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ORIGINAL PAPER

The Family of Peanut Fatty Acid Desaturase Genes and a Functional Analysis of Four ω-3 *AhFAD3* Members

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Abstract

The synthesis of α-linolenic acid (ALA) requires the activity of ω-3 fatty acid desaturases (ω-3 FADs). The quality of peanut oil would be much improved if the content of ALA could be increased. A scan of the peanut genome revealed that it harbored 36 *FAD* genes, mapping to 16 of the species' 20 chromosomes. A phylogenetic analysis concluded that these genes belonged to six sub-families, namely stearoyl-acyl-acyl carrier protein desaturases (*SAD*), *FAD2*, *FAD3*, *FAD4/5*, *FAD6* and *FAD7/8*. Of these, *FAD3* and *FAD7/8* encoded ω-3 FADs, while genes belonging to the other four sub-families encoded ω-6 FADs. Based on RNA-Seq data, each of the 36 *FAD* genes was shown to be transcribed in non-stressed plants, but there was variation between them with respect to which organs they were transcribed in. Four *ω-3 AhFAD3* genes were functionally characterized; when expressed in *Arabidopsis thaliana* protoplasts, each was localized mainly in the endoplasmic reticulum, while within peanut, the genes were more strongly transcribed in the developing seed than in either the root or the leaf. When constitutively expressed in *Arabidopsis thaliana*, both the total fatty acid content of the seed and the relative contribution of ALA were increased. The transgenic seedlings also exhibited an improved level of survival when challenged by salinity stress.

Keywords *Arachis hypogaea* L. · ω-3 fatty acid desaturase · Transcription profiling · Sub-cellular localization · Functional characterization · Salinity tolerance

Key Message Cultivated peanut genome harbors 36 *AhFAD* genes which belonged to six sub-families. Synthesis of α-linolenic acid requires the activity of ω-3 fatty acid desaturases (ω-3 FADs). Four *ω-3 AhFAD3* genes were functionally characterized and localized mainly in the endoplasmic reticulum. Over-expression in Arabidopsis increased α-linolenic acid content, improved salt-resistance.

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Introduction

The seeds of peanut (*Arachis hypogaea L.*) represent an important source of culinary oil, the fatty acid content of which is dominated by oleic acid (C18:1, about 50% of the total oil content) and linoleic acid (C18:2, about 30% of the total oil content); its content of linolenic acid (C18:3) is very low (about 0.1%) (Li et al. 2012). α-linolenic acid (ALA) is an essential fatty acid in the human diet as it cannot be synthesized in the body, since humans lack any ω-3 fatty acid desaturases (ω-3 FADs) (Baker et al. 2016). Increasing the ALA content of peanut oil therefore represents a potential means of improving its quality.

In plant cells, ALA represents not only a component of storage and membrane lipids, but also is a precursor of the phytohormone jasmonic acid, which is central to the plant response to abiotic and biotic stress (Weber 2002). Several *FAD3* and *FAD7/8* genes have been identified in plants (Arondel et al. 1992; Chi et al. 2017; Hernández et al. 2016; Radovanovic et al. 2014; Román et al. 2012; Teixeira et al. 2010; Venegas-Calerón et al. 2010; Vrinten et al. 2005; Xue et al. 2018; Yang et al. 2012; Yurchenko et al. 2014). A number of examples have been presented which confirm that the activity of ω-3 FADs affects the plant stress response: these include the up-regulation by low temperature of a *FAD* gene in *Arabidopsis thaliana* plants (Chen et al. 2018; Gibson et al. 1994) and a demonstration that the over-expression of an *ω-3 FAD* improves chilling tolerance in tomato (Yu et al. 2009). Many *FAD* genes are known to experience alternative splicing (AS), a post-transcriptional phenomenon which is important for plant growth, development, signal transduction, flowering and the response to various environmental cues (Blencowe 2006; Reddy et al. 2013; Remy et al. 2014; Stamm et al. 2005; Tang et al. 2016; Yang et al. 2014; Zhang et al. 2016). For example, in soybean, the abundance of specific splicing variants of both *GmFAD3A* and *GmFAD7* respond to low temperature stress (Román et al. 2012).

The genome sequences of the diploid progenitors of cultivated peanut (*A. duranensis* and *A. ipaensis*) (Bertioli et al. 2016; Chen et al. 2019), along with that of peanut itself, have recently been acquired (Bertioli et al. 2019; Zhuang et al. 2019), which now enables a systematic analysis of the complement of peanut *FAD* genes (*AhFAD*s). In addition, four ER-located ω-3 *AhFAD3* genes have been selected for an analysis of their gene structure and function.

Materials and Methods

Plant Materials

The peanut cultivar (cv.) 'Fenghua1' was used for gene cloning and the *A. thaliana* ecotype Col-0 for transformation.

Sequence Analysis of FADs

The complement of *FAD* genes in the genome of cultivated peanut was identified by using "fatty acid desaturase" as a search string in a search of www.peanutbase.org/ (Bertioli et al. 2019). Gene structures were derived using GSDS software (http://www.gsds.cbi.pku.edu.cn/), while TMHMM software (http://www.cbs.dtu.dk/services/TMHMM/) was used to locate transmembrane domains and ProtComp 9.0 (http://www.softberry.com/berry.phtml?topic=protcomppl&group=programs&subgroup=proloc) to predict the sub-cellular localization of gene products.

