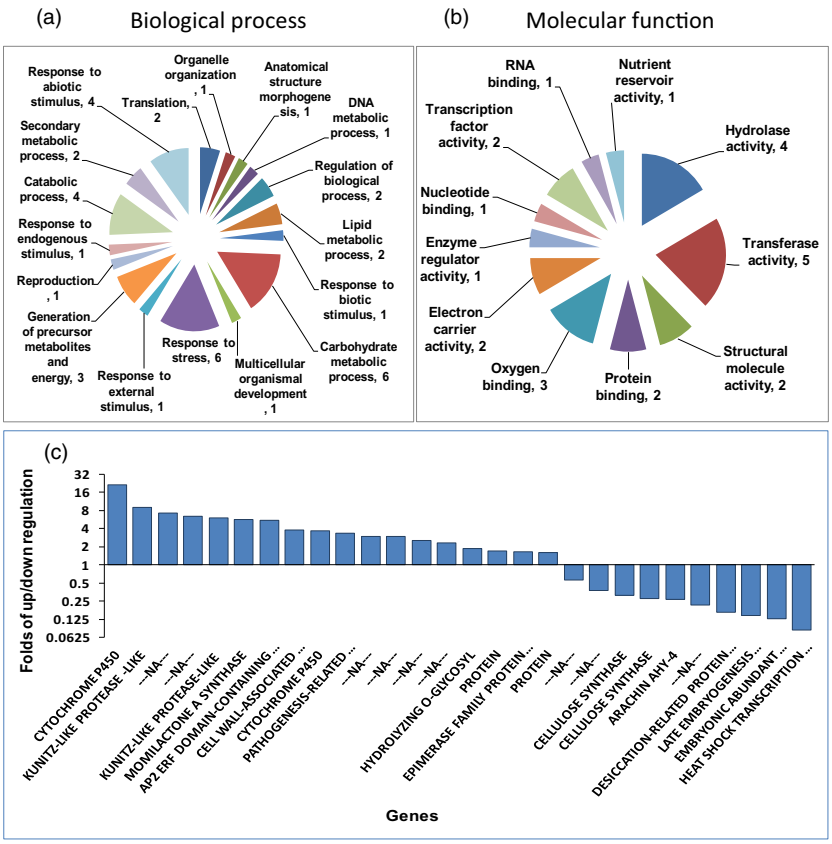

Figure 5 Distribution and confirmation of Ca^{2+}-induced genes selected by SSHaLL screening. (a, b): Showed the up- and down-regulated genes obtained by SSHaLL were classified according to the potential biological process and molecular function of corresponding proteins and the numbers in different groups. (c): Microarry analysis confirming the expression levels of 28 genes selected by SSHaLL. The folds of up or down-regulated expression ratio between Ca^{2+}-deficiency and sufficiency stresses were shown.

powder-like abortive seeds and fewer normal-size seeds (Figure 10e,f). The plants over-expressing *AhCYP707A* showed characteristics similar to those found in peanuts with abortive dry seeds that were grown under low-calcium conditions (Figure 1c). To confirm whether transgenic plants over-expressing *AhCYP707A4* are ABA deficient, we analysed the plants for *AhCYP707A4* expression and ABA content. As shown in Figure 10d,g, ABA content in the transgenic plants was reduced by about 75%. The *AhCYP707A4* was expressed strongly in the transgenic lines and not in wild-type plants. These indicated that transgenic plants constitutively expressing *AhCYP707A4* have increased 8′-hydroxylase activity and led to ABA degradation (Figure 10d,g). The results further demonstrated that the *AhCYP707A4* functioned as ABA 8′-hydroxylase. Ca^{2+} deficiency in soil causes embryo abortion in peanut because of the highly up-regulation of AhCYP707As, which resulted in the reduction of ABA level in early embryos, thereby causing embryo apoptosis and producing empty pods.

Discussion

SSHaLL is a convenient and efficient method for differential expression genes analysis

The obtained results demonstrated that SSHaLL is a simple, safe and efficient method for generating cDNAs highly enriched for differentially expressed genes. We have obtained representative differential genes occurred under Ca^{2+} deficiency with this method, although they were not screened on a large scale. However, SSHaLL can be a larger scale gene expression analysis method that could be used to comprehensively assess the main differentially expressed genes among various tissues or genes induced by different stresses. Using double-strand cDNAs as probes, which represent original differential expression profile, we have generated differential expression genes with natural difference expression pattern. We have successfully cloned many pericarp-specific genes and its upstream promoters in peanut and

