

Discovery, Identification, and Functional Characterization of Long Noncoding RNAs in *Arachis hypogaea* L.

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Abstract

Background

Long noncoding RNAs (lncRNAs), which are typically >200 nt in length, are involved in numerous biological processes. Studies on lncRNAs in the cultivated peanut (*Arachis hypogaea* L.) largely remain unknown.

Results

A genome-wide scan of the peanut (*Arachis hypogaea* L.) transcriptome identified 1,442 lncRNAs, which were encoded by loci distributed over every chromosome. Long intergenic noncoding RNAs accounted for 85.58% of these lncRNAs. Additionally, 189 lncRNAs were differentially abundant in the root, leaf, or seed. Generally, lncRNAs showed lower expression levels, tighter tissue-specific expression, and less splicing than mRNAs. The majority of the lncRNAs featured an exon/intron structure, and approximately 44.17% were alternatively spliced; this rate was slightly lower than the splicing rate of mRNA. Transcription at the start site event was the alternative splicing (AS) event with the highest frequency (28.05%) in peanut lncRNAs, whereas the occurrence rate (30.19%) of intron retention event was the highest in mRNAs. AS changed the target gene profiles of lncRNAs and increased the diversity and flexibility of lncRNAs, which may be important for lncRNAs to execute their functions. Additionally, a substantial number of the peanut AS isoforms generated from protein-encoding genes appeared to be noncoding because they were truncated transcripts; such isoforms can be legitimately regarded as a class of lncRNAs. The predicted target genes of the lncRNAs were involved in a wide range of biological processes. Furthermore, expression pattern of several selected lncRNAs and their target genes were examined under salt stress, results showed that all of them could respond to salt stress in different manners.

Conclusions

This study provided a resource of candidate lncRNAs and expression patterns across tissues, and whether these lncRNAs are functional will be further investigated in our subsequent experiments.

Background

The central dogma of molecular biology, which proposes the flow of information from DNA to RNA to protein [1], is no longer tenable with mounting number of RNAs not coding proteins. These noncoding RNAs (ncRNAs) have been classified in various ways in accordance with their locations, lengths, and biological functions [2]. Broadly, ncRNAs fall into two categories: house-keeping and regulatory [2, 3]. The latter can be divided into short ncRNAs (< 200 nt) and include short interfering RNAs (20–31 nt), small ncRNAs and long noncoding RNAs (> 200 nt, lncRNAs) [4]. Short interfering RNAs include small interfering RNAs, microRNAs (miRNAs), and PIWI-interacting RNAs; these sequences differ across the different eukaryotes where they are found [5]. lncRNAs are categorized into antisense lncRNAs, long intergenic

ncRNAs (lincRNAs), and intronic lncRNAs (incRNAs) on the basis of their genomic origin and/or their orientation relative to their neighboring protein-encoding transcripts [3, 6–8]

Undoubtedly, advances in genomics and bioinformatics, particularly the extensive application of next-generation sequencing, have boosted the identification and annotation of ncRNAs that have been determined to participate in a range of regulatory roles rather than simply represent transcriptional noise [4–8]. Although lncRNAs might be the least well-studied of these ncRNAs, a growing body of evidence suggests that they exert their functions at the transcriptional, post-transcriptional, and epigenetic levels in fungi, plants, and animals [5, 9–15]. In particular, animal lncRNAs have been associated with aging, hematopoiesis, pri-miRNA processing, muscle differentiation, neural development, and immune responses [16–21]. Xist, one of the best-studied lncRNAs, silences transcription by directly interacting with SHARP, recruiting SMRT, activating HDAC3, and deacetylating histones to exclude Pol II across the X chromosome during development in female mammals [12]. While studies on plants are limited compared with those on humans and animals, tens of thousands of lncRNAs have been identified via RNA-seq and bioinformatics analyses in several plants, such as Arabidopsis [22], Medicago [23] soybean [9], rice [24], wheat [25], maize [26], tomato [27], mulberry [28], poplar [29], and sea buckthorn [11]. Furthermore, available researches have suggested that a small quantity of lncRNAs perform regulatory functions in plants similar to those in animals [30–32]. For example, the lncRNA COOLAIR, a long intronic noncoding RNA, is required for establishing stable repressive chromatin at FLC through its interaction with PRC2 [31]. In rice, Ding et al. found that the lncRNA long-day-specific male-fertility-associated RNA is essential for the normal pollen development of plants grown under long-day conditions [30]. And also, more and more researches suggest that lncRNAs play important roles in the regulation of gene expression in response to various stresses [33–38]. An Arabidopsis lncRNA, DROUGHT INDUCED lncRNA (DRIR), was a new positive regulator of the plant response to drought and salt stress [37]. DRIR expressed at a low level under control conditions but increased significantly under drought and salt stress as well as ABA treatment. And also, *drir^D* mutant or overexpressing DRIR in Arabidopsis could increase tolerance to drought and salt stress of the transgenic plants, RNA-seq results demonstrated that DRIR can modulate the expression of a series of genes involved in ABA signaling, water transport, and other stress-relief processes. Although a growing body of evidence supports the diverse potential roles of lncRNAs in plants, studies on lncRNAs in the cultivated peanut (*Arachis hypogaea* L.) largely remain unknown. Recently, Zhao et al. identified 50,873 lncRNAs of peanut from large-scale published RNA sequencing data, which belonged to 124 samples involving 15 different tissues, and predicted the co-expressions of targeted genes and 386 hub lncRNAs [39].

The peanut is an allotetraploid species derived from natural hybridization between the wild diploids *A. duranensis* and *A. ipaensis* [40, 41]. The peanut ranks sixth among oilseed crops in terms of seed production; approximately two thirds of harvested peanut seed is used for oil production, with the residual seed cake used as a protein-rich meal for livestock [42]. The genomic sequences of both of the progenitor species of the peanut have now been acquired [43], thus providing an opportunity to analyze the contribution of lncRNAs to the plant's transcription and ultimately to its phenotype. Here, strand-specific

sequence data obtained from the peanut were scanned for lncRNA content, and bioinformatics analysis combined with experiment were applied to illustrate the range of biological processes in which lncRNA activity is likely involved. Some new viewpoints were put forward.

Results

Peanut Transcriptome Analysis

After trimming adapter sequences and low-quality reads, over 11.14 Gb of clean data were acquired from each of the four libraries (FH1-seed1, FH1-seed2, FH1-root, and FH1-leaf) for further analysis. The reads were resolved into 203.8 billion paired-end reads with lengths of 125 bp. The individual libraries' Q30 value ranged from 88.90–89.79%, and their GC content ranged from 44.48–50.48% (Additional file 1: Table S1). The raw data were deposited in the NCBI sequence read archive (<http://www.ncbi.nlm.nih.gov/sra>) with project PRJNA354652. Between 79.91% and 84.16% of the reads were successfully aligned with the peanut reference genome sequence (Additional file 2: Table S2). The majority of reads (66.60–74.25%) were mapped to the exonic sequence, with < 10% mapping to intronic sequence (Fig. 1A). The FPKM values associated with each library suggested that the distribution of lncRNA abundance in the four libraries was broadly similar, although some evidence showed that the FH1-root library differed marginally with respect to their sequence distribution and their abundance (slightly higher) (Fig. 1B). Three types of lncRNA sequences were identified: lincRNAs, incRNAs, and antisense-lncRNAs, and lincRNAs accounted for 85.60% of the full set (Fig. 1C).

Identification of LncRNAs

A total of 1,442 sequences remained after the imposition of the various criteria intended to identify putative lncRNAs (Additional file 3: Table S3; Fig. 1C); of these sequences, 1,007 were represented in FH1-seed1, 992 in FH1-seed2, 952 in FH1-root, and 917 in FH1-leaf (Fig. 2A). The number of sequences represented in all four libraries was 465, whereas the numbers of library-specific sequences were 86, 75, 78, and 50; 261 of the lncRNAs were specific to the developing seed (specific to either FH1-seed1 or FH1-seed2 or present in both libraries but absent from FH1-root and FH1-leaf). The number of lincRNAs represented in all four libraries was 387, whereas the numbers of library-specific lincRNAs were 77, 70, 70, and 42 (Fig. 2B). Substantial numbers of organ-specific intronic-RNAs and antisense-lncRNAs were also recognized (Fig. 2C, D). A total of 189 of the lncRNAs were classified as differentially abundant lncRNAs (DALs; Fig. 3; Additional file 4: Table S4). The number of DALs represented in all four libraries was 20, whereas the numbers of library-specific sequences were 16, 16, 11, and 3 (Fig. 2E).

Genomic Distribution of LncRNAs

The set of identified lncRNAs was encoded by sequences distributed across all 20 chromosomes of the peanut genome, although a higher number was associated with the B subgenome than with the A subgenome chromosomes (863 vs 579, Additional file 5: Table S5). Chromosome Araip.B02 encoded 112 of the identified lncRNAs against the 31 encoded by chromosome Aradu.A07. The mean number of

lncRNAs per hundred genes within the A subgenome was 2.57, whereas the equivalent statistic for the B subgenome was 3.37. The correlation between the number of lncRNAs and the number of transcribed genes (based on an FPKM threshold of 0.1) encoded by each chromosome was + 0.61 (significant at $P < 0.01$).

