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The Role of the Expansin Gene in the Process of Novel Subterranean Seed Development in *Arachis duranensis*

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ABSTRACT

Background: Plants in the genus *Arachis* produce flowers aerially that develop into gynophores that grow into the ground, where they develop into fruits. Although some aerial gynophores develop into pods aboveground, embryo or seed abortion occurs in these individuals. During pod development, the shell wall initially comprises the majority of the fruit volume, but then the seeds expand up to the total pod volume. Expansins are plant cell wall-loosening proteins involved in cell enlargement and a variety of other developmental processes in which cell-wall modification occurs.

Methods: In this study, we analyzed the expansin genes during seed development using RNA-seq data by bioinformatic approaches.

Results: We identified five expansin genes exhibited up-regulated expression, while four genes were down-regulated expression using *Arachis duranensis* RAN-seq data. Multiple transcription factors regulate *AdEXPs* throughout seed development. Genes co-expressed with *AdEXPs* are involved in metabolic processes throughout pod development into seed development, reframing the current understanding of the novel subterranean peanut fruits.

Key words: *Arachis*, Co-expression, Expansin, Gynophore, Transcription factor.

INTRODUCTION

Peanuts (*Arachis hypogaea*) are a major oil and protein crop worldwide. Peanuts originated South America through the formation of an allopolyploid hybrid formed between the wild diploid species *A. duranensis* and *A. ipaensis* (Bertioli *et al.,* 2016). Plants in the genus *Arachis* produce flowers aerially, which develop into gynophores that then grow into the ground, where they develop into fruits (Smith 1950). This novel developmental trait may protect seeds and help them access more water for germination (Bertioli *et al.,* 2016). *Arachis duranensis* and *A. ipaensis* genome sequences have been released (Bertioli *et al.,* 2016) and their publication has provided a helpful tool for understanding aerial flowers that develop into subterranean fruits. The genome size of *A. hypogaea* (~2.7 Gb) is similar to the combined *A. duranensis* (~1.25 Gb) and *A. ipaensis* genomes (~1.56 Gb) (Bertioli *et al.,* 2016), indicating that *A. duranensis* is an ideal choice for a research organism because it has a smaller genome.

Previous studies have examined the aerial flowers and subterranean fruits of *Arachis* using RNA-seq data. Zhang *et al.* (2016) found that differentially expressed genes are involved in light signalling and plant hormone metabolism in the embryonic and basal tissues of both aerial and subterranean gynophores. However, we have observed aerial pod development in greenhouse conditions and the field (Fig 1A). Development of the peanut shell wall of the pod initially occupies the majority of the fruit volume, but then the seed expands up to the total pod volume through a process called pod-filling (Gupta *et al.,* 2016). When pods cannot continue to expand, embryos or seeds abort. Accordingly, research into the process of seed development in pods can inform the current understanding of the development of aerial flowers into subterranean fruits. Expansins are plant cell wall-loosening proteins involved in cell enlargement and in a variety of other developmental processes in which cell-wall modification occurs (Marowa *et al.,* 2016). The present study was conducted with two main aims: (1) the identification of differentially expressed expansin genes between the Pattee 3 pod and Pattee 5 seed stages; (2) the identification of genes regulated by *A. duranensis* expansin genes (*AdEXPs*).

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MATERIALS AND METHODS

A total of RNA-seq datasets from 22 tissues have been released on the PeanutBase website (https://peanutbase.org/gene_expression/atlas). All details of the sequencing, transcriptome assembly and generation of expression values were previously described by Clevenger *et al.* (2016). The fragments per kilobase per million reads

mapped (FPKM) values for each gene were distinguished for both the *A. duranensis* and *A. ipaensis* genomes from cultivated peanut. Peanut kernel maturity can be future classified into one of 15 stages (Pattee *et al.,* 1974). Among these stages, Pattee stage 3 pods exhibited very watery pericarp tissues and very small embryos that were not easily removed and Pattee stage 5 seeds exhibited flat embryos that were white or just turning pink at one end (Pattee *et al.,* 1974). The differentially expressed genes between the Pattee stage 3 pods and Pattee stage 5 seeds were identified using the DESeq script in *R* program. If the observed value of the $\log_2$-transformed fold-change exceeded 2 or was less than -2 and the adjusted *p*-value was less than 0.05 for a particular gene, the gene was considered as differentially expressed.