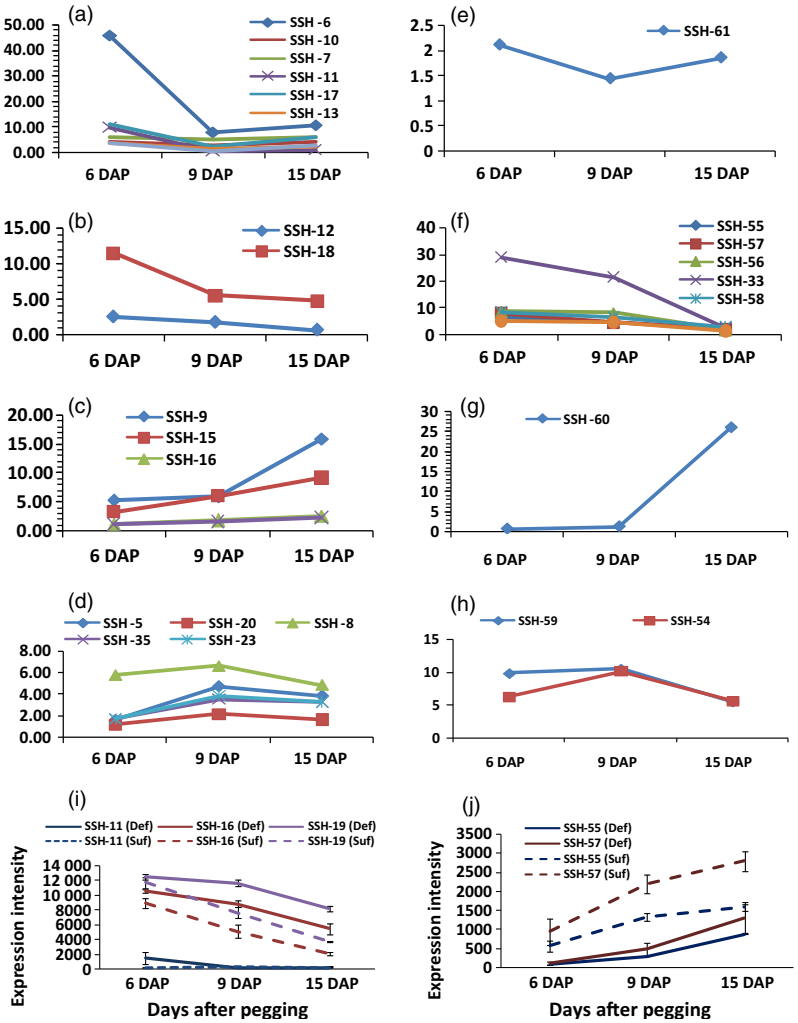

Figure 6 Expression patterns of up-regulated and down-regulated genes during early embryo development under deficient Ca^{2+} stress vs. sufficient Ca^{2+} stress by chip analysis. (a–h): There are eight expression patterns after a time course of pegging among these genes. (a–d), folds of up-regulated expression; (e–h), folds of down-regulated expression. (a, e): Up or down expression ratios peak at 6 DAP, followed by a decrease at 9 DAP, and an increase again at 15 DAP. (b, f): Up or down expressions peak at 6 DAP, followed by a decline. (c, g): Up or down expressions increase from 6 to 15 DAP. (d, h): Up or down expression increase from 6 DAP and peak at 9 DAP, followed by a decline. (i): Expressions of three cell wall-degraded genes showing much greater up-regulation in Ca^{2+} deficiency, SSH11 up-regulates only at 6 DAP. Def, deficient Ca^{2+}; Suf, sufficient Ca^{2+}. (j): Expression patterns of two cellular synthesis genes showed much higher down-regulation after pegging under deficiency Ca^{2+}. SSH-6, SSH-10: cytochrome p450s; SSH-7: dehydration-responsive element binding protein 2; SSH-11, SSH-16, SSH-19: cell wall-related degradation genes; SSH-5: pathogenesis-related protein 10; SSH-8: momilactone A synthase. SSH-9, SSH-15: kunitz-like protease; SSH-20: aluminum-activated malate transport 9; SSH-33: desiccation–related protein Pcc13-62. SSH-56: Arachin Ahy-4. SSH-59: embryonic abundant protein precursor. SSH-54: late embryogenesis abundant protein group 8. SSH-60: heat shock transcription factor. SSH-13,14,17,18,23,35, 46,58,61: Unknown genes.

root-specific genes, as well as its promoters in tobacco with this method (unpublished data). Differentially expressed genes picked by SSHaLL coupled with custom microarray or macroarray analysis will provide a powerful strategy to study the differential expression of genes in any given cell type, tissue or experimental paradigm.

There are a variety of strategies which have been used to identify differentially expressed genes, such as differential display (Liang *et al.*, 1994), microarray techniques (Heller, 2002), SAGE (Velculescu *et al.*, 1995), RDA (Lisitsyn *et al.*, 1993) and differential subtraction chain (Li *et al.*, 2005; Luo *et al.*, 1999), but all the above-mentioned methods have merits and drawbacks. Differential analysis of primary library expression (DAzLE) was a powerful technique designed to gain a direct and quantitative measure of gene expression and long sequence fragments from a cDNA library (Li *et al.*, 2004). However, DAzLE may not focus on the relatively specific expression of gene separation and needs more work. Microarray (Leelatanawit *et al.*, 2011), SAGE (Malig *et al.*, 2006) and RNA sequencing (Chen *et al.*, 2014) can be high-throughput and comprehensive analyses or also directly isolate differential genes, but they need specific instruments and more expensive, which cannot be carried by ordinary laboratories. SSH is a robust and widely used method in the market aiming to enrich rare transcripts and low-abundance genes to easily isolate differentially expressed genes of interest (Bassani *et al.*, 2004; Diatchenko *et al.*, 1996; Jang *et al.*, 2003). Recent studies on cDNA library construction by SSH are still one of the hot spots in identification of differentially expressed genes (Badapanda, 2013; Ragusa *et al.*, 2013; Sahu and Shaw, 2009). SSHaLL is a technique of SSH-associated macroarray library lift. It incorporates the beneficial SSH process of enriching rare, differentially expressed genes for ease of determination. Thus, screening work is reduced. Macroarray library lift, which uses film-grown colony screening, is simple, high-throughput and rapid. Macroarray library lift overcomes the defaults of dot-to-dot macroarray library screening method, which is a time-consuming and costly method based on PCR amplification of each clone (Chen *et al.*, 2011; Liu *et al.*, 2008; Zhou *et al.*, 2011). This work is geared to overcome the incomplete membrane blocking and removal of antibody-associated probes, which were associated with the use of DIG High Prime DNA Labeling and Detection Starter Kit II (which produced a black background). Therefore, we changed the prehybridization, hybridization and blocking systems and were able to produce a clear background at a much lower cost. Although DIG High Prime DNA Labeling and Detection Starter Kit II was used in cDNA arrays and Southern blot, it was not appropriate for colony hybridization. Therefore, SSHaLL can be applied in the common supplied laboratories to completely perform library screening with higher throughput in a short time. In the present study, rare and abundant differentially expressed genes, such as transcription factor, *AhCYP707A4* and *AhHsfA4a*, in