Comparison between mRNAs and lncRNAs

The sequences of 213,515 transcripts, generated from 55,621 genes, were acquired: this set of sequences, which included AS isoforms, is collectively referred here to “mRNAs”. The mean length of the mRNAs was 2,017 nt, with the majority of their lengths falling in the range of 400–2,600 nt (Fig. 4A), whereas the equivalent lengths of the lncRNAs were 1,074 nt and 400–1,400 nt. (Fig. 4B). The mean length of the open reading frames (ORFs) of the mRNAs was 240 nt, with the majority falling within the range of 100–300 nt, whereas that of the lncRNAs was 96 nt, with the length ranging from 50 nt to 150 nt (Fig. 4C, D). All of the lncRNAs, which included at least one intron, had fewer exons with a mean exon number of 2.77 than the mRNAs, which had a mean exon number of 5.48 (Fig. 4E, F). The abundance of the mRNAs was generally higher than that of the lncRNAs (Fig. 4G). The mRNAs generated a greater number of AS isoforms than did the lncRNAs (Fig. 4H).

Predicting the Target Genes of the lncRNAs

To investigate the potential functions of the lncRNAs, their target genes were examined in cis (Additional file 6: Table S6) and in trans (Additional file 7: Table S7). All the target genes were annotated with their integrated function on the basis of five protein databases. A total of 11,765 target genes were obtained (Additional file 8: Table S8), with 9326 in FH1-seed1, 9415 in FH1-seed2, 8742 in FH1-root, and 8497 in FH1-leaf. Among these annotated target genes, 6,550 were annotated in the COG database, 4,209 in the GO database, 1,922 in the KEGG database, 4,422 in Swiss-Prot, and 10,019 in NR. Differential expressed target genes (DETGs) between these four libraries were analyzed, and the GO terms were used as an example to analyze the functional classification of the DETGs. In total, 49,706 unigenes were assigned GO terms, and the enriched terms differed in diverse tissues (Fig. 5). The number of DETGs enriched between FH1-seed1 and FH1-seed2 was 244 (Fig. 5A), with 367 between FH1-root and FH1-leaf (Fig. 5B), whereas the number of DETGs enriched between FH1-root and FH1-seed1&FH1-seed2, FH1-leaf and FH1-seed1&FH1-seed2 were 50 and 54, respectively (Fig. 5C, D). All the GO terms were classified into three major categories (biological process, cellular component, and molecular function), implied that lncRNAs might well be important for the regulation of a wide range of biological processes.

Experimental Validation of lncRNAs

In order to verify the reality of the predicted lncRNAs, seven of the lncRNAs (lncRNA1-7) were validated through PCR (Additional file 9: Table S9). The results of Sanger sequencing showed that five sequences identity with the relevant lncRNA sequence was very high (99.33–99.86%) except two (lncRNA1 and lncRNA2, Additional file 10: Figure S1). lncRNA1 that shared only 85.68% identity with TCONS_00179561; a Blastn search of the genomic sequence located a highly homologous sequence

(sequence identity: 99.6%) on Aradu.A03 in segment 130713152–130713435, whereas TCONS_00179561 was located in Araip.B03 131673533–131674406; thus lncRNA1 could not have been a product of TCONS_00179561, maybe PCR amplified its homologous genes. lncRNA2 shared 95.81% identity with TCONS_00109592. Blastn search located this sequence in the expected chromosome position (TCONS_00109592) but highlighted a 9 nt indel and several nucleotide polymorphisms between the genomic sequence and the lncRNA. We speculated that the product might be an AS isoform of lncRNA2. The successful validation of six out of the seven lncRNAs indicated that the prediction of the majority of the lncRNAs was credible.

AS Events of lncRNAs

Comparing the lncRNA and genomic DNA sequences suggested that AS is likely involved in their transcription. On the basis of the RNA-seq data, five AS events (transcription start site, TSS; transcription terminal site, TTS; exon skipping, ES; intron retention, IR; alternative exon, AE) were investigated (Table 1). Overall, 1,811 AS events were involved in 637 lncRNAs, accounting for approximately 44.17% of the identified 1,442 lncRNAs. Meanwhile, approximately 47.82% of mRNAs produced alternative transcripts; this rate is higher than that for lncRNAs. TSS was the AS event with the highest frequency (28.05%) in peanut lncRNAs, whereas the occurrence rate (30.19%) of IR was highest in mRNAs. Additionally, the frequency of the ES event was the lowest in lncRNAs and mRNAs.

Table 1
The comparison of AS events between lncRNAs and mRNAs

AS	lncRNA		mRNA	
	AS events	Percentage	AS events	Percentage
TSS	508	28.05%	16942	19.87%
TTS	478	26.39%	13914	16.32%
ES	39	2.15%	10791	12.65%
IR	340	18.77%	25748	30.19%
AE	446	24.63%	17880	20.97%
Total	1811	100.00%	85275	100.00%

Using the genomic segment 94398232–94402918 on chromosome Araip.B05 as an example, three lncRNAs (TCONS_00217911, TCONS_00212806, TCONS_00209269) (Additional file 3: Table S3) were speculated as a group of AS isoforms and verified by PCR test. Five transcripts, namely T1–T5, were sequenced (Fig. 6, Additional file 11: Figure S2). T1 was identical to TCONS_00217911, T2 was an isoform of TCONS_00217911, and a TSS event was happened. T3 and T4 were AS isoforms of TCONS_00212806, and TSS and ES events were happened. T5 was an AS isoform of TCONS_00209269,

and IR and ES events were happened. These alignments demonstrated that AS was a universal phenomenon in lncRNA, and may play important roles in their function regulation.

Another phenomenon was found related to the AS events of lncRNAs; that is, different AS isoforms of one lncRNA have different target genes. For example, the target genes of three lncRNAs (TCONS_00217911, TCONS_00212806, TCONS_00209269) were used for comparison. All of them have no cis-target genes (Additional file 6: Table S6) but have many trans-target genes (Additional file 7: Table S7). Each of them has 12, 14, and 11 trans-target genes, respectively. There were five trans-target genes belonged to three of them. Eight similar trans-target genes belonged to TCONS_00217911 and TCONS_00212806; five similar trans-target genes belonged to TCONS_00217911 and TCONS_00209269; eleven similar trans-target genes belonged to TCONS_00209269 and TCONS_00212806. Only TCONS_00217911 had four specific target genes (Fig. 2F). Other examples included TCONS_00011553, TCONS_00013006, and TCONS_00013007, which were all located on genome Aradu.A01 (99975619–99981506, Additional file 3: Table S3). The results of gene structure analysis showed that they had different AS isoforms (Additional file 12: Figure S3A), and a total of 29 target genes were found (Additional file 6: Table S6, Additional file 7: Table S7). Among these target genes, 26 were represented in three of them, whereas only TCONS_00013006 had no specific target genes and the other two had only one (Additional file 12: Figure S3B). AS can change the target-gene profiles of lncRNAs and increase the diversity and flexibility of lncRNA. This may be an important way for lncRNAs to execute their function.

Protein-Encoding Genes as a Source of lncRNAs

Not all AS isoforms encode a protein and are noncoding to a certain extent. A substantial number of the peanut AS isoforms generated from protein-encoding genes appeared to be noncoding because they were truncated transcripts; such isoforms can be legitimately regarded as a class of lncRNA. Several papers reported that approximately one-third of the alternative transcripts were likely noncoding [44, 45]. Here, a protein-encoding gene Aradu.Z4DIZ, which generates five transcripts, was selected as an example for validating this point (Fig. 7, Additional file 13: Figure S4). Only two of the AS products (Aradu.Z4DIZ.1 and Aradu.Z4DIZ.3) were predicted to encode complete proteins. The Sanger sequencing results of the amplified product generated from a primer pair directed at this gene revealed the presence of five isoforms: L1.5 matched Aradu.Z4DIZ.4 given that both transcripts lacked the seventh exon. L1.1 encoded 232 amino acids (aa) and was 18 residues shorter than the predicted Aradu.Z4DIZ.1 product. L1.2 encoded 118 aa, L1.4 encoded 177 aa, and L1.3 encoded 240 aa; the three encoded proteins were incomplete. The predicted product of Aradu.Z4DIZ is a diacylglycerol O-acyltransferase that carries a conserved LPLAT domain within its C-terminus; the truncated transcripts all lack the determinants of a complete LPLAT domain. This characteristic thereby compromises the functionality of their translation product.

Validation of Expression Level of lncRNAs by qRT-PCR

To validate the abundance of lncRNAs, eight lncRNAs (lncRNA8-15) were randomly selected and analyzed through qRT-PCR (Additional file 9: Table S9). As shown in Fig. 8, the expression patterns of

these lncRNAs were relatively consistent with RNA-seq results; this consistency indicated that the lncRNA expression patterns based on RNA-seq data are reliable.

Coexpression of lncRNAs and Their Target Genes by RT-PCR

One lncRNA can interact with several gene targets and vice versa (Additional file 14: Figure S5). Five lncRNAs (lncRNA16-20) and their target genes (Additional file 9: Table S9) were selected to verify the relationships between lncRNA and mRNA, the relative position of the five lncRNAs and their target genes are shown in Fig. 9A. TCONS_00176941 was located far from its two target genes at 8.2 and 9.5 Kb upstream and downstream, respectively. In all of the six organs, the expression level of TCONS_00176941 was higher than that of its two target genes (Fig. 9B). TCONS_00015630 was close to its two target genes downstream (Fig. 9A), and showed similar expression pattern with Aradu.8P876 in the six organs (Fig. 9C). TCONS_00011551 had three cis-target genes and one trans-target gene which showed different expression patterns in the six organs (Fig. 9A, D). TCONS_00292946 and its two target genes showed similar expression patterns in stems, leaves, flowers, and seeds at 30 day after flower (DAF) and in seeds at 50 DAF (Fig. 9E). TCONS_00243464 and its three target genes showed different expression patterns in six organs (Fig. 9F).