AdEXPs were identified among differentially expressed genes reported by Guimaraes *et al.* (2017). The 2 kb *cis*-acting element sequence of the full-length expansin gene was retrieved from the PeanutBase website (https://peanutbase.org/genomes/jbrowse/?data=Aradu1.0). Transcription regulatory elements were predicted using NSITE with a *p*-value threshold of 0.05. Transcription factor annotation was retrieved from the PlantTFDB 4.0 database. Genes co-expressed with the expansin were detected among 22 tissues using a weighted gene co-expression network analysis (WGCNA) script in *R*. Differentially expressed genes with a $\log_2$ fold-change of greater than 2 or less than -2 were used for WGCNA analyses. A soft threshold (β) value of 12 was used in the transformation of the adjacency matrix in order to meet the scale-free topology criteria.

Gene ontology (GO) annotations for genes in each module containing the expansin gene were extracted from the *A. duranensis* genome available on the PeanutBase website (https://peanutbase.org/files/genomes/Arachis_duranensis).

RESULTS AND DISCUSSION

AdEXPs play a crucial role in pod development into seed development

Nine *AdEXP*s were identified as differentially expressed between the Pattee stage 3 pod and Pattee stage 5 seed tissues in *A. duranensis*. These genes, as named by Guimaraes *et al.* (2017), were *AdEXPA8*, *AdEXPA11*, *AdEXPA17*, *AdEXPA18*, *AdEXPA21*, *AdEXPB5*, *AdEXPB6*, *AdEXLA1* and *AdEXLB8*. Among these genes, five were up-regulated, while four were down-regulated (Fig 1B). Among up-regulated genes, *AdEXPA18* had the highest expression (up to 8-fold, Mann–Whitney U test, $P < 0.05$). Among down-regulated genes, *AdEXLB8* had the lowest expression (up to 9-fold, Mann–Whitney U test, $P < 0.05$).

The tissue expression analysis showed that six *AdEXPs* were expressed at high levels, but three *AdEXPs* were expressed at low levels among 22 tissues (Fig 1C). In the low expression group, *AdEXPA18* was expressed at a high level at different periods in seed tissues, compared to other tissues (Fig 1C). This indicated that *AdEXPA18* is possibly involved in seed development in *A. duranensis*. In contrast, in the high expression group, *AdEXPB5* and *AdEXLB8* were expressed at low levels at different periods in the development of seed tissues compared to other tissues (Fig 1C). The expression of *AdEXPB5* is higher in the androecium, Pattee stage 3 pod, aerial gynophore tip and subterranean gynophore tip tissues relative to other tissues, indicating that *AdEXPB5*

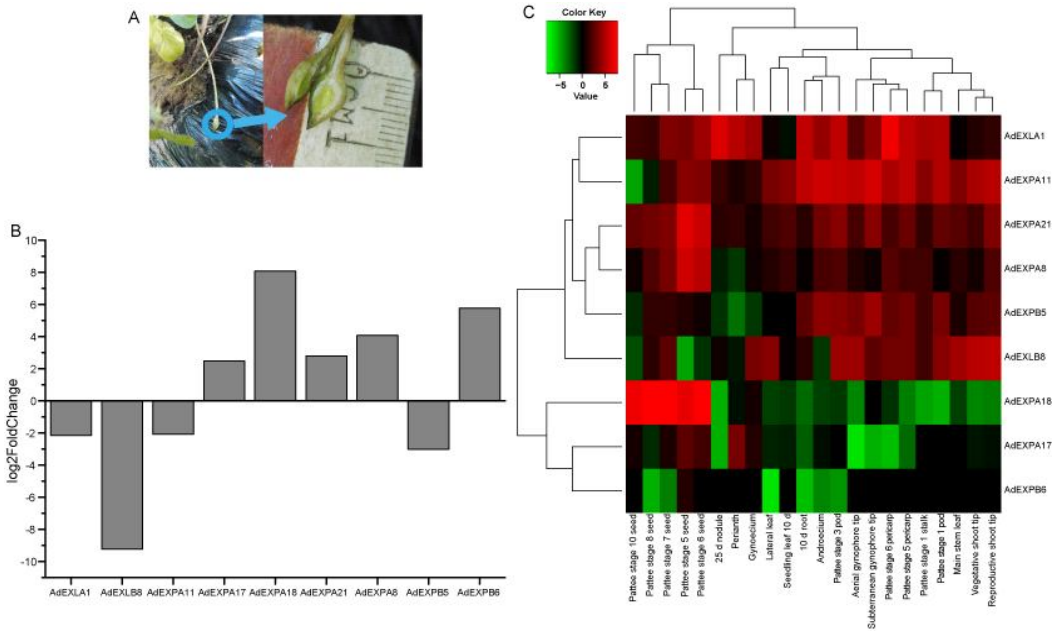


Fig 1: *AdEXPs* involved in pod duvelopment.