Phylogenetic Analysis

The Phytozome database (http://phytozome.jgi.doe.gov/pz/portal.html) was scanned for plant FAD protein sequences using "FAD" as a search string. Following the removal of incomplete sequences, an alignment was performed using the ClustalW program (http://www.genome.jp/tools-bin/clustalw). Mega6 software was used to construct a plant FAD phylogeny, based on the neighbor-joining method; statistical confidence in the clade branching points was obtained by running 1000 bootstrap replicates (Hall 2013).

Transcription Profiling Based on RNA-Seq Data

The abundance of *AhFAD* transcripts was estimated from an RNA-Seq based peanut transcriptome (NCBI Sequence Read Archive SRP093901). The four sources of cDNA used for this purpose were prepared from the root and leaf of 12 day old cv. 'Fenghua 1' seedlings, and from seed of the same cultivar harvested 30 days after flowering (seed1) and 50 days after flowering (seed2). After the removal of adapter sequence and low quality reads, the remaining sequence data were aligned with the genome sequence of cv. 'Tifrunner' (www.peanutbase.org/peanut_genome) (Bertioli et al. 2019) using TopHat2 software (Kim et al. 2013). The aligned reads were assembled using the Cufflinks v2.2.1 program, and estimates of transcript abundance were based on the FPKM (fragments per kilobase million) parameter (Trapnell et al. 2010).

Isolation of *AhFAD3* Genes

Four *ω-3 AhFAD3* genes harbored by peanut cv. 'Tifrunner' (*Arahy.BC0JZ1*, *Arahy.40PHQK*, *Arahy.0JDQ22* and *Arahy.ZDHF3I*) (https://www.peanutbase.org/) were isolated from cv. 'Fenghua1' and functionally analyzed: they have been redesignated here as *AhFAD3-1* through *-4*. The *AhFAD* coding sequences were amplified in RT-PCRs based on the primer pairs FAD3-1/4-F/-R and FAD3-2/3-F/-R) (Table S1). The template provided in these reactions was cDNA prepared from seed harvested 30 days after flowering.

Each 25 μL reaction contained 1 μL template (100 ng/μL), 2 μL 2.5 mM dNTP, 2.5 μL 10×*TransTaq*@ HiFi Buffer (http://www.transgenbiotech.com), 1 μL *TransTaq*@ HiFi DNA polymerase, 1 μL of each primer (10 μM) and 16. 5 μL ddH_2O. The reactions were given an initial denaturation (94 °C/5 min), followed by 28-30 cycles of 94 °C/30 s, 60 °C/30 s, 72 °C/35 s, and a final elongation of 72 °C/10 min. The amplicons were electrophoretically separated through a 1% agarose gel and appropriate fragments purified and submitted for sequencing to Sangon Biotech (Shanghai, China).

Total RNA Isolation and Fluorescence-Based Semi-Quantitative PCR

The root, stem, leaf and flower of cv. 'Fenghua 1' plants were sampled at 15, 30, 45 and 60 days after flowering to prepare total RNA, using a DP441 RNAprep Pure Plant kit (Tiangen, Beijing), and the resulting RNA converted into cDNA using a Thermo Scientific RevertAid First Strand cDNA Synthesis kit (https://www.thermofisher.com/cn/zh/home.html). An RT-PCR assay supplied by Agilent Technologies (Agilent Technologies, California, USA) was used to profile *AhFAD3* transcription; the relevant primer pairs were FAD3-1/4-F/−R to amplify a fragment of *FAD3-1* and *-4*, and FAD3-2/3-F/−R a fragment of *FAD3-2* and *-3* (Table S1). The primer pair Actin11-F/-R (Table S1) was used to amplify the reference *Actin* sequence (GenBank number GO264911). 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 ddH_2O. 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 (Livak and Schmittgen 2001). Each reaction was run in triplicate.

Sub-Cellular Localization of AhFADs

The open reading frames (lacking the stop codons) of *AhFAD3-1* and *-4,* and *FAD3-2* and *-3* were PCR-amplified using the primer pairs PBSK-1/4-F/-R and PBSK-2/3-F/-R) (Table S1), respectively, and inserted into the multiple cloning site (MCS) of the pBSK+-35S-EGFP plant transient expression vector (kindly provided by Weicai Yang, Institute of Genetics and Developmental Biology, Chinese Academy of Science). The constructs were then transformed into *A. thaliana* mesophyll protoplasts using the PEG-calcium transfection method (Yoo et al. 2007). Fluorescence generated by the expression of *GFP* was observed using confocal laser scanning microscopy (Leica TCS SP5).

A. thaliana Transformation

Primer pairs AN-1/4-F/-R and AN-2/3-F/-R were used to amplify the open reading frames of, respectively, *FAD3-1* and *-4,* and *FAD3-2* and *-3*. The amplicons were inserted into the plant binary vector PRI101-AN (kindly provided by Minqin Wang, Shandong University, China) and from thence into *Agrobacterium tumefaciens* strain LBA4404 (Poirier et al. 2000). *A. thaliana* plants were transformed using the inflorescence infection method (Clough and Bent 1998). Progeny of putative transformants were plated on half strength Murashige and Skoog (1962) (MS) medium (Murashige and Skoog 1962) containing 100 mg/L kanamycin to select for T_1 plants carrying the transgene, and the same selection procedure was imposed on the T_2 and T_3 generations. The derivation of seed fatty acid content and composition was carried out using seed set by transgene homozygous T_3 plants. All primer sequences are given in Table S1.

Fatty Acid Composition

The fatty acid content of both non-transgenic and T_3 transgenic *A. thaliana* was obtained using the gas chromatography protocol described by Zheng et al. (2017).