Expression Pattern Analysis of lncRNAs and its Target Genes Under Salt Stress

lncRNAs play important role in stress resistance. Here, three lncRNAs and their target genes (Additional file 9: Table S9) were selected to test their expression patterns in different organs under salt stress using qRT-PCR (Fig. 10). Results showed that these three lncRNAs all responded to salt stress. In the roots, the expression level of TCONS_00292946 decreased within 12 h and then increased at 24 h (Fig. 10A). In the leaves, the expression level fluctuated (Fig. 10D). TCONS_00176941 showed opposing expression levels in the roots and leaves (Fig. 10B, E). The expression level of TCONS_00011551 slowly increased along with salt stress (Fig. 10C), but the expression level fluctuated in the leaves (Fig. 10F).

lncRNAs and their target genes showed different expression patterns in response to salt stress. TCONS_00292946 and its two target genes showed similar expression patterns in the roots (Fig. 10A) and opposing expression patterns in the leaves (Fig. 10D). TCONS_00176941 and Araip.BU32W showed similar expression patterns in the roots (Fig. 10B) and different expression patterns in the leaves (Fig. 10E). TCONS_00011551 and Aradu.ISE5U showed similar expression patterns in the roots and leaves, whereas TCONS_00011551 and Aeadu.Y8Q46 showed different expression patterns in the leaves (Fig. 10C, F). The action mode of these lncRNAs in response to salt stress will be further studied.

Discussion

Improvements in nucleotide sequencing technology have revealed the existence of lncRNAs, some of which act as the regulators of a range of eukaryotic cellular processes [15, 37, 46–48]. The number of lncRNAs identified by transcriptomic analyses reflects the depth of the sequencing method applied. A set of > 13,000 Arabidopsis lncRNAs [2], > 20,000 maize lncRNAs [26], and > 2,000 rice lncRNAs has been

reported [49]. Here, a survey of the peanut transcriptome revealed 1,442 lncRNAs. The low number of identified sequences may simply reflect the small scale of the experiment, which involved only four libraries as opposed to the 200 libraries underlying the above set of Arabidopsis lncRNAs. In addition, the selection criteria applied here were more stringent than those commonly used. For example, by removing the restriction placed on the number of exons, > 10,000 lncRNAs would have been predicted (data not shown), among which a large number would have been false positives. When a small sample (seven sequences) of the set of lncRNAs was subjected to PCR-based validation, > 70% proved to be genuinely represented in the peanut transcriptome.

AS is adopted universally for post-transcriptional gene regulation [50, 51]. In plants, it is deployed to control aspects of growth, development, signal transduction, flowering, circadian clock function, and environmental cue responses [49, 52–55]. Similar to protein-encoding genes, the coding sequences for numerous lncRNAs developed AS variants in animals and plants [56–58]. For example, lncRNA-PXN-AS1, which lacks exon 4, binds to the coding sequences of PXN mRNA and inhibits PXN mRNA translation. By contrast, lncRNA-PXN-AS2, which contains exon 4, preferentially binds to the 3' untranslated region of PXN mRNA, protects PXN mRNA from degradation, and thereby increases PXN expression [58]. Here, a number of AS isoforms were predicted from peanut RNA-seq data, and experiments via PCR validated these isoforms were real (Fig. 6, 7). Like AS of protein-coding genes, all kinds of AS types of protein-coding genes were found in lncRNAs (Table 1), but remarkable difference was also existed. We found that IR was the most occurred AS events in mRNA, with TSS the highest frequency in lncRNAs. AS events of lncRNAs can also increase the diversity of lncRNAs. It can change the targeted genes of a lncRNA (Fig. 2F, Additional file 12: Figure S3), thus increase the flexibility of lncRNA. It may be an important regulation of lncRNAs.

Additionally, numerous protein-encoding genes transcribe diverse variants; several of these transcripts are unable to encode a functional protein because they represent the truncated forms of a fully functional mRNA (Fig. 7). These sequences could be regarded as another class of lncRNAs or pseudogene-derived lncRNAs in some sense [59]. Protein-coding mRNA transcripts can crosstalk with other mRNA transcripts by competing for common microRNAs [59]. The pseudogene PTENP1 has been hypothesized to be biologically active in prostate cancer cells by competitively binding to miR-17, miR-19, miR-21, and miR-26 families to regulate the cellular levels of PTEN and exert a growth-suppressive effect [60]. lncRNAs that were thought not to encode proteins could also translate small polypeptides to exert their function but not through the lncRNA itself [61, 62]. For example, Anderson et al. discovered a conserved micropeptide encoded by a skeletal muscle-specific RNA that was annotated as a putative lncRNA plays an important role in muscle performance [16]. Similarly, experimental evidence exists for the production of functional truncated polypeptides from AS variants derived from a protein-encoding gene. For example, the *A. salina* CCA1 generates two isoforms, one of which is a full-size transcript (CCA1 α), whereas the other is a truncated form (CCA1 β) that lacks a functional N-terminal MYB DNA-binding domain [63]. The latter transcript inhibits CCA1 α and LATE ELONGATED HYPOCOTYL transcription factors by forming nonfunctional heterodimers and is modulated by low temperatures. The human USP2 gene generates seven AS variants, two of which are not translated into a functional protein [64]. The

zebrafish gene LGP2 forms three AS transcripts, of which only the full length version can confer protection against viral infection [65]. The distinction between coding and noncoding RNA is not as sharp as previously thought. Some protein-encoding genes generate noncoding AS variants, which can be recognized as lncRNAs; these sequences may be involved in gene regulation. With technological progress and unremitting effort, additional possible regulatory mechanisms of lncRNAs will be explicitly studied.

A growing body of research suggests that lncRNAs play important roles in plant stress resistance [33–36, 38, 47]. In this study, some peanut lncRNAs with the target genes were tested under salt stress, and the results showed that all of them changed their expression levels (Fig. 10). In poplar, more than 10,000 lncRNAs were identified, and approximately 40% of them responded to salt stress with tissue-specific expression patterns [34]. In cotton, lncRNA973 was localized in the nucleus and increased by salt treatment. The overexpression of lncRNA973 in *Arabidopsis* could increase salt tolerance, whereas the knockdown of lncRNA973 in cotton could reduce salt tolerance [36]. Furthermore, many studies reported that lncRNAs play important roles in plant defense against pathogens [66–68]. In tomato, a plausible model for TYLCV-induced diseases and host antiviral immunity was uncovered; that is, lncRNAs interact with the IR-derived vsRNAs to control disease development during TYLCV infection, which provide an effective strategy for the control of plant viral pathogens [67]. In rice, the connection between lncRNAs and the JA pathway in the regulation of bacterial blight was confirmed, which provided a novel insight into plant disease resistance [68]. lncRNAs have three salient features: low expression, lack of conservation between species, and tissue-specific expression patterns [23]. These features indicated that specific prevention and cure methods can be developed for a specific disease, according to specific species and lncRNAs. This will be a development direction for plant disease resistance research in the future.

Conclusion

We identified 1,442 lncRNAs in peanut transcriptome with strand-specific RNA-Seq technique. Among them, 189 lncRNAs were differentially abundant in the root, leaf, or seed. The majority of the lncRNAs featured an exon/intron structure, and approximately 44.17% were alternatively spliced. This rate was slightly lower than the splicing rate of mRNA. AS changed the target-gene profiles of lncRNAs and increased the diversity and flexibility of lncRNAs. This may be an important way for lncRNAs to execute their functions. Additionally, protein-encoding genes produced many truncated transcripts by AS, which may be another source of lncRNAs. Some lncRNAs and their target genes were selected for salt response experiments. The results showed that all of them could respond to salt stress in different manners. Identification of peanut lncRNAs will have a strong impact on peanut development, growth, and stress resistance breeding.

Materials And Methods

Materials

Peanut plants (cultivar 'Fenghua-1') were grown in a growth chamber with a photoperiod cycle of 16 h light at 26 °C and 8 h dark at 24 °C. Roots and leaves were collected from 12-day-old seedlings (names as FH1-root and FH1-leaf), and developing seeds were collected from plants at 30 days after flowering (DAF) (named as FH1-seed1) and 50 DAF (named as FH1-seed2).

Library Construction and Sequencing

Total RNAs were isolated from FH1-root, FH1-leaf, FH1-seed1 and FH1-seed2 samples. whole transcriptome library preparation and deep sequencing were performed as previously described [69]. Briefly, whole transcriptome libraries were prepared using NEBNext® Ultra™ Directional RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) following the manufacturer's recommendations. Ultimately, sequencing was performed by imposing a paired-end 125 cycle rapid run on a HiSeq2500 platform (Illumina, San Diego, CA, USA).

Transcriptome Assembly

Sequence data were stripped of adapter sequences and low-quality reads. The sequence data acquired from each library were aligned separately with peanut genome sequences (<https://www.peanutbase.org/>) using TopHat2 software [70]. The aligned reads were assembled into a full transcriptome using the Cufflinks v2.2.1 program [71].

Genome-wide Identification of LncRNAs and Alternative Splicing Events in peanut

The assembled transcripts were annotated using the Cuffcompare facility (<http://cufflinks.cbc.umd.edu/>). Transcripts with lengths of more than 200 nt and at least two exons were selected as lncRNA candidates. Subsequently, the transcripts were analyzed using the coding potential calculator (score < 0) [72], the coding-noncoding index (score < 0) [73], the coding potential assessment tool [74], and Pfam (E-value < 0.001) [75] to remove all likely remaining protein-encoding genes. Only sequences that passed all of these four scans were considered as likely lncRNA candidates. Alternative splicing (AS) events were identified using ASTALAVISTA program (<http://genome.crg.es/astalavista/>). The diverse categories of AS events (TSS, TTS, ES, IR and AE) [76] were identified using a Perl script developed in house.