A. Gynophores expanding onto the ground in the field. **B.** Nine *AdEXPs* were differentially expressed between Pattee stage 3 pod and Pattee stage 5 seed tissues. **C.** Nine *AdEXPs* expressed in 22 tissues.

may be involved in reproduction. Accordingly, *AdEXLB8* is a likely component of a regulatory hub.

Multiple transcription factors regulate *AdEXP* genes in seed development

The 2 kb sequence segments upstream of the initiation codons of eight *AdEXPs* were extracted for all *AdEXPs* except *AdEXPA21* because it is within a portion of the genome with poor sequencing coverage. The NSITE results indicated that various transcription factors regulate these eight *AdEXPs* (Table S1). The *cis*-acting elements of *AdEXPB5* and *AdEXPA8* genes contained embryo- and epicotyl-specific nuclear transcription factor binding sites, respectively. Moreover, various transcription factors regulated *AdEXPs*; bZIP transcription factors were the most common regulator of these *AdEXPs*, followed AP2/ERP, bHLH and WRKY (Table S1). In this study, we also identified a NAC transcription factor binding site in *AdEXPA11* and *AdEXPB5* as well as a LBD transcription factor binding site in *AdEXLA11*. These results indicated that NAC and LBD transcription factors may regulate the process of seed development from pods in *A. duranensis*.

Genes co-expressed with *AdEXPs* were involved in metabolism-associated and signal transduction processes

The co-expression analysis revealed that the genes differentially expressed between the Pattee stage 3 pod into the Pattee stage 5 seed tissues could be classified into five modules involved in 2,340 total genes, including 1042, 604, 400, 189 and 105 genes in modules 1, 2, 3, 4 and 5, respectively (Fig 2). Modules 1, 2, 3 and 5 contained three (*AdEXPA11*, *AdEXPA17* and *AdEXLA1*), two (*AdEXPA8* and *AdEXPA21*), two (*AdEXPA18* and *AdEXLB8*) and one (*AdEXPB5*) *EXPs*, respectively. *AdEXPB6* was absent from all modules, indicating that this gene was not co-expressed with other genes. Further, we determined the most similar patterns of expression between *EXPs* and other genes in each module (Fig 3). The most closely matching expression pairs were *AdEXPA8* and Aradu.5N10F, *AdEXPA11* and Aradu.M76UB, *AdEXPA17* and Aradu.U39XE, *AdEXPA18* and Aradu.KM0LY, *AdEXPA21* and Aradu.L5D7V, *AdEXPB5* and Aradu.22FIV, *AdEXLA1* and Aradu.X70GU and lastly, *AdEXLB8* and Aradu.8I3T0 (Fig 3).

EXPs in modules 1, 3 and 5 had similar GO terms, including GO:0005576 (extracellular region) and GO:0009664 (plant-type cell wall organization; Table S2). However, *AdEXLA1* and *AdEXPA8* lacked GO annotation and *AdEXLB8* was only associated with GO:0005576 (extracellular region; Table S2). *AdEXPB5*, in module 5, was associated with GO:0019953 (sexual reproduction), indicating that this gene is involved in the process of sexual reproduction. The GO terms of genes with the most similar

Table S1: The transcription factor binding sites from the *cis*-acting elements of *AdEXPs*.