Salinity Tolerance of Transgenic *A. thaliana*

Seed of both non-transgenic and T_3 transgenic *A. thaliana* were sterilized and plated on half strength MS medium for 2-3 weeks. Uniform seedings were then transferred to the same medium containing either 0, 100, 125, 150, 175, 200 or 225 mM NaCl. The seedling survival rate was estimated after a two week exposure. The experiment was run in triplicate.

Promoter Cloning and GUS Staining

Genomic DNA was prepared from peanut plants using a Plant Genome DNA Extraction kit (TIANGEN Biotechnology Co., Beijing, China). The primer pairs FAD3-1-F/-R, FAD3-2-F/-R, FAD3-3-F/-R and FAD3-4-F/-R (Table S1) were used to amplify the up-stream sequence of each of the four *FAD* genes. The fragments were inserted to the MCS site of the pCAMBIA1381Z plasmid which also harbors the *GUS*. The resulting constructs were transformed into *A. tumefaciens* strain LBA4404, and from thence into *A. thaliana,* as described above. Progeny of putative transformants were raised on half strength MS medium containing 100 mg/L kanamycin to select for T_1 plants carrying the transgene, and the same selection procedure was repeated in the T_2 and T_3 generations. Whole plants, flowers, pods and seeds of transgene homozygous T_3 plants were subjected to GUS staining (Jefferson et al. 1987). All primer sequences are given in Table S1.

Results

FAD Genes in the Cultivated Peanut Genome

The search for *AhFAD* genes in the cultivated peanut genome identified 36 candidate genes distributed over 16 of the 20 chromosomes (Table S2); each of the two subgenomes harbored 18 genes. Four of these genes mapped to each of chromosomes Arahy.17 and Arahy.19, while Arahy. 04, Arahy.07, Arahy.10 and Arahy.14 each harbored only one gene. The predicted product length of the set of genes varied from 75 (Arahy.3BHF0I.1) to 456 (Arahy.722ASC.1) residues (Table S2). The *Arahy.3BHF0I.1* product was an incomplete protein, while those of both *Arahy.NPA3T0.1* and *Arahy.ZRK7H3.1* were shorter than the other *AhFAD2s*. Each of the *AhFAD* sequences belonged to one of the six subfamilies, namely *SAD*, *FAD2*, *FAD3*, *FAD4/5*, *FAD6* or *FAD7/8* (Fig. 1). Both *FAD3* and *FAD7/8* genes encode ω-3 FADs, while those belonging to the other four subfamilies encode ω-6 FADs. The largest subfamily was the *SADs* (12 members, including three pairs of homeologs: *Arahy.XVN2S5 / Arahy.9C266U*, *Arahy.E24IL6/Arahy.BR0SNA* and *Arahy.7G971V/Arahy.H654N0*) and three sets of paralogs (*Arahy.RN8HKV/Arahy.7J26PX*, *Arahy.JF7GVB/ Arahy.1R1L75* and *Arahy.KNYU1K/Arahy.072NVT*). The *FAD4/5* subfamily comprised only three members, including the homeologs *Arahy.K3W3ZZ/Arahy.GU7MJ6*. The *FAD2* subfamily comprised seven members, including three pairs of homoelogs (*Arahy.BY45PL/Arahy.8TPQ4A*, *Arahy.S1Y1PZ/Arahy.9P5B67* and *Arahy.42CZAS/*