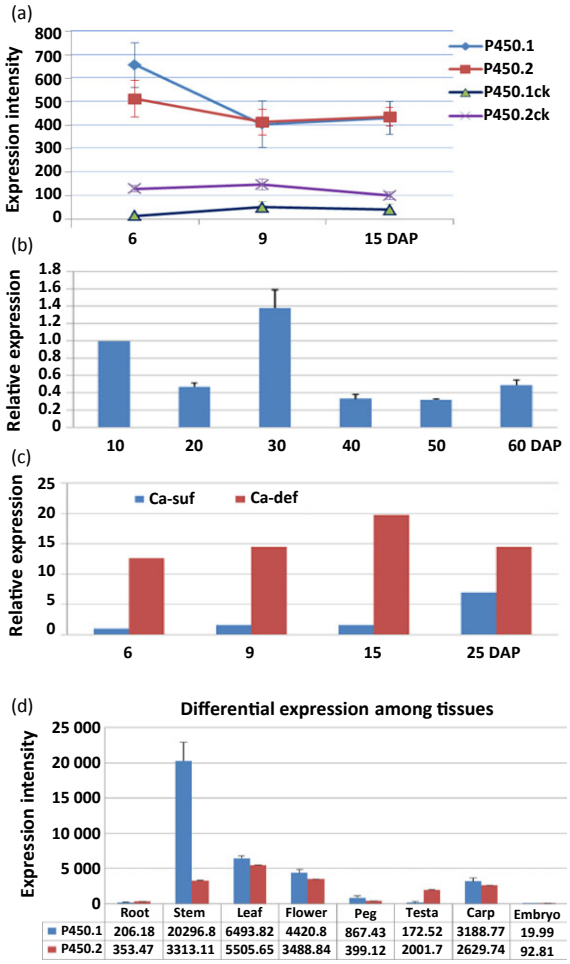

Figure 7 In silico or qRT-PCR analysis of two cytochrome P450 genes expressions. (a): Patterns of expression intensity of two cytochrome P450 genes in different time courses after pegging under deficiency and sufficiency Ca^{2+} conditions by microarray. Both were up-regulated significantly from 6 to 15 DAP in deficiency Ca^{2+}. (b): Pattern of *CYP707A4* transcripts in seeds from 10 to 60 DAP under Ca^{2+} sufficiency condition in comparison with the expression level at 10 DAP, analyzed by qRT-PCR. (c): *CYP707A* transcripts in early developing seeds (6–25 DAP) under deficiency and sufficiency Ca^{2+} stresses compared with Ca^{2+}-sufficient expression level at 6 DAP, analyzed by qRT-PCR; much higher expression induced under low Ca^{2+}. Ca-suf in the figure represents sufficient Ca^{2+}, Ca-def represents deficient Ca^{2+}. (d): The expression intensity of two cytochrome P450 genes in different tissues by chip analysis. Both were expressed weakly in root and embryos, and appeared similar temporal and spatial characteristics of expressions.

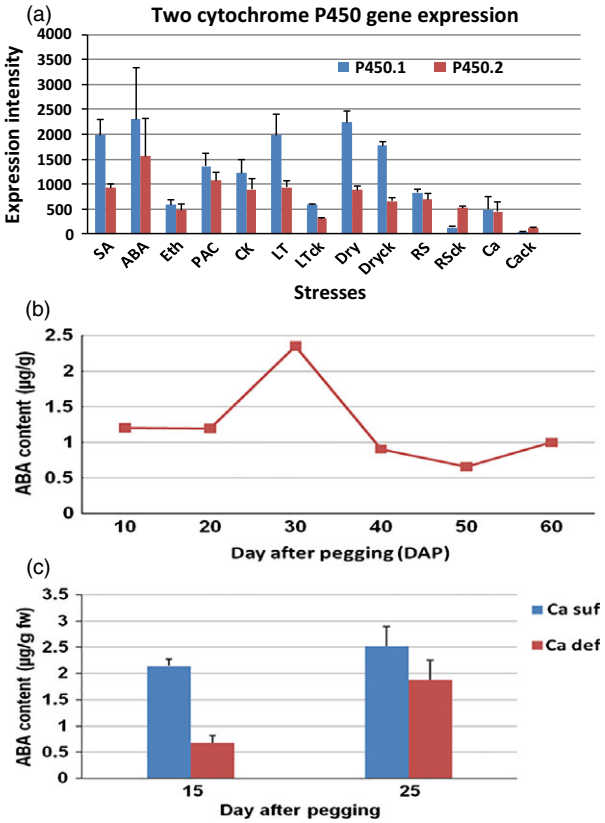

Figure 8 Expression changes of *AhCYP707A4* genes under biotic and abiotic stresses and ABA content in peanut seeds in various developmental stages and under calcium stresses. (a): The expression intensity of the two cytochrome P450 genes under various biotic and abiotic stresses by chip analysis. (b): Changes in ABA contents in developing peanut seeds under sufficient Ca^{2+} condition. (c): Lower ABA contents in young seeds under low Ca^{2+}, resulted from up-regulation of Cytochrome P450s. Ca suf. represents Ca^{2+} sufficiency treatment; Ca def. represents Ca^{2+} deficiency.

early embryos grown under Ca^{2+}-deficient conditions were isolated. Many of the genes isolated in the screening process are unknown.

Differential genes isolated by SSHaLL provide an account for low Ca^{2+}-induced embryo abortion

Sixty-two differentially expressed genes induced by Ca^{2+} deficiency were screened with SSHaLL. They are involved in many biological processes, including response to stresses, regulation, DNA and RNA metabolism, translation, carbohydrate and lipid metabolisms, and morphogenesis. Many Ca^{2+} deficiency-induced up-regulation genes were hydrolysis genes, such as cell wall-associated hydrolase genes, cellulase genes, hydrolysing o-glycosyl genes and glycolysis-related genes; stress-response genes, such as P450s, aluminium-activated malate transporter 9-like genes, 14-3-3-like protein genes, mannose/glucose-binding lectin genes and pathogenesis-related protein ten genes; and rRNA intron-encoded homing endonuclease genes (Figures 5 and 6). However, several embryo development-related genes, such as desiccation-related protein pcc13-62, late embryogenesis-abundant group 6, embryonic abundant protein usp92, arachin ahy-4, cellulose synthases and HsfA4a, were down-regulated (Figure 6). These changes demonstrated that Ca^{2+} deficiency affects many biological pathways and regulations, which made embryo development-required gene expressions diminished, but hydrolysis metabolism increased. By microarray analysis, we confirmed 28 SSHaLL-selected gene expression patterns and found out several cell wall metabolism-related genes showed differed expression patterns during Ca^{2+} stress (Figure 6i,j). The up-regulation of cell wall hydrolases and down-regulation of cellular synthases resulted in the embryonic cell wall degradation. This could also explain the rotten pods or pegs happened under serious Ca^{2+} deficiency. Therefore, these results were consistent with the observed cell apoptosis marked by cell wall