Name	Binding factor
AdEXPA11	BPC1(BBR-BPC family, AGAAAGAGA); IDD8 (C2H2 Family,CTTTTGTCC); RTCS (LBD family,TCGCCGCT); TCP8 (TCP family,GTGGGCCC); WRAF1/2 (AP2/ERF family,CTAAAGAAAATTC)
AdEXPB5	embryo protein factor(AACCCA); ABF(bZIP family, CACGTGGC); ABI3(B3 family, CATGCG); ABI5(bZIP family, GACACGTGGC); AFL(LFY family,GACACGTGGC); bZIP(bZIP family, GCCACGTGTC); DPBF4(bZIP family, ACACGTGGC); GBF1(bZIP family, TGCCACGTGTCC); GT-1(Trihelix family, AAACCGtTAAAA); HY5(bZIP family, GGACACGTGGCA); MYB88(MYB family, GGCGCGCC); MYC2 (bHLH family, GGACACGTGGCA); NAC092 (NAC family, GTTGCGTGT); STK-SEP3(MADS family, CTAAAAATTGG); WRI1(AP2/ERF family, AAAAAGTAtGAGTTT)
AdEXPA17	BPC1(BBR-BPC family, AGAAAGAGA); GT-1(Trihelix family, TTATTGTAAAAT); GT-2A(Trihelix family, GTTTAGTTAAT ATG); IDD8(C2H2 family, CTTTTGTCC)
AdEXPB6	BPC1(BBR-BPC family, GGAGAGAGA); Dof11(Dof family, ATAAAGAATAAAGATA); GT-3a(Trihelix family, AATGTTACT TT); ORA59(AP2/ERF family, GCAGCCTCT); TSAR1(bHLH family, CTCTCGTGGA); WRKY26 (WRKY family, ATTT TGACCATTT); ZFHD1(ZF-HD family, AGTAATTAATTCATAAATTA)
AdEXLB8	bZIP(bZIP family, CCACGCA); HsfA2(HSF family, TCAATTCAAGAAACTA); HsfA3(HSF family, TGGAAGTTTCAA)
AdEXLA1	ABI4 (AP2/ERF family, GCCACCG); bHLH(bHLH family, CATCATCTGCCA); bZIP1(bZIP family, CTGACGTGGC); GBF1(bZIP family, TGACGTGG); HBP-1b(bZIP family, CCACGTCA); HY5(bZIP family, GCTGACGTGGCT); NAC(NAC family, GCCACGTCAGC); NF-YB3(NF-YB family, CCAAT); OBF4(bZIP family, GCTGACGTGGC); OBF5(bZIP family, GCTGACGTGGC); RAP2.6(AP2/ERF family, TGCCACCGC); ROM2 (bZIP family, GCCACGTCAGCA); STY1 (SRS family, GTACTCTACTC); TGA1(SBP family, TGACGTGG); WRKY1(WRKY family, TTGACTTTTGACT); WRKY11(WRKY family, GTTTTGACTTTTG)
AdEXPA18	BZR1(BES1 family, GCCATGTGCT)
AdEXPA8	epicotyl-specific nuclear factor (TCATCTACACTTTTCAA); ABF(bZIP family, TCCACGTCAC); BPC1(BBR-BPC family, AGAGAGAGA); CBF(AP2/ERF family, ATGCCCCtCA); E2F/DP(E2F/DP family, TCTCCCTCC); E2F2(E2F/DP family, GGCGCCAAA); GBF1(bZIP family, TGACGTGG); TGA1(SBP family, TGACGTGG); HBP-1b(bZIP family, CCACGTCA); HBP-11(bZIP family, CCACGTCA); LFL1(B3 family, CATGCATG); MYB9(MYB family, TTCAAGTGCT); OBF4(bZIP family, TGTGACGTGGA); OBF5(bZIP family, TGTGACGTGGA)

Note: Contents of bracket indicate gene family and binding site, respectively.