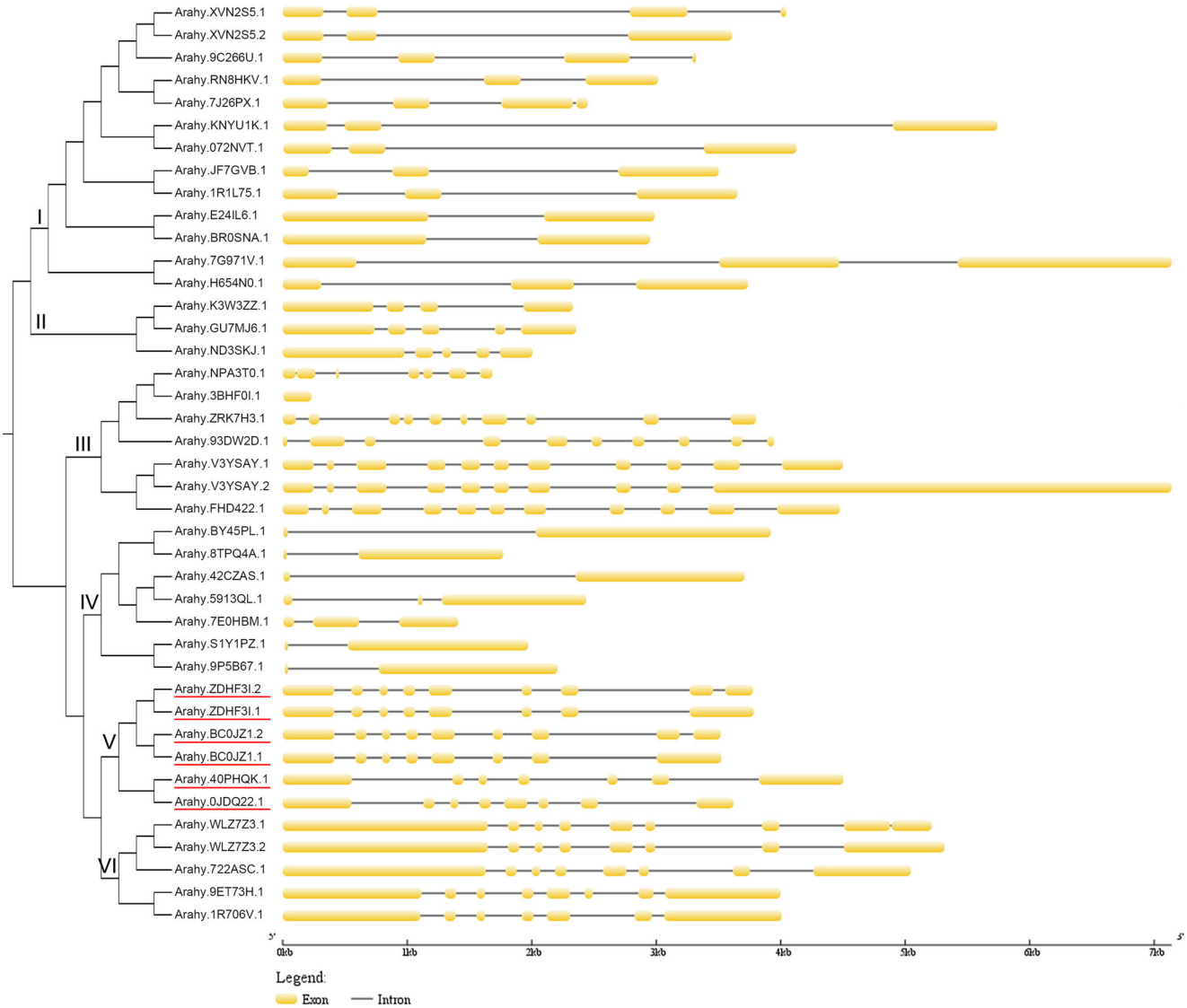

Fig. 1 The gene structure of *AhFADs*. Clade I: *SAD* subfamily; clade II: *FAD4/5* subfamily; clade III: *FAD6* subfamily; clade IV: *FAD2* subfamily; clade V: *FAD3* subfamily; clade VI: *FAD7/8* subfamily. The phylogenetic tree was generated using amino acid sequence of the proteins. Red lines marked the four genes studied in this paper

Arahy.5913QL). The *FAD6* subfamily included six members, including one pair of homeologs (*Arahy.V3YSAY*/*Arahy.FHD422*). The *FAD3* subfamily comprised the two pairs of homeologs *Arahy.ZDHF3I*/*Arahy.BC0JZ1* and *Arahy.40PHQK*/*Arahy.0JDQ22*. Finally, the *FAD7/8* subfamily had four members, represented by two pairs of homeologs (*Arahy.WLZ7Z3*/*Arahy.722ASC* and *Arahy.9ET73H*/*Arahy.1R706V*). Five of the *FADs* showed evidence of AS: each of *Arahy.XVN2S5*, *Arahy.V3YSAY*, *Arahy.ZDHF3I*, *Arahy.BC0JZ1* and *Arahy.WLZ7Z3* generated two isoforms (Table S2). None of the splicing variants differed from its non-spliced isoform in terms of the number of transmembrane domains encoded.

The number of introns present in the various genes varied (Fig. 1). In members of the *SAD* subfamily, the number ranged from one to three, with alternative splicing affecting the exon number contributing to the transcripts formed by *Arahy.XVN2S5.1* and *Arahy.XVN2S5.2*. Among members of the *FAD2* subfamily, the number was one or two, while among members of the *FAD4/5* subfamily, it was three or four. The highest number of introns was associated with members of the *FAD6* subfamily (six to ten) (except for *Arahy.3BHF0I.1*).