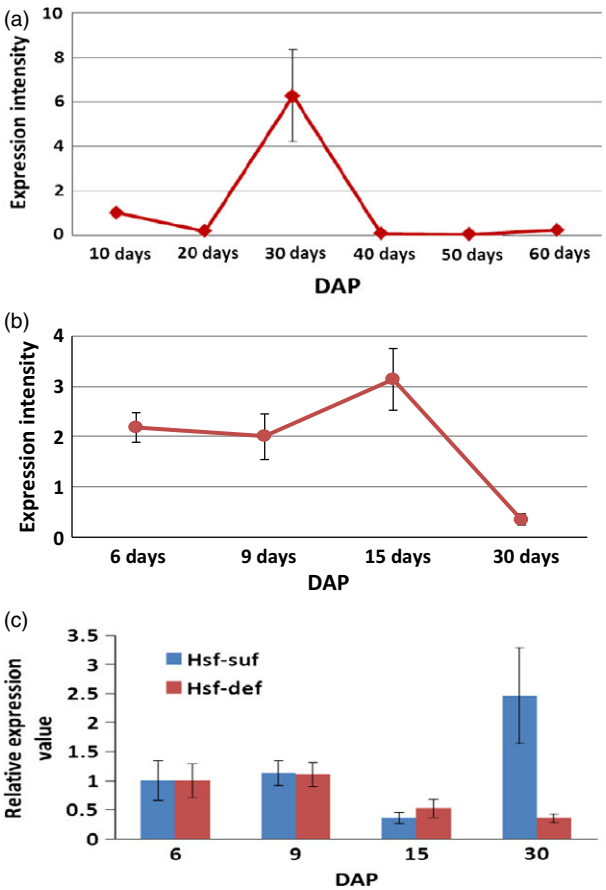

Figure 9 *AhHsfA4a* gene expression in peanut embryos under sufficient and deficient Ca^{2+} stresses in the soil. (a): Transcript level of *AhHsfA4a* changed with developing seeds with drastic increase at 30 day after pegging (DAP) in peanut grown under Ca^{2+} sufficiency. (b): Transcript levels of *AhHsfA4a* were higher from 6 to 15 DAP, but much lower at 30 DAP in Ca^{2+} deficiency stress in comparison with that of Ca^{2+} sufficiency trial of the same stage. (c): Compared with the transcript level of *AhHsfA4a* at 6 DAP, the expression patterns of *AhHsfA4a* between deficient and sufficient Ca^{2+} trials were similar from 6 to 15 DAP, but it differed greatly at 30 DAP with Ca^{2+} deficiency embryos showing significantly lower expression level in contrast to that of Ca^{2+} sufficiency embryos. Hsf-Z indicates sufficiency Ca^{2+} trial and Hsf-Q indicates deficiency Ca^{2+} trial.

degradation and disordered cell structure under Ca^{2+} deficiency (Figure 1). In addition, many unknown genes were also found to be involved in embryo abortion and their functions require further characterization.

A recent study on the transcriptome sequencing by 454 reported the association between the up-regulation of three genes (two senescence-related genes and one late embryogenesis-abundant gene) and early embryo abortion inside aerial pegs of peanut (Chen *et al.*, 2013). The results did not agree with our results probably because the former study used not only embryos, but also pegs or young pods, which are mainly composed of other tissues. Usually, ovules inside the pegs remain dormant for a long time prior to pegging into the soil under drought, but these ovules develop normal pods after penetrating into the soil under humid conditions. Therefore, stage differential expression during early pod development is indicated.

AhCYP707As played a key role in regulating Ca^{2+} deficiency-induced early embryo apoptosis in peanut

Low expression of AhCYP707As in embryo should be necessary to embryo development in peanut

The *P450.1* and *P450.2* were selected by SSHaLL as up-regulated under Ca^{2+} deficiency. They were both annotated as *AhCYP707A4* which encodes ABA 8′-hydroxylase (Figure S10; Table S1), a key enzyme in ABA catabolism producing phaseic acid (Okamoto *et al.*, 2009). Spatio-temporal expressions showed *P450.1* and *P450.2* demonstrated similar features under sufficient Ca^{2+} conditions (Figure 7d). They expressed the least in embryo and weakly in root and pegs, but much higher in stem, leaf, flower and pericarp, indicating a high content of ABA was needed for embryo development (Yang and Zeevaart, 2006). Both *P450.1* and *P450.2* in leaves showed up-regulated response to ABA, SA, low temperature, drought and *R. solanacearum* inoculation to varied extents, but down-regulated response to ethylene (Figure 8a). The *P450.1* showed higher expression levels than *P450.2*, thereby implying that these genes may have different origins given that peanut is a tetraploid species. Nevertheless, *AhCYP707A* expression in embryos was observed up-regulated the most ratio only under deficient Ca^{2+} which led to embryo apoptosis (Figure 8a). These results indicate that low *CYP707A4* expression maintains a higher level of ABA, which should be essential for embryo development in peanut.