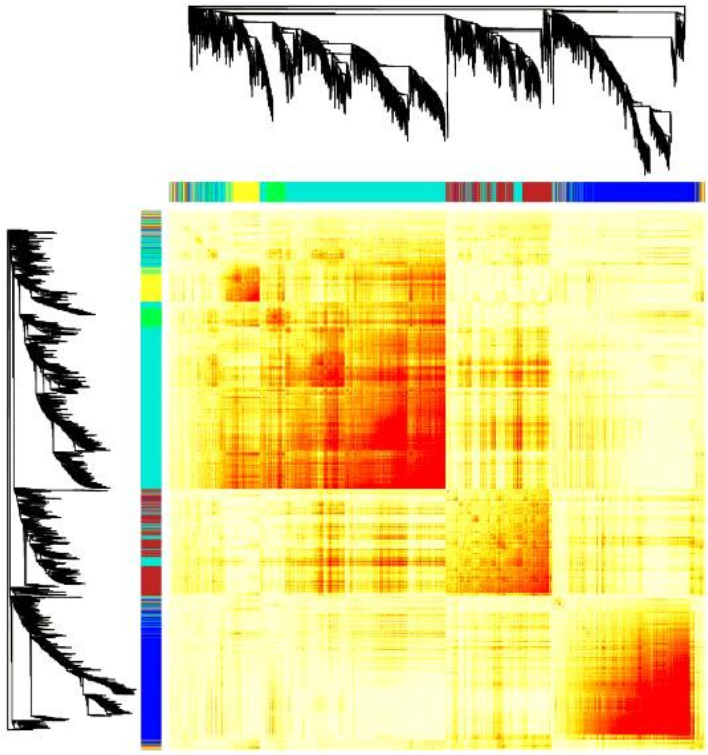


Fig 2: Network heat map of the nine *AdEXPs* and co-expressed genes in 22 tissues.

Table S2: The GO annotation of *AdEXPs* and their related most similar genes in four modules.

Name	Annotation	GO term
Module 1		
AdEXPA17	Cell wall protein	GO:0005576 (extracellular region); GO:0009664 (plant-type cell wall organization)
Aradu.U39XE	DNA-binding pseudobarrel domain	GO:0003677 (DNA binding)
AdEXPA11	Cell wall protein	GO:0005576 (extracellular region); GO:0009664 (plant-type cell wall organization)
Aradu.M76UB	AUX/IAA protein	GO:0005634 (nucleus)
AdEXLA1	Cell wall protein	No detection
Aradu.X70GU	Unknown protein	No detection
Module 2		
AdEXPA8	Cell wall protein	No detection
Aradu.5N10F	Fatty acid desaturase	GO:0006629 (lipid metabolic process); GO:0055114 (oxidation-reduction process)
AdEXPA21	Cell wall protein	GO:0005576 (extracellular region); GO:0009664 (plant-type cell wall organization)
Aradu.L5D7V	Unknown protein	No detection
Module 3		
AdEXPA18	Cell wall protein	GO:0005576 (extracellular region); GO:0009664 (plant-type cell wall organization)
Aradu.KM0LY	Ethylene response factor	No detection
AdEXLB8	Cell wall protein	GO:0005576 (extracellular region)
Aradu.8I3T0	Peroxidase superfamily protein	GO:0004601 (peroxidase activity); GO:0006979 (response to oxidative stress); GO:0020037 (heme binding); GO:0055114 (oxidation-reduction process)
Module 5		
AdEXPB5	Cell wall protein	GO:0005576 (extracellular region); GO:0019953 (sexual reproduction)
Aradu.22FIV	ATP-citrate synthase alpha chain protein	No detection

expression patterns among *EXPs* revealed that genes in module 1 were associated with DNA binding and nuclei, genes in modules 2 and 3 were involved in the oxidation–reduction process and genes in module 5 lacked GO annotation (Table S2). The top ten GO terms among biological processes in modules 1, 2, 3 and 5 were mainly involved in metabolic processes and signal transduction (Table S3). In general, *AdEXPs* were associated with producing energy and co-regulation of multiple genes. In modules 1, 2, 3 and 5, the GO terms "protein phosphorylation" and "oxidation–reduction process" were particularly frequent. Expansin proteins can be classified into four subfamilies,