Target Gene Prediction and Functional Annotation

Whether the lncRNAs acted in cis or in trans was predicted. The putative functions of the target genes were also predicted. Coding genes lying within 100 kb either at the 5' upstream or 3' downstream of each lncRNA were identified as potential cis targets [77] on the basis of whether the Pearson and Spearman correlation coefficients between the expression levels of these genes were ≥ 0.6 or ≤ -0.6 , and $P < 0.05$, whereas potential trans targets were predicted with Pearson correlation $r > 0.9$, $P < 0.05$; a gene coexpression network was constructed using the LncTar program [78], which searches for sequence complementarity between mRNAs and lncRNAs. Subsequently, the target genes were subjected to functional annotation analysis using the following databases: NCBI nonredundant protein sequences

[79], KOG/COG [80], Swiss-Prot [81], KEGG [82], and GO [83]. The KOBAS software with default parameters was used to test the statistical enrichment of differentially expressed genes in KEGG pathways following the methods described by Mao et al. [84].

Differential Abundance of LncRNAs

Cufflinks v2.2.1 software was used to calculate FPKM values associated with lncRNAs and coding genes within each library. The analysis was performed using the EBseq (2010) R package [85]. P-values were adjusted to q-values [86]. Only the lncRNAs that met the criteria q-value < 0.01 and log₂ (fold change) > 1 were considered as DALs.

Validation of LncRNAs by PCR

Seven putative lncRNAs (lncRNA1-7) identified from RNA-seq data were validated using PCR assay (Additional file 9: Table S9). An aliquot of the total RNA used to construct the libraries was reverse-transcribed using a RevertAid First Strand cDNA Synthesis kit (Thermo Scientific™, Waltham, MA) following the manufacturer's recommendations. Subsequently, the reaction products were diluted 20-fold to serve as the template in a PCR driven by the primer pairs given in Supplementary Table S9. Each 50 µL reaction comprised 5 µL of 10 × TransTaq® HiFi Buffer II (Transgen Biotech, Beijing, China), 4 µL of 2.5 mM dNTP (each 10 µM), 1 µL of the forward primer and 1 µL of the reverse primer (each 10 µM), 4 µL of the cDNA template (50 ng/µL), 1 µL of TransTaq® HiFi DNA Polymerase (Transgen Biotech), and 35 µL of ddH₂O. The reactions were initially denatured (94 °C/120 s), then subjected to 35 cycles of 94 °C/30 s, T_m/47–60 °C s (T_ms shown in Supplementary Table S1), 72 °C/60 s, and completed with a 72 °C/5 min final extension. The amplified product was purified (TIANGel Midi Purification Kit DP209, TIANGEN Biotech, Beijing, China) and subcloned into the pEASY-T1 Cloning Vector (Transgen Biotech) for Sanger sequencing.

Quantification of LncRNAs and Target Genes Abundances Using Real-Time Quantitative PCR

The real-time quantitative PCR (RT-qPCR) platform was used to validate the abundance of a selection of the lncRNAs (lncRNA8-20, Supplementary Table S1) and adjacent target genes (Araip.73735, Araip.BU32W, Aradu.K3YEA, Aradu.8P876, Araip.36N6E, Araip.9LF7H, Aradu.NR2UV, Aradu.R9S8R, Aradu.Y8Q46, Aradu.ISE5U, Araip.4W7P2, Araip.WRS65, Araip.WU69J, Supplementary Table S9). We extracted total RNA of the tissues for qRT-PCR using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the first-strand cDNA was synthesized by using MMLV reverse transcriptase with random hexamer primers according to the manufacturer's protocol. RT-qPCR analyses were conducted in triplicate using SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) on ABI 7500 FAST real-time PCR platform. The 20 µL reaction volume contained 10 µL SYBR® Green Realtime PCR Master Mix, 0.5 µL template cDNA and 0.5 µL primer was processed in three stages: 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The specificity of PCR products was verified through melting curve analysis, and lncRNA and gene expression were quantified by using the

$2^{-\Delta\Delta Ct}$ method [87], with the abundance of transcript of the gene Actin-1 (XP_015966232.1) used for normalization. The primer sequences are shown in Supplementary Table S1.

AS Isoforms Validation of LncRNAs by PCR

Three pair of specific primers (911-F/R, 269-F/R and 806-F/R, Additional file : Table S9) was designed to test for the presence of the AS isoforms of three lncRNA with the same genomic location (TCONS_00217911, Araip.B05 94398232–94402710; TCONS_00209269, Araip.B05 94398710–94402916; TCONS_00212806, Araip.B05 94398630–94402918). A reaction based on KOD-Plus-neo enzyme (TOYOBO, Osaka, Japan) was conducted according to manufacturers' recommendations. The reactions were initially denatured (94 °C/120 s); then subjected to 35 cycles of 98 °C/10 s, 60 °C/30 s, and 68 °C/60 s; and completed with a 68 °C/5 min final extension. After purification, the amplified product was transferred as above for sequencing.

LncRNAs from Protein-Encoding Genes

A pair of primers (Aradu.Z4DIZ-F/R, Additional file 9: Table S9) was designed to identify the AS isoforms generated from the gene Aradu.Z4DIZ. The PCR was based on the KOD-Plus-neo enzyme (TOYOBO, Osaka, Japan) with 50 µL reaction volume and comprised an initial denaturation (94 °C/120 s) cycle; followed by 40 cycles of 95 °C/15 s, 56 °C/30 s, and 72 °C/60 s; and ending with a 72 °C/5 min extension cycle following manufacturers' instructions. Amplicons were purified and transferred as above and sequenced.

Salt Treatment and Validation by qRT-PCR

For salt treatment, peanut seedlings with a uniform growth status (2 weeks old, approximately 8 cm in height) were treated with 1% NaCl. Roots and leaves were harvested at 0, 0.5, 12, and 24 h after treatment. The harvested materials were snap-frozen in liquid nitrogen and stored at – 80 °C until required for RNA extraction. Total RNAs were prepared using a DP441 RNeasy Pure Plant kit (Qiagen, Beijing), and the resulting RNA converted into cDNA using a RevertAid First Strand cDNA Synthesis kit (K1621, Thermo Scientific™). RT-PCR analyses were conducted in triplicate using SYBR® Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan) on ABI 7500 FAST real-time PCR platform.

Three groups of lncRNAs and their target genes (primers in Additional file 9: Table S9) were used for identification. Each 20 µL reaction contained 10 µL TaqMan Fast qPCR Master Mix, 0.4 µL of each non-labeled primer (10 µM each), 0.4 µL of fluorescently-labeled primer (10 µM), 2 µL cDNA (100 ng/µL) and 6.8 µL ddH₂O. The reactions were given an initial denaturation (94 °C/3 min), followed by 45 cycles of 94 °C/5 s, 57 °C/15 s, 72 °C/30 s, and a final elongation of 72 °C/10 min. Relative transcript abundances were estimated using the $2^{-\Delta\Delta Ct}$ method [88]. Each reaction was run in triplicate.

Abbreviations

AE, Alternative exon ends; AS, Alternative splicing; DAF, day after flower; DETGs, Differential expressed target genes; ES, Exon skipping; FPKM, Fragments per kilobase of exon per million fragments mapped; Go, Gene Ontology; incRNAs, intronic lncRNAs; IR, Intron retention; KEGG, Sequences Kyoto Encyclopedia of Genes and Genomes; KOG/COG, Clusters of Orthologous Groups of proteins; lincRNAs, long intergenic ncRNAs; lncRNAs, long noncoding RNAs; miRNAs, microRNAs; ncRNAs, noncoding RNAs; Nr, non-redundant protein sequences; ORFs, Open reading frames; PCR, Polymerase Chain Reaction; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; TSS, Transcription start site; TTS, Transcription terminal site.

Declarations

Ethics approval and consent to participate

Peanut cultivar 'Fenghua1' was kindly provided by Prof. Yongshan Wan, Shandong Agricultural University. 'Fenghua1' is a good peanut cultivar and widely planted in North China, and the seeds can be bought and sold at will.

Consent for publication

Not applicable.

Availability of data and materials

Transcriptome raw data of *A. hypogaea* are available at NCBI project PRJNA354652 with accession number SRR5053815, SRR5054076, SRR5054058 and SRR5054059.

Competing Interests

The authors declare no competing interests.

Author Contributions

HT and FG acquired data, JM and HD analyzed and interpreted data, ZZ performed PCR amplification and sequencing, XL, SW and ZP participated in design and drafting of the manuscript, revised the manuscript and gave final approval of the version to be published. All authors read and approved the final manuscript.

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Additional Files

Additional file 1: Table S1. Overview of the four total RNA-seq data sets.

Additional file 2: Table S2. RNA-seq data production and alignment results of four samples.

Additional file 3: Table S3. The detailed informations of 1,442 lncRNAs.

Additional file 4: Table S4. 189 differentially expressed lncRNAs.

Additional file 5: Table S5. The distribution of lncRNAs in peanut chromosomes.

Additional file 6: Table S6. *Cis*-target genes of lncRNAs.

Additional file 7: Table S7. *Trans*-target genes of lncRNAs.

Additional file 8: Table S8. Integrated function annotation of the target genes.

Additional file 9: Table S9. Primers used in this paper.

Additional file 10: Figure S1. Sequence alignment of seven lncRNAs (lncRNA1-7) with their corresponding RNA-seq sequences.

Additional file 11: Figure S2. Sanger sequencing of five lncRNAs (T1-5).

Additional file 12: Figure S3. AS Events of lncRNAs and different AS isoforms had different target genes.

Additional file 13: Figure S4. Sanger sequencing of five lncRNAs (L1.1-1.5).

Additional file 14: Figure S5. The lncRNAs-protein interaction networks.