ABA level controlling early embryo development is regulated by AhCYP707A4 under low calcium

The *AhCYP707A4* expressions and ABA levels were analysed comparatively in embryos under deficient and sufficient Ca^{2+}. qRT-PCR showed embryo *AhCYP707A4* expressed higher from 10 to 30 DAP and decreased to a stable level after 30 DAP (Figure 7b). Investigating the dynamics of ABA metabolism under sufficient Ca^{2+} condition showed a curve with single peak at about 30 DAP (Figure 8b), corresponding to the rapid embryo cell growth and reserve accumulation (Zhuang *et al.*, 1992). This indicates a balance between ABA synthesis and degradation during seed development. This pattern was also conformed to the results of bean (Yang and Zeevaart, 2006). Furthermore, both microarray and qRT-PCR showed *AhCYP707A4* expression was much higher under low Ca^{2+} conditions than under sufficient Ca^{2+}, especially at 6 DAP (Figure 6a; Figure 7a,c). The *P450.1* showed up-regulation over 45-fold at 6 DAP under low Ca^{2+} conditions as determined by the hybridization of microarray with double strains of cDNA (Figure 7a). Correspondingly, ABA level was reduced significantly, especially during the early stages (prior to 15 DAP) under the deficiency Ca^{2+} (Figure 8c). Thus, up-regulated *AhCYP707A4* led to ABA degradation in early embryo under deficiency Ca^{2+}. A number of studies have shown that ABA is essential for seed development and maturation and for the prevention of precocious germination (Kermode, 1990; Matakiadis *et al.*, 2009). Catabolism of ABA under water stress and during rehydration in bean was regulated by *PvCYP707As* at the transcriptional level (Yang and Zeevaart, 2006). CYP707As regulate seed dormancy through catabolism of ABA both during seed development and germination (Matakiadis *et al.*, 2009). These suggest that ABA content during peanut embryo development is regulated by *AhCYP707A* at the transcriptional level under deficient calcium condition.

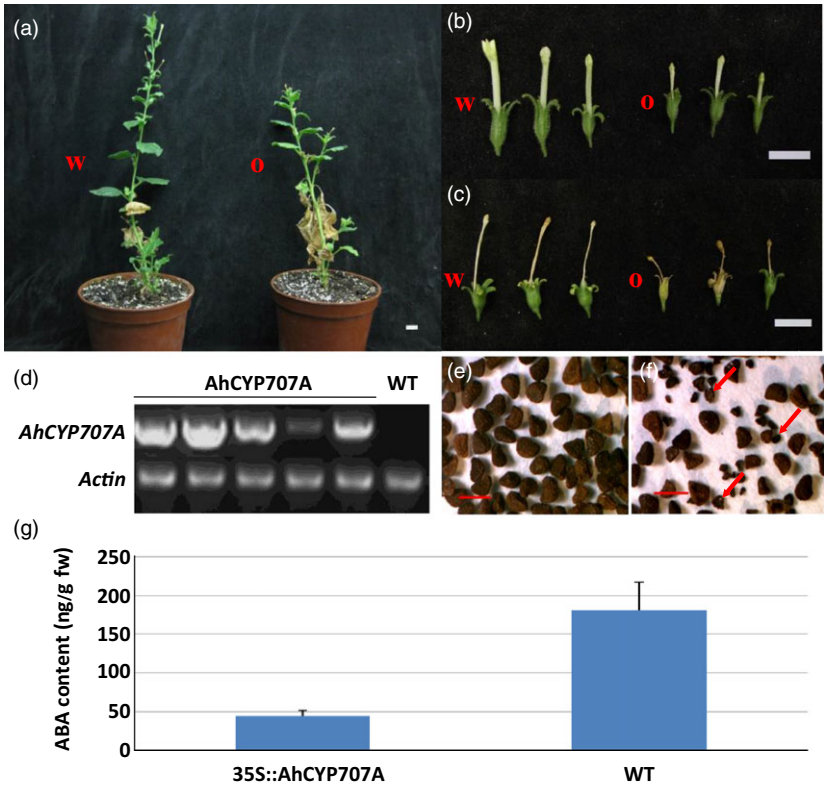

Figure 10 Phenotype of representative *Nicotiana benthamiana* plants that constitutively express *AhCYP707A* genes. (a–f): Phenotypes of *35S::AhCYP707A* transgenic and wildtype plants after 35 days. Transgenic plants show easy to wilt under drought (a-o); flowers show smaller and develop slowly in transgenic plants (b-o); fruits are much smaller (c-o) with tiny and high degree of steril seeds and less bigger seeds (c-o; f), arrows indicate abortive embryos. Wildtype plants have normal size of flowers (b-w), fruits (c-w) and seeds (e). (d): Constitutive expression of *AhCYP707A* was seen on transgenic plants but not on wide type. (g): ABA content is significantly lower in plants with *35::AhCYP707A*. White bars indicate 1 cm; red bars indicate 500 μm.

Up-regulated AhCYP707As decreasing ABA under Ca^{2+} stress lead to embryo abortion in peanut

Over-expression of *AhCYP707A4* in *N. benthamiana* definitely showed that *AhCYP707A4* degradation of ABA resulted in a high number of aborted embryos. ABA is a growth regulator controlling stress signals and vegetative growth; ABA also reportedly determines cell fate (Chandrasekaran and Liu, 2014; Young and Gallie, 2000). Genetic and biochemical studies in maize showed that the onset of programmed cell death was accelerated in developing endosperm of ABA-insensitive *vp1* and ABA-deficient *vp9* mutants (Young and Gallie, 2000). Some studies showed that ABA regulated embryogenesis and endosperm development and was associated with the biosynthesis of storage reserves (lipids, proteins and starch) at the seed filling stage (Carrier *et al.*, 1999; Chandrasekaran and Liu, 2014). These findings agreed with our results in peanut. Transgenic *Nicotiana sylvestris* plants over-expressing *PvCYP707As* displayed a wilt phenotype with partially dead leaves and produced small pods with low seed number (Yang and Zeevaart, 2006). Transgenic *N. benthamiana* plants with *35S::AhCYP707A4* showed a strong expression of *AhCYP707A4* and a significantly reduced ABA content compared with the wild-type plants, which were consistent with those in peanut under deficient Ca^{2+}. These transgenic plants showed phenotypes of easier wilt under dry conditions, lower flower growth, and small flowers and fruits with numerous aborted seeds (Figure 10b–f). We concluded that embryo abortion induced by Ca^{2+} deficiency resulted from the up-regulation of *AhCYP707As* in the early stage of development, which reduced ABA level and consequently led to embryo abortion via apoptosis. Thereby, we first demonstrated reason of low Ca^{2+}-induced embryo abortion in peanut, which could provide foundations for further studies on signal pathway of how AhCYP707A4 links the calcium and ABA signals in controlling embryo development and the reserves filling.