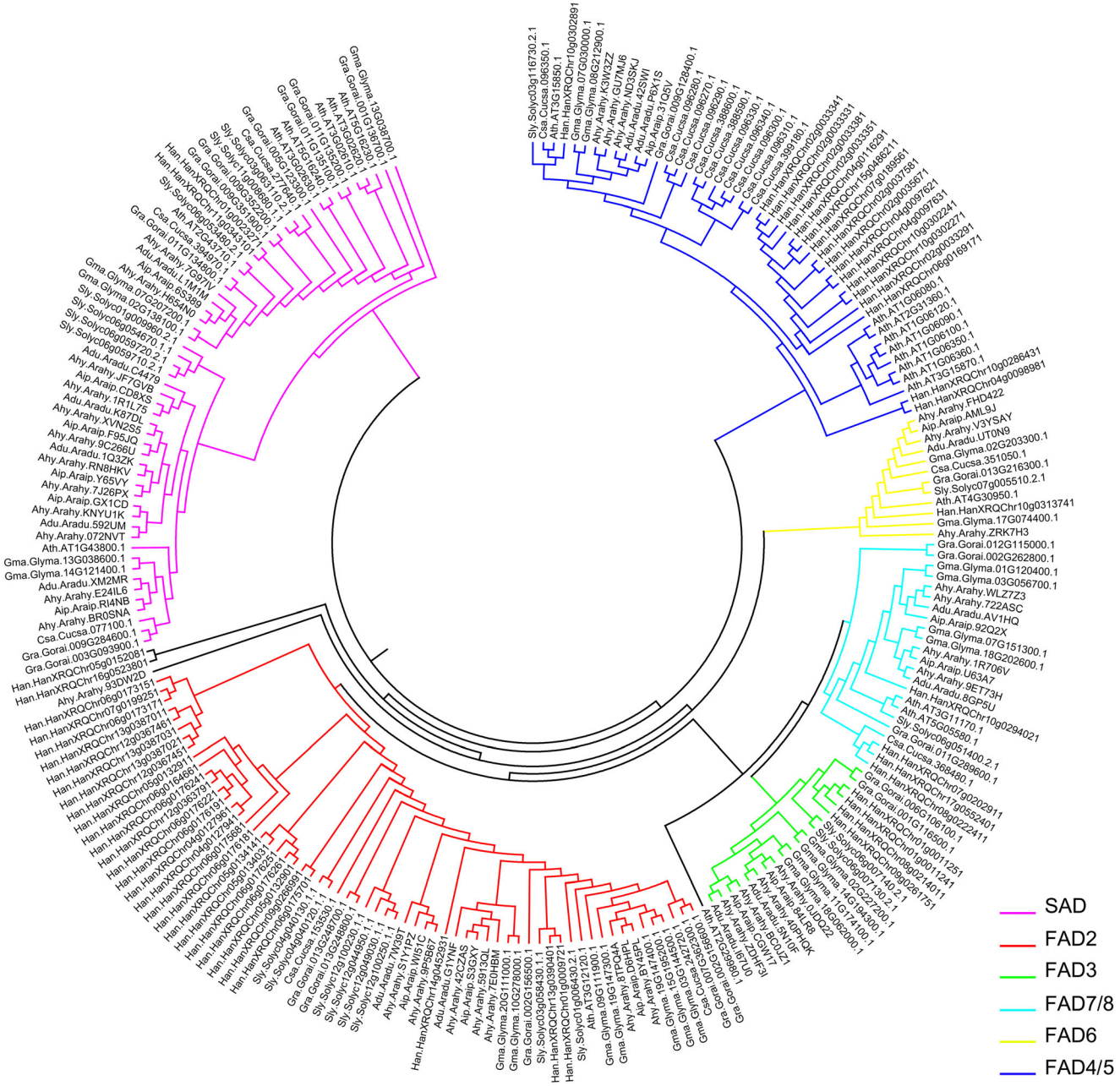

Fig. 2 The phylogeny tree of plant *FAD* genes. Ath: *A. thaliana*, Gra: *Gossypium raimondii*, Csa: *Cucumis sativus*, Sly: *Solanum lycopersicum*, Han: *Helianthus annuus*, Gma: *Glycine max*, Ahy: *A. hypogaea*, Adu: *A. duranensis*, Aip: *A. ipaensis*

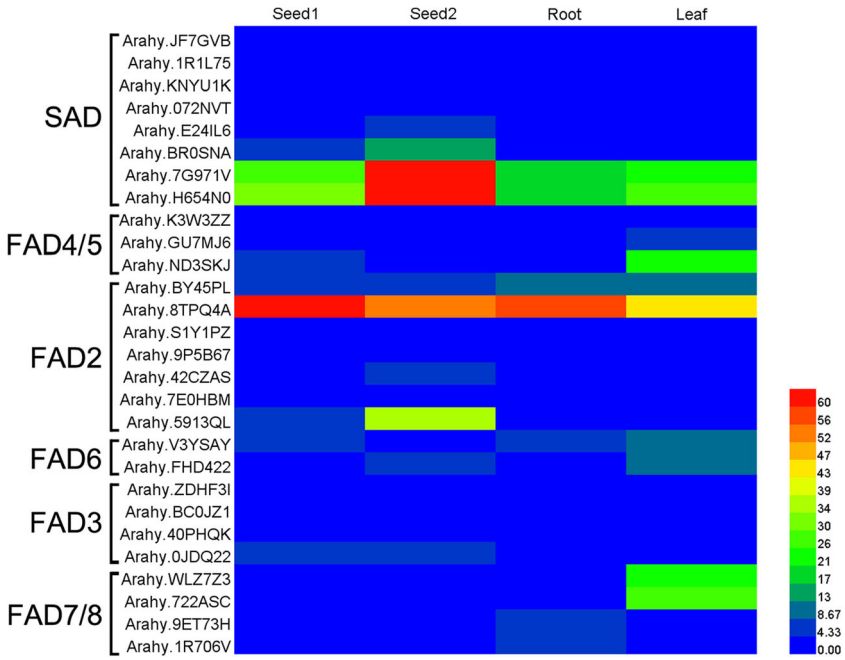

Fig. 3 Transcription profiling of 28 *AhFAD* genes in various organs of the plant. Seed1, 30 days after flowering; Seed2, 50 days after flowering; Root and Leaf, 12 day old of cv. 'Fenghua 1' seedlings

Genes belonging to the *FAD3* and *FAD7/8* subfamilies were interrupted by six to eight introns, with AS affecting the exon number contributing to some of the transcripts.

All but one *SAD* subfamily member (the exception was *Arahy.BR0SNA.1*) encoded no transmembrane domains. All but one of the *FAD2* genes encoded five or six transmembrane domains; the exception was *Arahy.7E0HBM.1* (three transmembrane domains). *FAD3* genes encoded four or five transmembrane domains, while *FAD4/5* genes encoded between two and four. Of the six *FAD6* genes, two encoded four transmembrane domains, but the other four encoded fewer than this, probably because these latter sequences were incomplete. The *FAD7/8* genes all encoded four transmembrane domains (Table S2).

With respect to the predicted location of expression, the *FAD2* and *FAD3* gene products likely were deposited in the endoplasmic reticulum (ER), whereas those belonging to either the *SAD*, *FAD4/5*, *FAD6* or *FAD7/8* subfamilies were expressed in the chloroplast (Table S2).