Figures

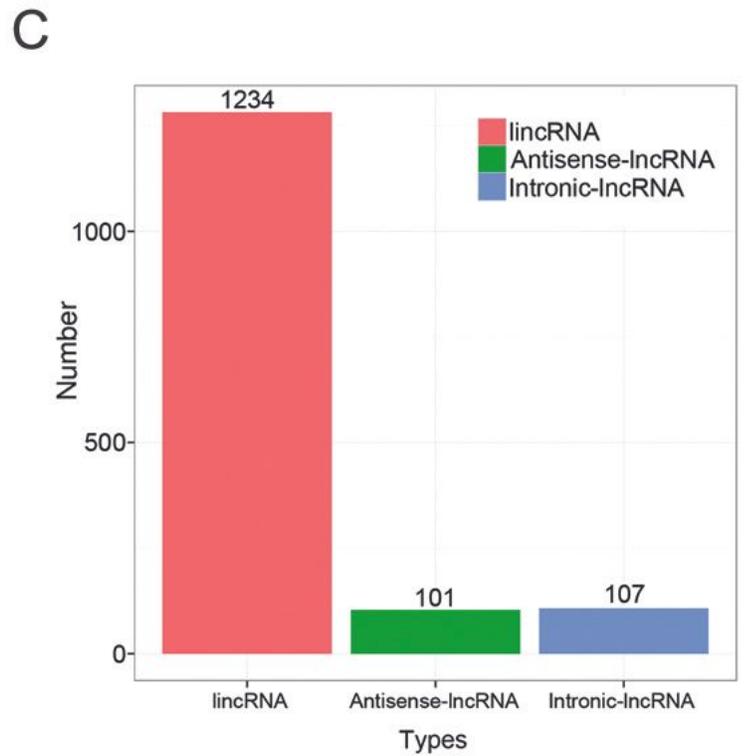
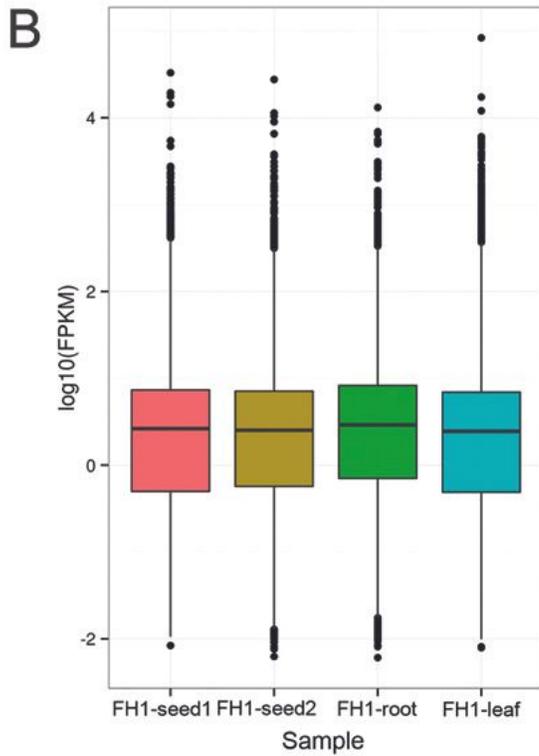
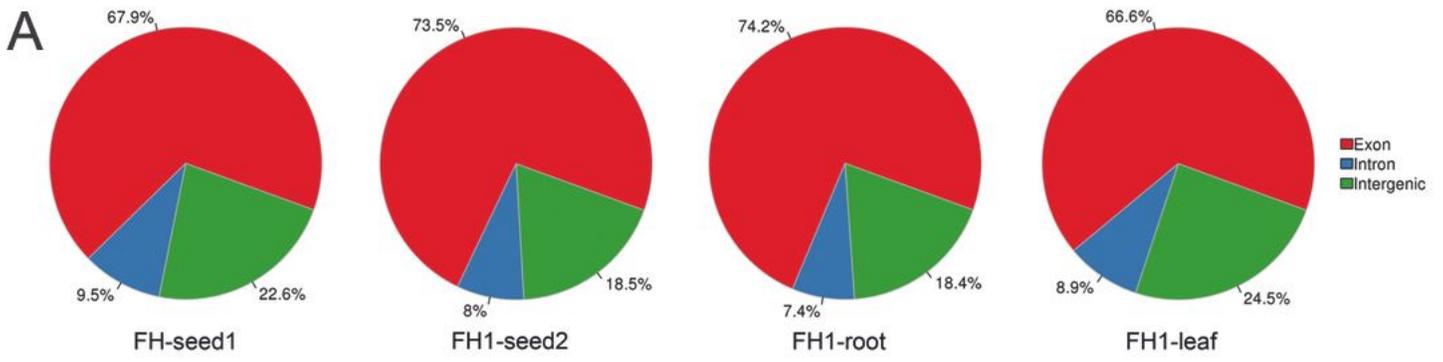


Figure 1

Long noncoding data compared with peanut reference genome sequence. A, The distribution of mapped reads across the peanut reference genome; B, FPKM_boxplots of each of the four libraries; C, The abundance of each class of lincRNAs.

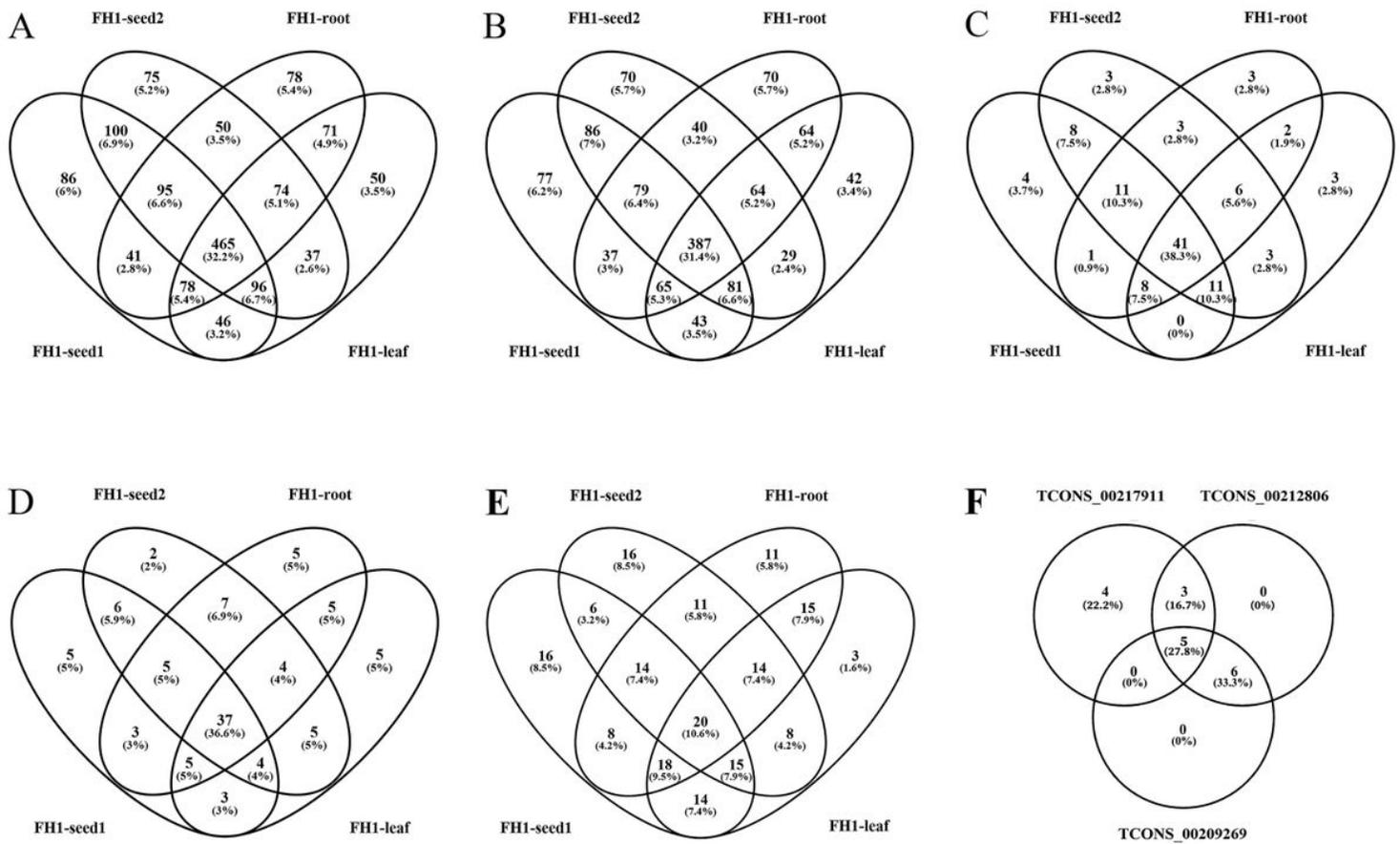


Figure 2

Overlap and uniqueness of the lncRNAs between organs. A, The full set of lncRNAs; B, lincRNAs; C, incRNAs; D, antisense-lncRNAs; E, DALs.