AhHsfA4a may participate in embryo abortion at the downstream region of *AhCYP707A4*

The *AhHsfA4a* was identified to be important in peanut postembryo development. The *AhHsfA4a* expression decreased, as observed by optical SSHaLL selection and by microarray analysis, and was down-regulated by over 10-fold in early embryos under Ca^{2+} deficiency. Quantitative expression analysis showed that *AhHsfA4a* was greatly expressed after 20 DAP and reached its highest level at 30 DAP (Figure 8). After 30 DAP, *AhHsfA4a* expression decreased back to trace level (Figure 8a). Cell fission of peanut embryo and its cotyledons was active from 15 to 30 DAP, during which reserve substances, such as lipids and proteins, accumulated rapidly and cell size expanded dramatically (Zhuang *et al.*, 1992). These changes were consistent with the strong expression activity of *AhHsfA4a* in this stage, indicating *AhHsfA4a* is greatly required for cell growth and reserves accumulation. It was demonstrated that *AhHsfA4a* participates in many biological processes in response to stresses (external, substance and chemical stimuli), regulating transcription towards biosynthesis and postembryonic development (Figures S7–S9). This elucidates that the deficient Ca^{2+}-decreased expression of the *AhHsfA4a* prohibits embryo development and leads to the embryo retardation in peanut.

Furthermore, HsfA4 had been characterized to act as antiapoptotic factor in tomato by controlling reactive oxygen species homoeostasis, and the status of its associated with pro-apoptotic HsfA5 is related to cell type-specific functions connected with the control of cell death, which is triggered by pathogen infection and/or reactive oxygen species (Baniwal *et al.*, 2007). The *Arabidopsis* plants expressing a dominant-negative mutant form

of HsfA4a were defective in their responses to oxidative stress (Davletova *et al.*, 2005; Miller and Mittler, 2006). Expression of HsfA4a in rice and wheat enhanced Cd tolerance (Shim *et al.*, 2009). Mouse embryos whose mothers lacked HSF1 were unable to develop properly beyond the zygotic stage (Christians *et al.*, 2000). We suggest that AhHsfA4a plays an important role in controlling embryo development, and its down-regulated expression under low Ca^{2+} conditions would lead to embryo abortion via apoptosis in peanut. In comparison with AhCYP707As, which were up-regulated significantly starting from 6 DAP, AhHsfA4a should participate in regulating embryo abortion downstream in relation to low ABA content.

In conclusion, we have developed SSHaLL, a simple and cost-efficient method for identifying differential expression genes under numerous conditions. By SSHaLL, we identified 62 differentially expressed genes associated with embryo abortion under low Ca^{2+} stress. Low Ca^{2+} induced the empty pods via embryo apoptosis marked with cell wall degradation and structure disorder. The cell wall degradation resulted from the changed expressions of cell wall-related genes. The up-regulation of P450s in early embryos leading to ABA degradation was the key player for low Ca^{2+}-induced cell death, and reduced ABA should in turn change gene expression resulting in embryo abortion. The down-regulated *AhHsfA4a* presumably plays a function for embryo apoptosis downstream of P450s. The obtained data first elucidate the basic reason of embryo abortion under low Ca^{2+} conditions, which will provide strategies of great importance to genetic enhancement of peanut adaptable to lower calcium. SSHaLL thereby provides a simple and high efficient method for screening important differential genes of interest.

Experimental procedures

Plant growth and treatments

The *A. hypogaea* cv. 'Minhua 6' was obtained from Fujian Agriculture and Forestry University and grown in the field. The Ca^{2+}-deficient soil in Pingtan, Fujian Province of China, was used for stress treatments. The exchangeable Ca^{2+} content in the soil was 0.245 cmol/kg soil. Peanut grown in this soil was used as Ca^{2+} deficiency-treated materials, and peanut grown in the same soil fertilized with 75 kg 667/m^2 plaster used as Ca^{2+} sufficiency-treated materials. The exchangeable Ca^{2+} content in the soil after fertilization was 1.298 cmol/kg soil, and generally, the critical value of Ca^{2+} content in soil that can result in peanut embryo abortion was <1.20 cmol/kg soil. Embryos (6, 9, 15, 21 and 30 DAP; Figure 1) were collected to construct the SSH cDNA library and for microarray analysis, qRT-PCR and ABA content assay.

Light microscope observation

Early embryos (9, 15 and 21 DAP) were fixed in 3% (v/v) glutaric dialdehyde in 0.1 M phosphate buffer (pH 7.3) for 4 h at 4 °C and postfixed in 1% (w/v) osmic acid of same buffer for 12 h. The embryos were dehydrated using graded ethanol series and embedded in Epon 8. Sections (1 μm thick) were cut by glass knife and stained by PAS for 20 min to detect polysaccharides after pretreatment with 0.5% (v/v) of periodic acid in 0.3% (v/v) of nitric acid for 10 min. The stained sections were washed for 5 min with sodium metabisulfite solution and for several minutes in distilled water prior to microscopic observation.

Total RNA extraction and mRNA purification

Total RNAs were extracted from early embryos harvested at three different stages (6, 9 and 15 DAP) under Ca^{2+} deficiency and sufficiency treatments using the improved CTAB method. Frozen samples were ground into powder in liquid N_2 and transferred into 1.5 mL CTAB extraction buffer [2% (w/v) CTAB, 2% (w/v) PVP, 100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 2 M NaCl and 4% (v/v) β-mercaptoethanol]. The lysate was extracted with an equal volume of chloroform–isoamyl alcohol [24 : 1 (v/v)] and precipitated for RNA at −80 °C for 1 h by adding 1/3 volumes of 10 M LiCl. The sediment was then dissolved in 600 μL DEPC-treated water and precipitated again by LiCl. The RNA sediment was washed and dissolved in DEPC-treated water. After quality analysis, mRNAs were isolated using Oligotex™-dT30<Super> mRNA Purification Kit (Takara, Dalian, China). The total RNAs of three different stages were mixed equally and used for mRNA isolation as tester and driver RNAs, respectively.