Phylogenetic Analysis of the FADs

The scan of the Phytozome database resulted in a set of 227 potential *FAD* sequences (Fig. 2), harbored by nine

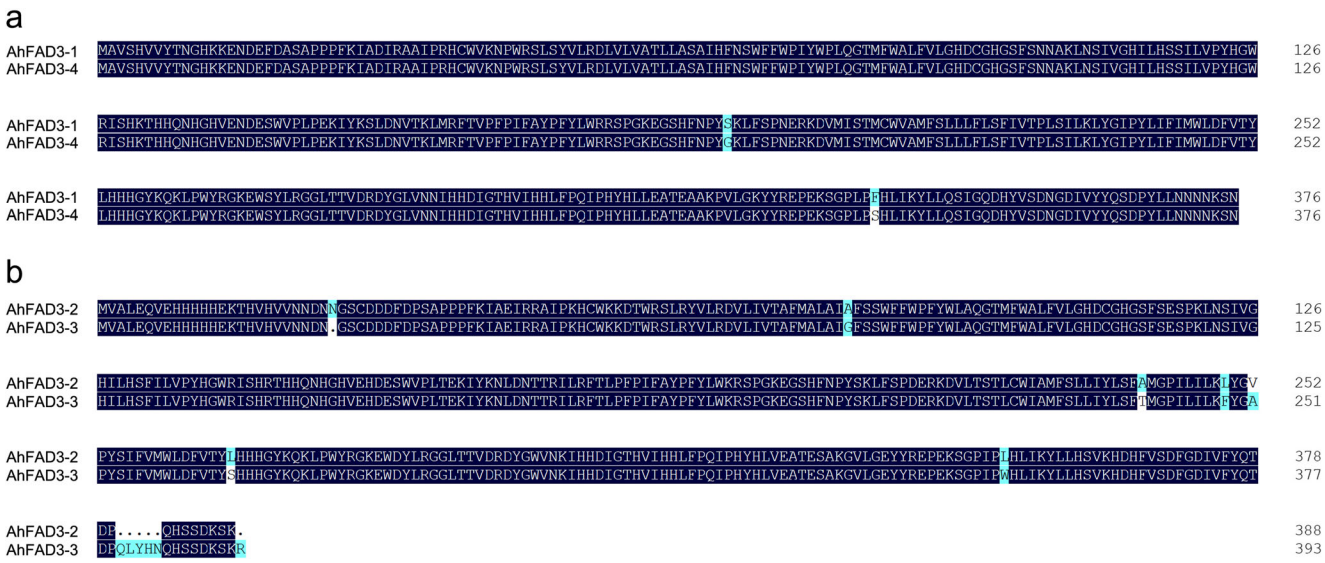

Fig. 4 Amino acids alignment of the four cloned *AhFAD3* products. **a** AhFAD3-1 vs AhFAD3-4, two amino acids differences. **b** AhFAD3-2 vs AhFAD3-3, eight single amino acids differencs and one deletion/insertion fragment

Table 1 General information of *AhFAD3s*

Gene name	Correspondig genes in peanutbase	ORF length (bp)	Amino acids (AA)	Conserved domains	Transmembrande domains	PI/MW	Genbank ID
AhFAD3-1	*Arahy.BC0JZ1.2*	1131	376	22-327	4	9.16/43.9 kD	MH085086
AhFAD3-2	*Arahy.40PHQK.1*	1167	388	35-341	5	7.94/45.6 kD	MH085087
AhFAD3-3	*Arahy.0JDQ22.1*	1182	393	34-340	5	8.15/46.4 kD	MH085088
AhFAD3-4	*Arahy.ZDHF3I.2*	1131	376	22-327	4	9.16/43.7 KD	MK757485

dicotyledonous species, namely *A. thaliana*, *Gossypium raimondii*, *Cucumis sativus*, *Solanum lycopersicum*, *Helianthus annuus*, *Glycine max* and the three *Arachis* species *hypogaea*, *duranensis* and *ipaensis*. The analysis revealed two large clades, one of which clustered the genes encoding the soluble, transmembrane domain-lacking SADs, and the other the insoluble FADs, which included at least one transmembrane domain. The 12 *Arachis* spp. *SAD*s were clustered into two secondary clades, of which one contained exclusively *Arachis* spp. sequences. Five secondary *FAD* clades were recognized: the *FAD4/5*s occupied a phylogenetic position closest to the root, indicating this group's more ancient origin, while the *FAD3*s and *FAD7/8*s appeared to be the most recently evolved groups. Although the *FAD3s* and *FAD7/8*s shared both a similar gene structure and nucleotide sequence, most of the *FAD3s* encoded a product deposited in the ER whereas the *FAD7/8* products were expressed in the chloroplast. The number of *FAD2* and *FAD5* sequences present in the sunflower genome is notably greater than in any of the other species surveyed.

Transcriptional Behavior of the Peanut FADs

The transcriptional behavior of each of the 36 *AhFADs*, as deduced from RNA-Seq data, is illustrated in Fig. 3. No transcript was detected for eight of the genes. Both *Arahy.7G971V* and *Arahy.H654NO* were strongly transcribed in each of the organs surveyed, and especially so in the seed2 sample, suggesting that their products make a substantial contribution to seed development and the accumulation of oil. *Arahy.E24IL6* and *Arahy.BR0SNA* were also strongly transcribed in both the seed1 and seed2 samples, implying they too may encode products important for lipid synthesis. Transcript of all seven *FAD2* members was detected, in particular that of *Arahy.8TPQ4A*, which was strongly transcribed in all four organs. Of the three *FAD4/5*s detected, *Arahy.ND3SKJ* and *Arahy.GU7MJ6* were notable in that they were well represented in the leaf sample. Transcript of only two of the six *FAD6s* was detected, and the abundance of these transcripts was highest in the leaf sample. Transcript of all four *FAD3*s was detected, most notably that of *Arahy.0JDQ22*. Transcript of the four *FAD7/8*s was represented in all four organs: *Arahy.WLZ7Z3* and *Arahy.722ASC* transcript was particularly abundant in the leaf.