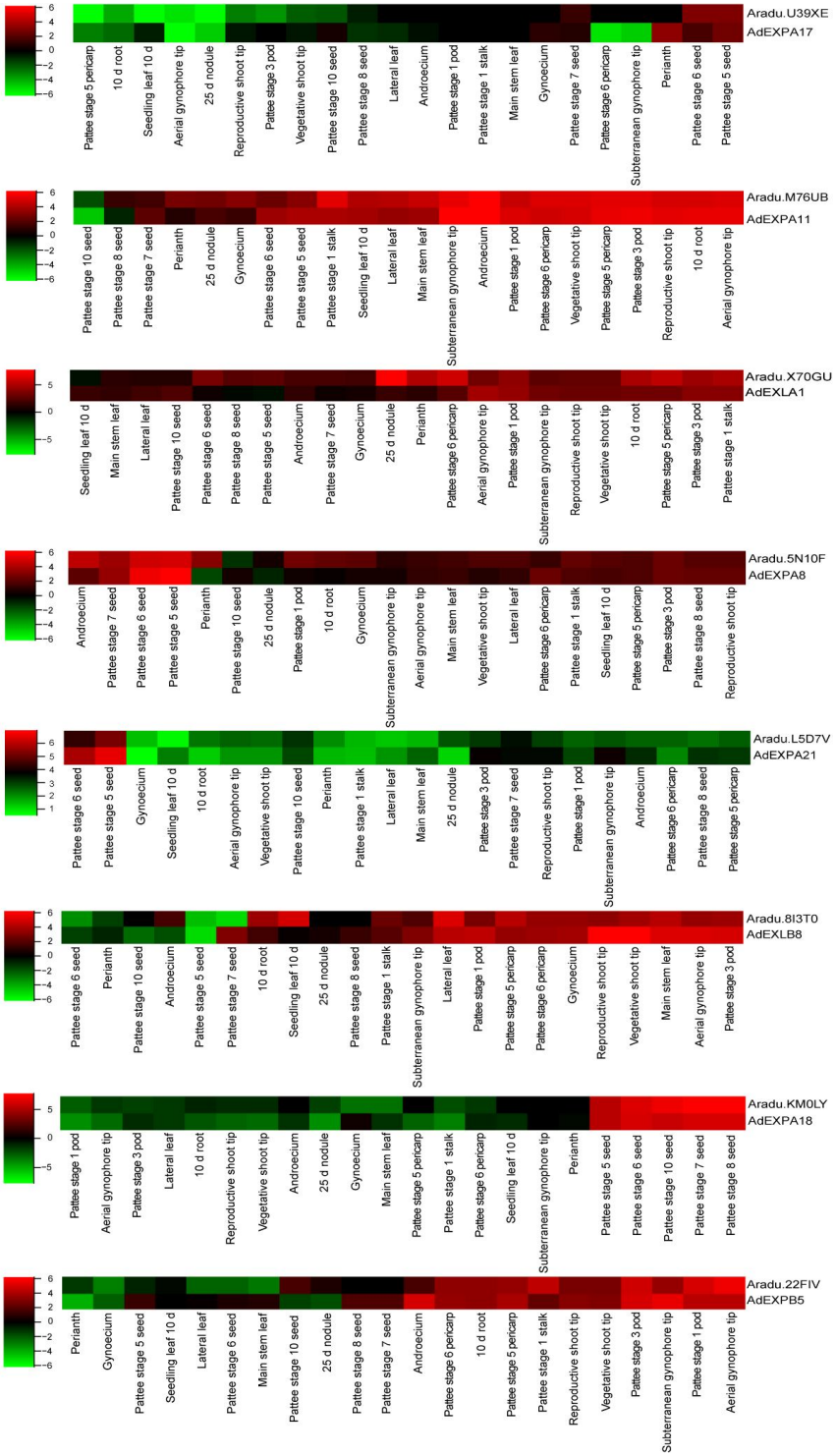


Fig 3: The gene expression pattern of *AdEXPs* and co-expressed genes in 22 tissues.

including α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) according to their phylogenetic relationships and a standardized nomenclature (Sampedro and Cosgrove 2005). Genome-wide identification of expansin proteins revealed that EXPA and EXPB proteins were detected in lower plants, but EXPLA and EXPLB proteins were only found among angiosperms (Carey *et al.,* 2013). While *EXPA* and *EXPB* are involved in cell wall-loosening, *EXPLA* and *EXPLB* genes had broader and more uncertain roles (Cosgrove 2015; Marowa *et al.,* 2016). Boron *et al.,* (2015) over-expressed *AtEXLA2* in *Arabidopsis thaliana*, cell wall strength decreased. In the present study, we found that *AdEXLA1* and *AdEXLB8* were possibly down-regulated between pod and seed development.