Construction of SSH cDNA library

Suppression subtractive hybridization was performed using the PCR-Select™ cDNA Subtraction Kit according to the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). When Ca^{2+} deficiency-induced early embryos were used as the testers, Ca^{2+} sufficiency-induced early embryos were used as the drivers, and vice versa. The tester and the driver cDNAs were prepared from 1 μg mRNA using M-MLV RTase cDNA Synthesis Kit (Takara, China). The tester cDNA was digested with *Rsa* I and ligated to adaptors 1 (5′-CTAATACGACTCACTATAGGGCTCGAGCGGC CGCCCGGGCAGGT-3′/5′-TGGACGGGCC-3′) and 2R (5′-CTA ATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3′/ 5′-TGGAGC CGGC-3′) in separate reactions at 16 °C. The driver cDNA was added to each of the tester samples for the first hybridization, and then, freshly denatured driver cDNA was added to the mixed testers for the second hybridization. PCR amplification with two different nested primers (Nested PCR primer1: 5′-TCG AGCGGCCGCCCGGGCAGGT-3′; Nested PCR primer2R: 5′-AG CGTGGTCGCGGCCGAGGT-3′) was conducted to amplify the differentially expressed cDNAs. The subtracted cDNAs for differentially expressed genes were inserted into pGEM-T Easy vector (Promega, Madison, WI) and transformed into *Escherichia coli* DH5α with eletrotransporation (BTX ECM 630) and cultured overnight on LB medium (containing 100 mg/L Amp and X-gal/IPTG) at 37 °C.

SSH library screening

Subtracted double-strand cDNAs of Ca^{2+} deficiency- and sufficiency-treated early embryos, 2 μg each, were labelled as probes with digoxigenin-dUTP by random prime DNA (Roche, Switzerland), respectively, according to the protocol of DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Switzerland).

Bacteria containing the Ca^{2+}-deficiency or Ca^{2+}-sufficiency embryo cDNA library were screened according to Amersham Hybond-N^+ nylon filter protocol. The library was applied to nylon filters (≈2000 colonies per plate) on agar plates with ampicillin and incubated at 37 °C. The colonies were transferred onto filters of duplicate replicas, and the original main filter was kept in 4 °C for picking positive clones after hybridization. Differential screening of the subtracted clones was performed using two methods in comparison. One was performed according to DIG High Prime DNA Labeling and Detection Starter Kit II protocol (Roche, Switzerland), and the other was carried out using our macroarray

library colony hybridization method. Our method proceeded as follows. The filters were briefly washed in sodium phosphate/SDS solution [2% (w/v) SDS, 0.15 M sodium phosphate, pH 7] in hybridization oven. Preheated hybridization solution [1% (w/L) BSA, 1 mM EDTA, 7% (w/L) SDS, 0.5 M sodium phosphate, pH 7] was added at 30 mL per bottle to the filters and prehybridized for 1 h at 65 °C. The hybridization solution was poured off. Freshly preheated hybridization solution at 20 mL (containing 50 ng/mL digoxigenin-labelled probes) was added. The filters were layered into the solution one at a time, so that each filter comes in contact with the probe solution. Hybridization proceeded with shaking or rotation at ~3 rpm for 16–18 h (overnight) at 65 °C. The probe solution was poured off and saved. The probe solution was stored frozen and reused several times. Filters were washed thrice for 10 min each at 65 °C in sodium phosphate/SDS solution and followed by thrice for 5 min each in TNT solution [0.1% (v/v) Tween-20, 0.5 M NaCl, 25 mM Tris-Cl, pH 7.5] at room temperature. Later, the filters were washed once for 5 min in TNT plus milk [0.1% (v/v) Tween-20, 4% (w/L) powered milk, 0.5 M NaCl, 25 mM Tris-Cl, pH 7.5] at room temperature and then in TNT solution plus milk containing a 1 : 10 000 dilution of anti-digoxigenin alkaline phosphatase were incubated for 1 h. The filters were layered into the solution one at a time so that each filter comes in contact with the antibody solution and were washed thrice for 10 min each with excess TNT at room temperature to remove nonreacted antibodies and finally placed into the detection buffer for the subsequent CSPD reaction according to DIG High Prime DNA Labeling and Detection Starter Kit II protocol.

The hybridized probes were immunodetected with anti-digoxigenin-AP fab fragments and were visualized with the chemiluminescence substrate CSPD (ready-to-use). Enzymatic dephosphorylation of CSPD by alkaline phosphatase resulted in a light emission at a maximum wavelength of 477 nm, which was recorded on X-ray films in complete dark condition. As most forward hybridization colonies showed stronger intensity of dark spots than those of the reverse hybridization after SSHaLL, the bacterial colonies showing different intensities with more than fivefold up-regulated or down-regulated expression on X-ray film were selected, cultured and sequenced.

SSHaLL

SSHaLL is based on the screening of a SSH cDNA library with the probes containing cDNAs labelled directly from the second PCR-amplified products of SSH cDNA or from the reverse transcription (Figure 2). This method involves the construction of SSH libraries, the preparation of macro array colony films with subpooled cDNA libraries, and the library screening with digoxigenin (DIG)-labelled cDNA probes after or prior to SSH. After hybridization, differentially expressed clones were picked as primary positive genes, which can be arrayed on filter for reverse northern blotting or can be sequenced directly for further identification.

Analysis of cDNA Insert and Blast2GO search

The cDNA inserts of the selected clones were sequenced by 3730xl sequencer [Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China] using T7 or Sp6 primers. The obtained DNA sequences were analysed for homology in NCBI databases using BLASTX (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997). Differentially expressed genes in Ca^{2+} deficiency-induced young embryo were isolated by SSHaLL. Blast2GO suite (Gotz *et al.*, 2008) was used to characterize the function of the selected genes. Biological functions of *AhCYP707A4* and *AhHsfA4a* were specifically analysed by Blast2GO.