Isolation of *AhFAD3* Sequences and their Transcriptional Behavior

The four *AhFAD3* genes *Arahy.BC0JZ1, Arahy.40PHQK, Arahy.0JDQ22* and *Arahy.ZDHF3I* (equivalent to *AhFAD3-1* through *-4*) were targeted for PCR-based cloning from cv. 'Fenghua 1' plants. Their sequences have been submitted to GenBank under accession numbers MH085086-88, MK757485. *AhFAD3-1* and *-4* map to the homoelogs Arahy.17 and Arahy.07, respectively (Table S2); their sequences shared a nucleotide identity of 99.6% and their predicted translation products differed by just two residues (Fig. 4a). Both gene products included the same conserved domains and featured a single transmembrane domain, but differed with respect to both their pI and their molecular weight (Table 1). *AhFAD3-2* and *-3* similarly map to a pair of homoelogs (chromosomes Arahy.11 and Arahy.01); their level of nucleotide identity was 96.7%, as was the level of peptide similarity between their predicted products (Fig. 4b).

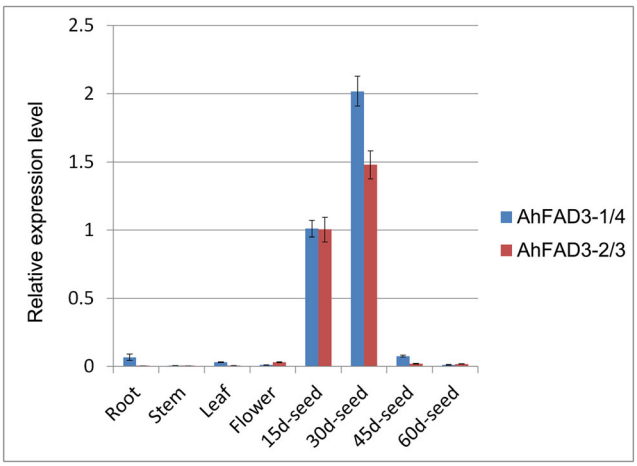

Fig. 5 Transcription profiling of the four *AhFAD3* genes in various organs of the plant. It was difficult to discern the abundance of *AhFAD3-1* and *-4* transcript, and similarly that of *AhFAD3-2* and *-3*, so the blue column represented the summary of *AhFAD3-1* and *-4*, and the red one represented that of *AhFAD3-2* and *-3*

A stretch of five residues lying close to the protein's C terminus was present in AhFAD3-3, but not in -2. The two polypeptides harbored the same conserved domains, but differed with respect to the number of transmembrane domains present, their pI and their molecular weights (Table 1). Given the high sequence similarity between each pair of genes, an analysis of their transcription in the vegetative tissue and in developing seed was based on aggregating the abundance of *AhFAD3-1* and *-4* transcript, and similarly that of *AhFAD3-2* and *-3* (Fig. 5). Both pairs of homeologs were most strongly transcribed early during seed development (15–30 days after flowering). The highest abundance of *AhFAD3-1/4* transcript was present in the root sample, whereas that of *AhFAD3-2/3* was in the flower. Neither pair was strongly transcribed in the stem.

Functional Analysis of the *AhFAD3*s

When each of the *AhFAD3-GFP* fusion transgenes was expressed in *A. thaliana* protoplasts, most of the GFP signal was concentrated in the ER, but was also detected in the cell membranes and the cytoplasm (Fig. 6). Subsequently, each of the genes was constitutively expressed in *A. thaliana* to determine their contribution to the seeds' FA content and