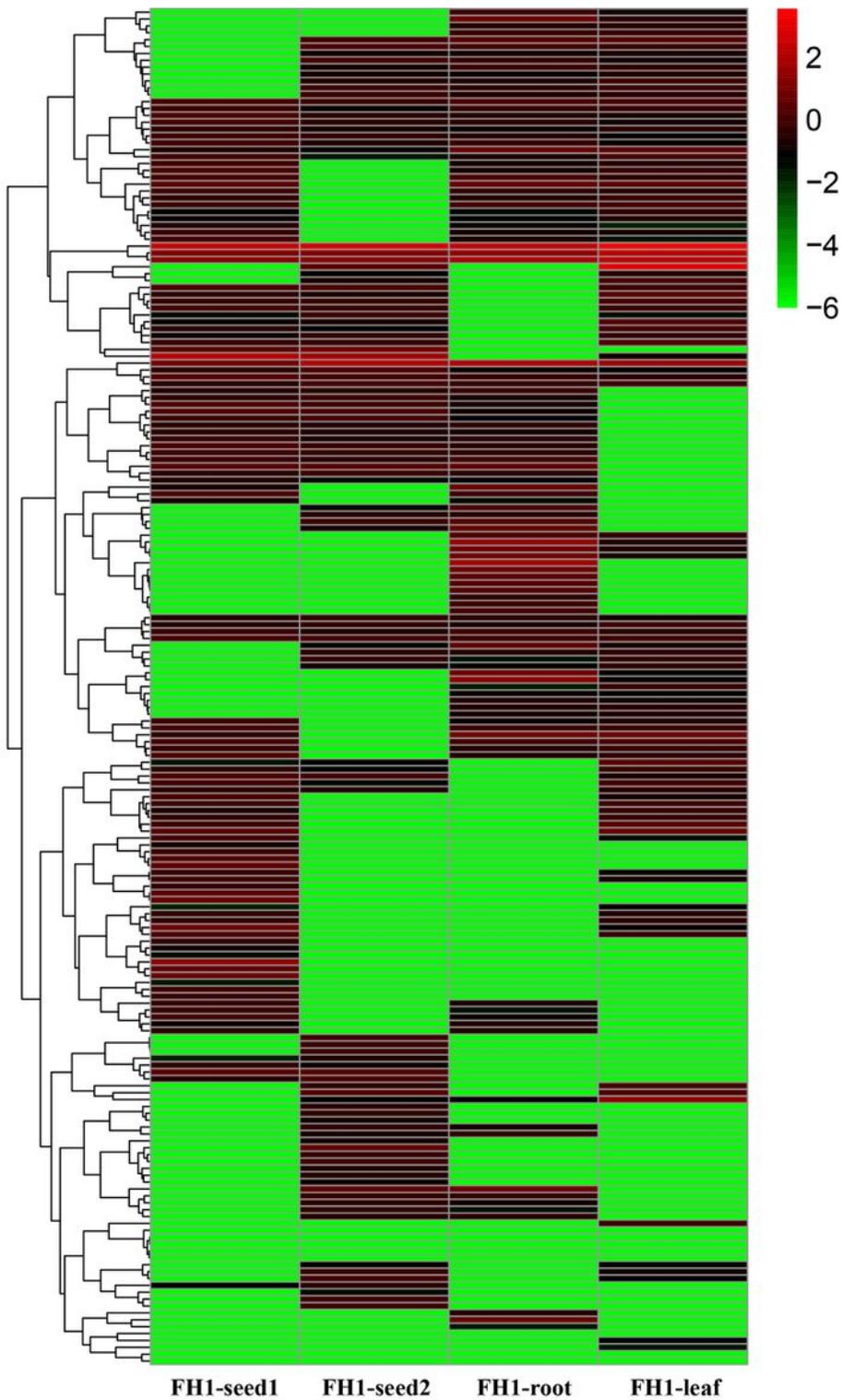


Figure 3

The abundance of the DALs across organs. The columns of the heat map represent each of the four libraries and the rows show the 189 DALs. The abundance of the DALs is indicated by the intensity of the color.

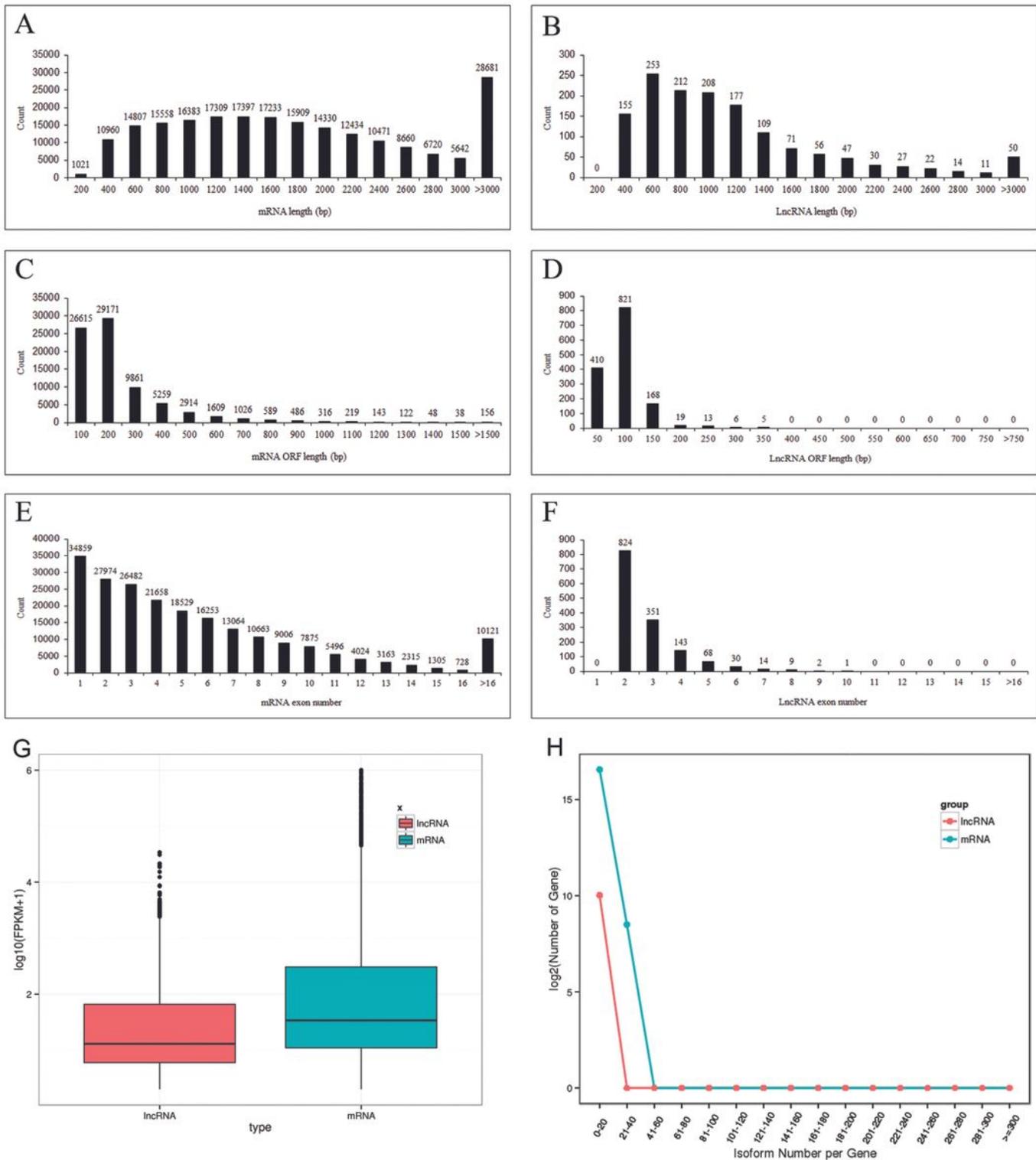


Figure 4

The population of peanut mRNAs and lncRNAs. A vs B, comparisons of transcript length; C vs D, ORF length; E vs F, exon number; G, transcript abundance; H, isoform density. The number on the bar (A-F) above the bar shows the transcript number.