Microarray analysis

Most SSHaLL-selected genes were evaluated by microarray analysis as probes. In silico analysis of peanut genes, eight organs and tissues at different growing stages and samples from embryos and leaves that underwent biotic and abiotic stresses, such as low temperature and drought, low Ca^{2+} stresses and hormone treatments (ethylene, ABA, salicylic acid and paclobutrazol), and *R. solanacearum*, were collected for microarray analysis. On a slide, unigene probes of 60-base oligonucleotides were arranged with a density of 12 × 135K probes. Total RNA was extracted using CTAB-LiCl method, and double strands were synthesized and further purified using NucleoSpin® RNA clean-up (MACHEREY-NAGEL, Düren, Germany) for hybridization. Hybridization, washing, scanning and data analysis were carried out according to the NimbleGen's Expression user's guide and performed at CapitalBio Corporation (Beijing, China). The expression intensity of all hybridizations was analysed, and expression-level estimates were calculated among different tissues and under diverse stress conditions. The expression data were normalized using quantile normalization (Bolstad *et al.*, 2003), and expression data of genes were generated using the Robust Multichip Average (RMA) algorithm (Irizarry *et al.*, 2003a,b). For all experiments, three replicates were performed.

The expression levels of 28 highly induced genes were identified and compared after the time courses of pegging under Ca^{2+}-deficient versus Ca^{2+}-sufficient conditions. The expressions of *P450.1 and P450.2* selected by SSHaLL were performed in the above microarray studies and analysed by Excel for expression features among different tissues and under various stresses.

Quantitative real-time RT-PCR

Total RNAs were extracted respectively from peanut young embryos (6, 9, 15, 30 DAP) induced by Ca^{2+} sufficiency and deficiency in soil, and from embryos of different development stages (10, 20, 30, 40, 50, 60 DAP) under Ca^{2+} sufficiency soil using the modified CTAB method for quantitative expression analysis of *AhHsfA4a* and *AhCYP707A*. Total RNA (3 μg) was subjected to cDNA synthesis using PrimeScript reverse transcriptase (TaKaRa, Dalian, China). Quantitative real-time PCR was performed in Mastercycler ep realplex (Eppendorf, Hamburg, Germany) using the SYBR Premix Ex Taq kit (Takara, Dalian, China) according to the manufacturer's instructions. The *AhHsfA4a* cDNA was amplified using specific primer pairs: AhHsfA4a_RT_F (5′-CAAGCTCCACTGACAGAATCAG-3′) and AhHsfA4a_RT_R (5′-CAGGTTTCTGCAGTACACTGGA-3′). Primer pairs of AhCYP707F (5′-TGAAGCAATCAAAGCAGAGCAGATG-3′) and AhCYP707AR (5′-GATGCTTGCCATCCTTAGGCTTTCC-3′) were used for amplification of AhCYP707A. A peanut actin gene was used as an internal reference in PCR amplification, and its cDNA was amplified using the following specific primers: actin-F: 5′-TCACACTGGTGTGATGGTTG-3′; and actin-R: 5′-TCCAGCTCTTGCTCATA GTC-3′. The relative transcript levels were calculated against the controls and expressed as ΔΔCT (Schmittgen and Livak, 2008) The calculation formula was $\Delta\Delta Ct = (CT_{gene} - CT_{actin})_{treat} - (CT_{gene} - CT_{actin})_{control}$. For detecting the relative transcript levels of *AhHsfA4a* and *AhCYP707A* under different treatments in peanut, *Ahactin* was

used as an internal reference. Data represented means and standard errors of four replicates.

Measurement of ABA

The procedures for extraction, purification and quantification of ABA were conducted as described previously (Hou *et al.*, 2013). Embryos at different stages or with calcium stresses or transgenic plants were used as samples. ABA was extracted using ethanol solution (80%, V/V) under low-temperature condition. The extracting sample was separated by HPLC (Hewlett-Packard, Palo Alto, CA) with a C18 ODS column (250 mm × 4.6 mm, 5 μm) using methanol–0.6% acetic acid (50 : 50, V/V) as the mobile phase. The signal was detected by Photodiode Array (PDA), and the external standard method was used for quantitative analysis. Analyses of ABA were repeated three times with similar results.

Transgenic plants

Fusion gene *35S::AhCYP707A4* was constructed on pBI121-GUSA binary vector (an improved pBI121 by our laboratory) at the *XbaI* and *AscI* sites and was transformed into *N. benthamiana* leaf discs mediated by *A. tumefaciens*. The leaf discs were transferred to Murashige and Skoog medium supplied with 2 mg/L benzyladenine, 0.1 mg/L naphthylacetic acid and 50 mg/L kanamycin for adventitious shoot induction. The same medium without any hormones was used for rooting. The rooted plants (T_0) were transferred to soil and grown in a growth chamber under the conditions of 14 h light at about 300 μmol/m^2/s a day at 25 °C. For confirmation of insertion, genomic DNA from transgenic plants was isolated and PCR amplification was performed. The segregation of the offsprings from T_0 to T_3 generations was characterized and selected by PCR. The inserted gene expression was identified by RT-PCR with primers located in 35S promoter and *AhCYP707A4*.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figures S1–S9 Pathway maps for 62 SSH genes, cytochrome P450.1, and *AhHsfA4a*, including of biological processes, molecular functions and cellular components.
Figure S10 ABA catabolism by AhCYP707A.
Figure S11 Sequence comparison of conserved proteins.
Tables S1–S3 Blast2GO results for the screened sequences.
Tables S4–S9 Functions of *AhCYP707A4* and *AhHsfA4a* by Blast2GO.
Data S1 Sequences of SSHaLL-isolated genes.
Data S2–S4 Folds of 28 SSH genes expression changes induced by deficiency Ca^{2+} screened by SSHaLL.