In this study, we found multiple transcription factors regulate *AdEXP* genes in seed development such as bZIP, AP2/ERP, bHLH, WRKY, NAC and LBD transcription factors. The previous studies have determined bZIP, transcription factors AP2/ERP, bHLH and WRKY can regulate seed development processes (Jakoby *et al.,* 2002; Rushton *et al.,* 2010). In peanut, Gao *et al.* (2017) found that bHLH regulates pod development. Previous studies had reported that LBD, NAC and HB12 transcription factors regulate *EXPs*. LBD transcription factors have been shown to regulate banana *MaEXP1/2* during fruit ripening and induce *AtEXPA14* during lateral root development (Cosgrove 2015). Overexpression of the NAC transcription factor (SPEEDY HYPONASTIC GROWTH) in transgenic *A. thaliana* can activate several *EXPs* to enhance waterlogging-triggered hyponastic leaf movement and cell expansion in abaxial cells within the basal petiole region, while both traits were sharply diminished in *shyg* knockout mutants (Rauf *et al.,* 2013). AtHB12 can activate *cis*-acting elements of *AtEXPA10* and thus promote leaf growth (Hur *et al.,* 2015).

Arachis originated east of the Andes in the area comprising southeastern Bolivia and northwestern Argentina (Bertioli *et al.,* 2016). *Arachis* produces flowers aerially, which develop into gynophores that grow into the ground and develop into fruits underground; this novelty may protect seeds and provide more access to more water for germinating seeds (Smith 1950). This novelty has accordingly been a focus of research; however, to date, the molecular mechanisms that underlie this novel trait have remained unclear. Although several papers have reported differential gene expression between aerial gynophores and subterranean gynophores, these studies have provided little insight into the causal molecular mechanism. Here, we have proposed that one possible key mechanism is the inability of pods to continually expand, thus resulting in embryo or seed abortion in the pod development process. Aerial gynophores are unable to develop into fruits (or seeds) on the ground because some environmental cues such as light may suppress *EXP* expression. While no studies have yet supported this specific hypothesis, this framework may be helpful in studying this novel trait in peanut. The research focus should shift to the reasons for embryo or seed abortion rather than sticking to comparisons of gene expression between aerial gynophores and subterranean gynophores. As such, we examined the expression of expansin genes during the Pattee stage 3 pod and Pattee stage 5 seed development. Beyond the scope of this study, comparisons among leguminous genomes have shown that the cultivated

Table S3: The top ten biological processes of co-expressed genes among four modules.

GO term	Name
Module 1	
GO:0006468	Protein phosphorylation
GO:0055114	Oxidation-reduction process
GO:0006952	Defense response
GO:0008152	Metabolic process
GO:0007165	Signal transduction
GO:0055085	Transmembrane transport
GO:0006629	Lipid metabolic process
GO:0005975	Carbohydrate metabolic process
GO:0006810	Transport
GO:0006508	Proteolysis
Module 2	
GO:0006468	Protein phosphorylation
GO:0055114	Oxidation-reduction process
GO:0006260	DNA replication
GO:0007018	Microtubule-based movement
GO:0005975	Carbohydrate metabolic process
GO:0006334	Nucleosome assembly
GO:0008152	Metabolic process
GO:0006281	DNA repair
GO:0006508	Proteolysis
GO:0007067	Mitosis
Module 3	
GO:0055114	Oxidation-reduction process
GO:0055085	Transmembrane transport
GO:0005975	Carbohydrate metabolic process
GO:0008152	Metabolic process
GO:0006468	Protein phosphorylation
GO:0006810	Transport
GO:0006508	Proteolysis
GO:0006629	Lipid metabolic process
GO:0006979	Response to oxidative stress
GO:0006952	Defense response
Module 5	
GO:0055114	Oxidation-reduction process
GO:0008152	Metabolic process
GO:0006468	Protein phosphorylation
GO:0055085	Transmembrane transport
GO:0006508	Proteolysis
GO:0006810	Transport
GO:0006855	Drug transmembrane transport
GO:0005975	Carbohydrate metabolic process
GO:0016567	Protein ubiquitination

peanut genome contains many peanut-specific genes (Song *et al.*, 2019). Genes surely control the transition from aerial flowers into subterranean fruits. However, taken together, we propose that this novel trait may be the product of a complex network rather than several genes of large effect. In this study, although we did not directly reveal the molecular underpinnings of this novel trait, we did identify and analyze the biological functions of the *AdEXPs* that were differentially expressed between the development of the Pattee stage 3 pod and Pattee stage 5 seed tissues. In future research, these nine *AdEXPs* may provide insight into understanding the evolutionary and developmental origins of this novel trait.

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