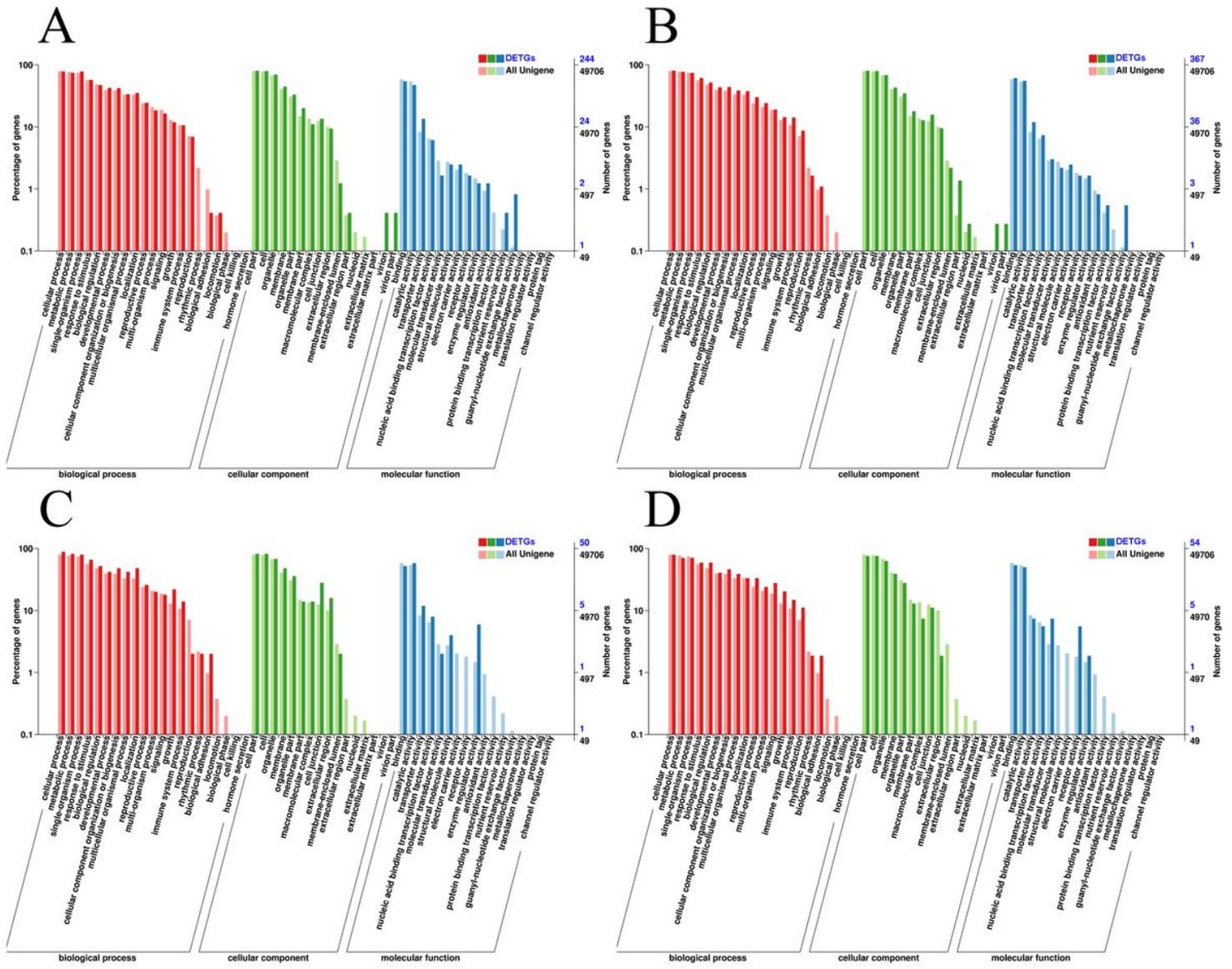
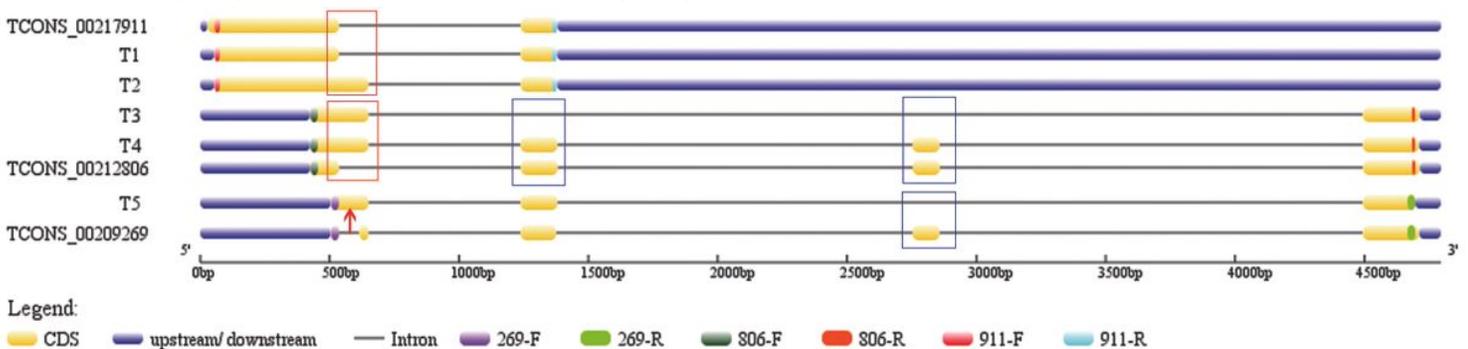


Figure 5

GO classification analysis of differentially expressed target genes of lncRNAs. A, FH1-seed1 and FH1-seed2; B, FH1-root and FH1-leaf; C, FH1-root and FH1-seed1&FH1-seed2; D, FH1-leaf and FH1-seed1&FH1-seed2. The ordinate is the enriched GO term and the abscissa is the number of differentially expressed target genes for the GO term. Different colors were used to distinguish biological processes (BPs), cellular components (CCs), and molecular functions (MFs).



Legend:

■ CDS
■ upstream/downstream
— Intron
■ 269-F
■ 269-R
■ 806-F
■ 806-R
■ 911-F
■ 911-R

Figure 6

Identification of the AS isoforms of peanut lncRNAs. Gene structures of lncRNA AS isoforms. T1-T5: PCR products from Sanger sequencing; TCONS_00217911, TCONS_00209269 and TCONS_00212806 from the RNA-seq. Red rectangles indicate TSS type and blue ones show ES type. The red arrow shows IR type.

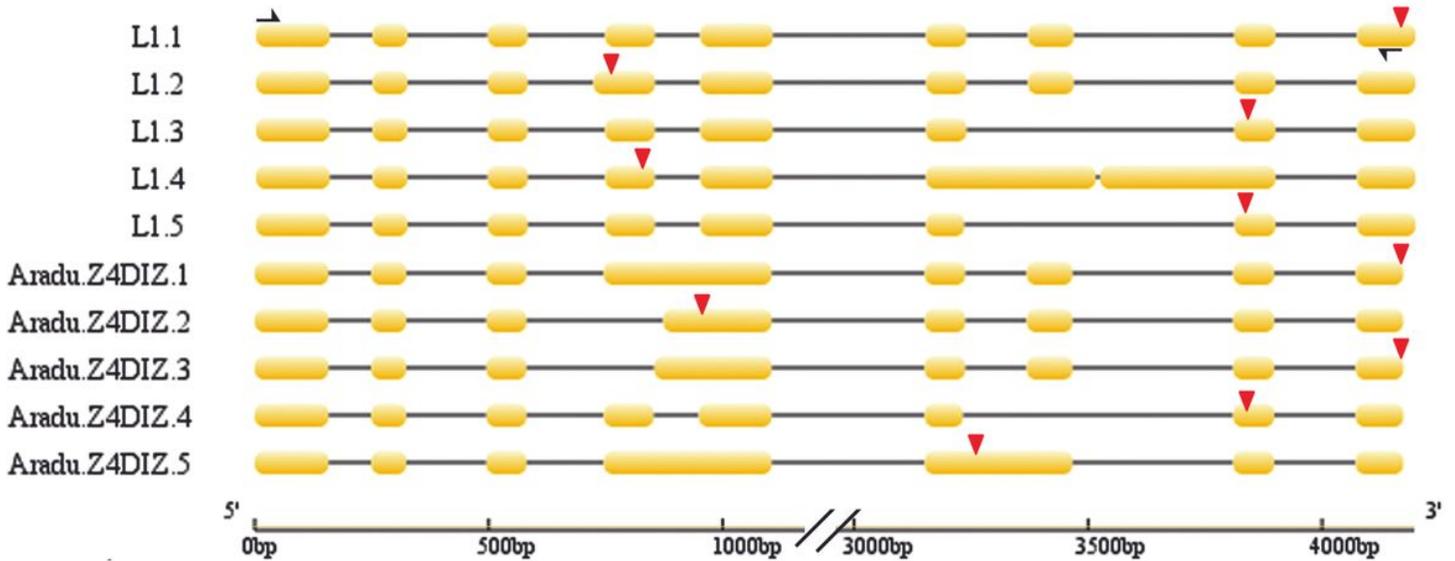


Figure 7

The gene structure of Aradu.Z4DIZ and its AS isoforms. L1.1-L1.5: PCR products from Sanger sequencing; Aradu.Z4DIZ.1-5: AS isoforms present in the transcriptomic sequence. Red triangle: stop codon. The black arrows show the location of the primers used for the PCR-based experimental validation.

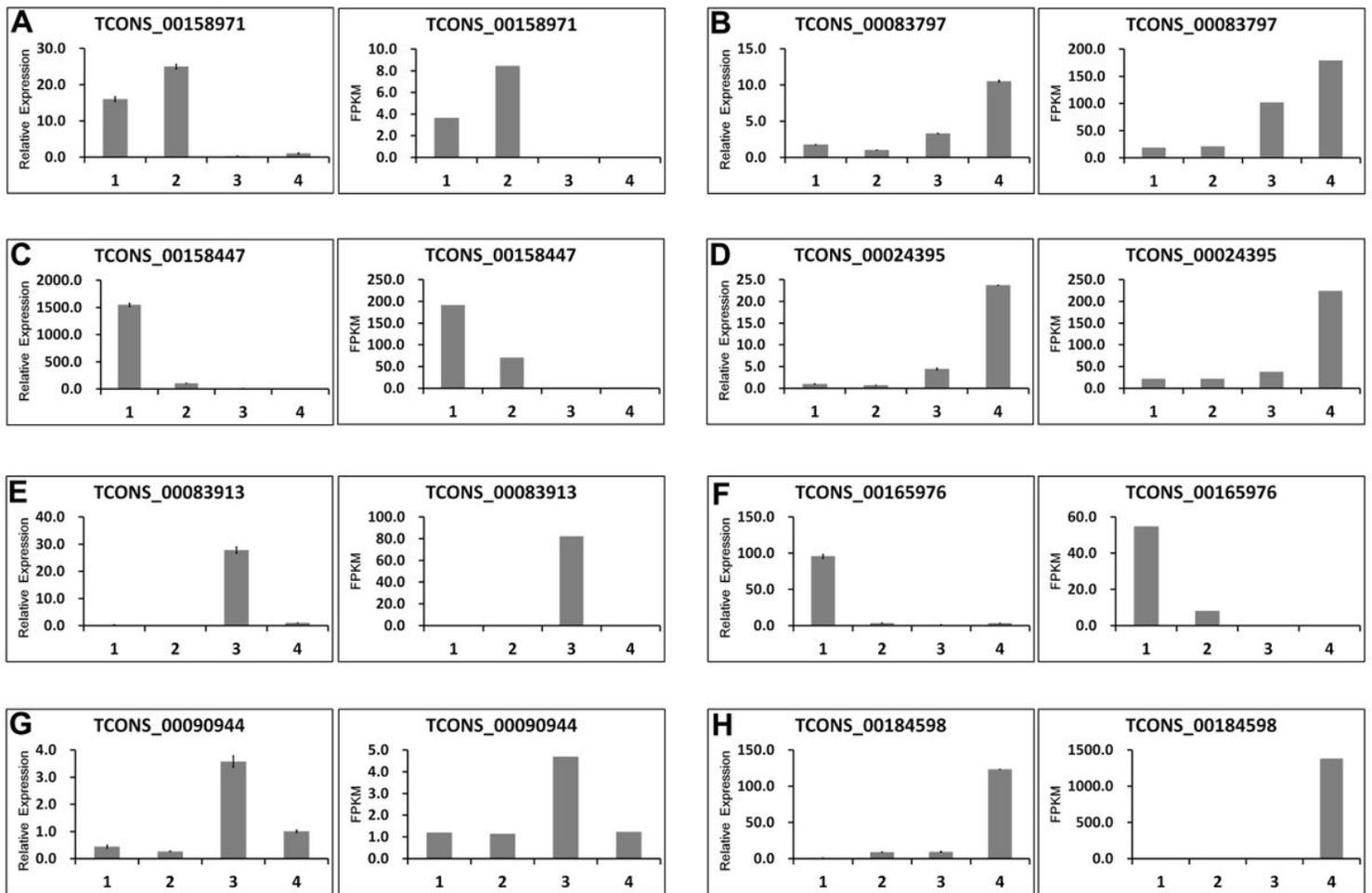


Figure 8

Validation of lncRNA expression using qRT-PCR. The abundance of each lncRNAs was deduced from RT-qPCR data (left-hand column) and from the RNA-seq data (right-hand column). The number 1, 2, 3, and 4 in the tissue label indicated FH1-root, FH1- leaf, FH1-seed1 nd FH1-seed2, respectively.

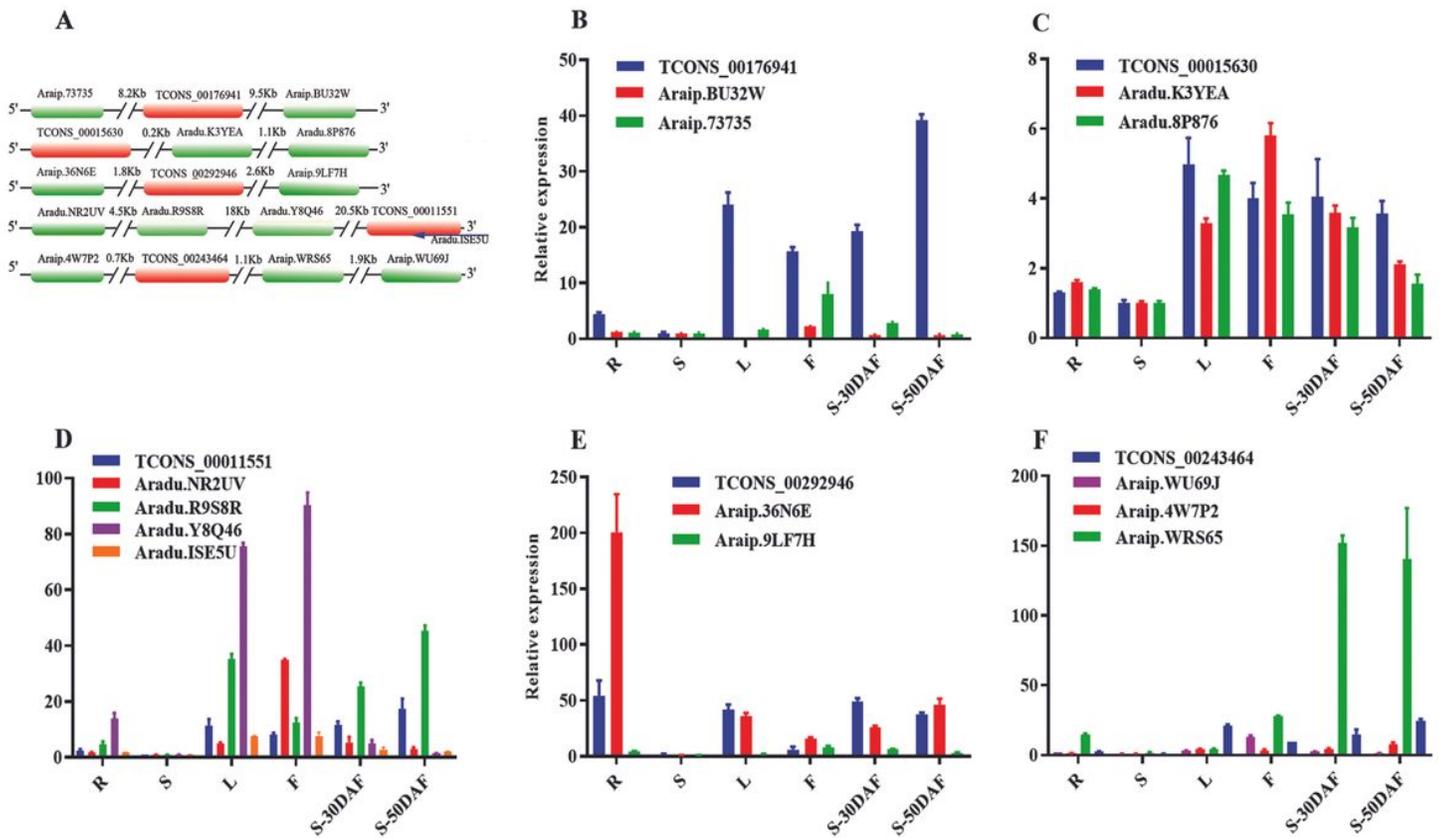


Figure 9

Comparison of the expression patterns of lncRNAs and the target genes by RT-PCR. A, The relative position of the five lncRNAs and their target genes. The blue arrow indicated Aradu.ISE5U which base-paired to the 3'-end of TCONS_00011551. B-F, Expression levels of five lncRNAs and their corresponding target genes in different tissues. R, S, L, F, S-30 DAF and S-50 DAF indicated root, stem, leaf, flower, seed from 30 DAF and 50 DAF, respectively.

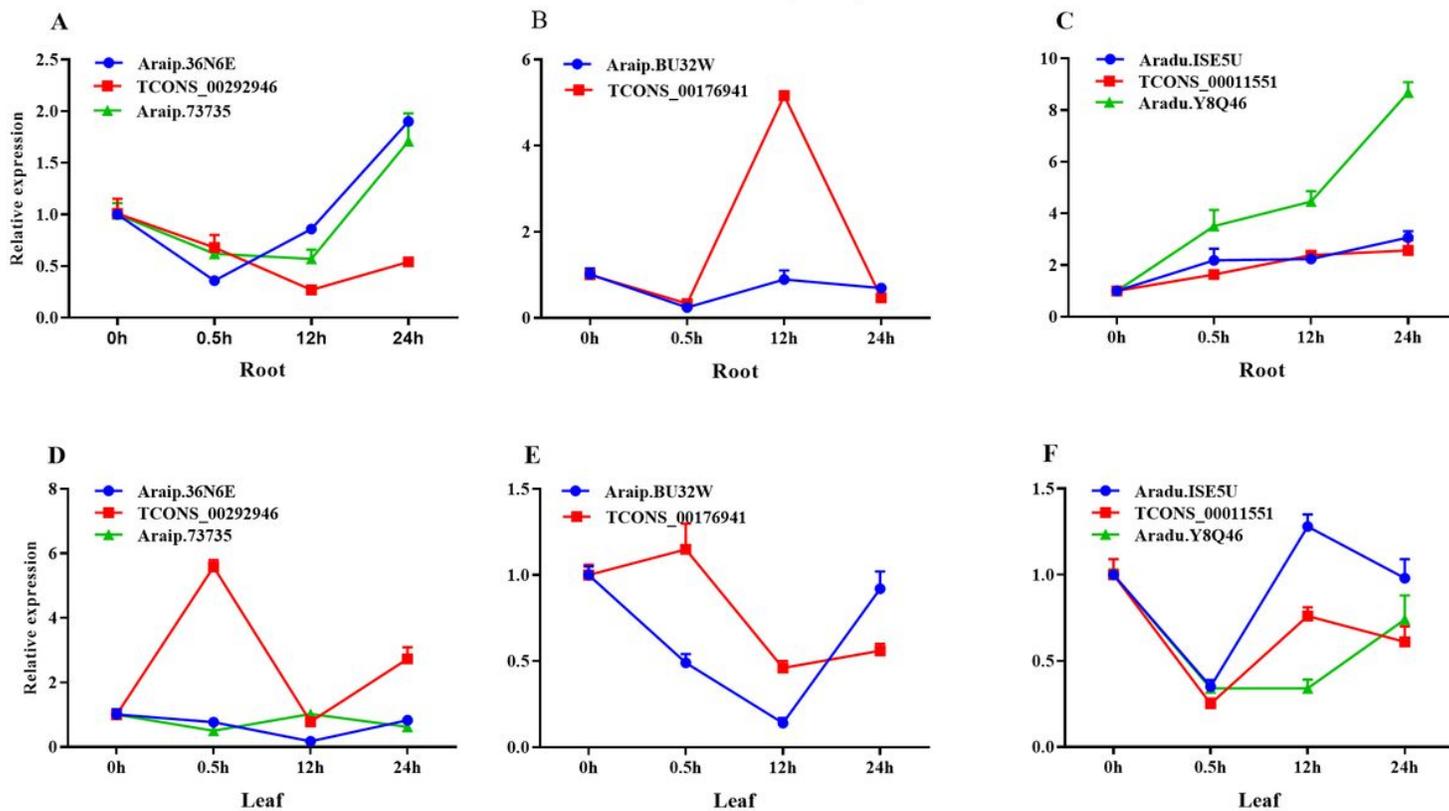


Figure 10

Expression patterns of three lncRNAs and their corresponding target genes under 1% NaCl stress in root (A-C) and leaf (D-F), respectively. Data are presented as means \pm SD of three independent replicates. Ahactin was used as the reference gene.

Supplementary Files

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