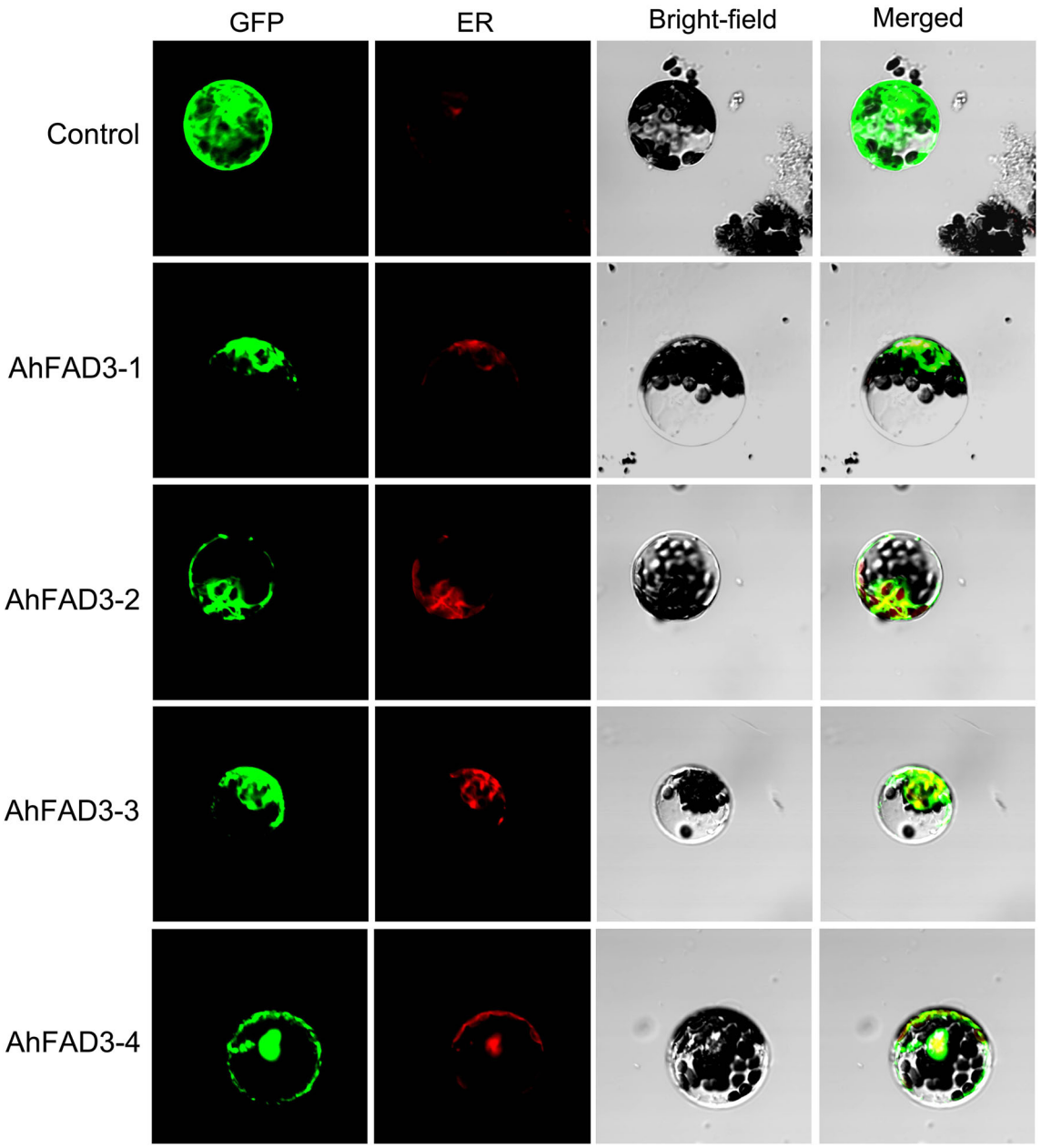

Fig. 6 The sub-cellular localization of AhFAD3-GFP fusion protein in transgenic *A. thaliana* protoplasts. "GFP": green fluorescent protein signal, "ER": red fluorescence of endoplasmic reticulum, "Bright-field": bright field image, "Merged": merged set of images

composition. In both non-transgenic and transgenic seeds, nine fatty acids were detected, namely C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C18:3(linolenic acid), C20:0 (arachidic acid), C20:1 (eicosenic acid), C20:2 (eicosadienoic acid) and C22:1 (erucic acid) (Fig. 7). The most abundant fatty acids were C18:2 (30.5%) and C18:3 (19.1%). The effect of the transgenes was to raise the total FA content of the seed by between 24.8% and 33.3%, reflecting a rise in the content of each of the individual FAs, but especially that of C18:3 (Fig. 7a). The relative contribution of the various fatty acids was also altered, with that of C18:3 increasing markedly and that of C18:2 decreasing (Fig. 7b). An analysis of the *AhFAD3* promoter sequences was carried out by linking each to *GUS* and expressing the resulting transgenes in *A. thaliana*. The whole plant, the flowers, the siliques and the seeds all tested positive for GUS activity (Fig. 8), consistent with the promoters all being capable of driving constitutive expression.

Over-Expressing AhFAD3s Increased the Salinity Tolerance of the Transgenic *A. thaliana*

In transgenic *A. thaliana* seedlings raised in the presence of less than 150 mM NaCl, there was no significant difference in the survival rate of non-transgenic and transgenic plants, both of which mostly (>99%) survived unscathed (Fig. 9). At higher concentrations of NaCl, however, the survival rate of the transgenic seedlings was higher than that of the non-transgenic ones: in the presence of 175 mM NaCl, the survival rate of the transgenic seedlings was about 10.2–20.5% greater than that of the non-transgenic ones; at 200 mM NaCl, the survival rate of the transgenic seedlings, though much reduced, remained 5.6–9.0% higher than that of the non-transgenic ones.

Discussion

Cultivated peanut is an allotetraploid formed following a natural hybridization between the two diploid species *Ar. duranensis* and *Ar. ipaensis* (Bertioli et al. 2016). The completion of the genome sequences of both progenitor species and of the cultivated peanut itself offers the opportunity to characterize the membership of gene families such as the *FAD*s (Bertioli et al. 2016; Chen et al. 2019). A previous report has documented that *A. duranensis* harbors 15 *FAD* genes, while *A. ipaensis* harbors 16 (Ruan et al. 2018). Here, a similar analysis of the cultivated peanut genome has revealed 36 *FADs*, with 18 represented in each of the two subgenomes (Table S2). The additional five genes comprised one *FAD2* (*Arahy.7E0HBM*) and four *FAD6s* (*Arahy.NPA3T0*, *Arahy.ZRK7H3*, *Arahy.3BHF0I* and *Arahy.93DW2D*). The structure of the latter genes differed sufficiently from that of

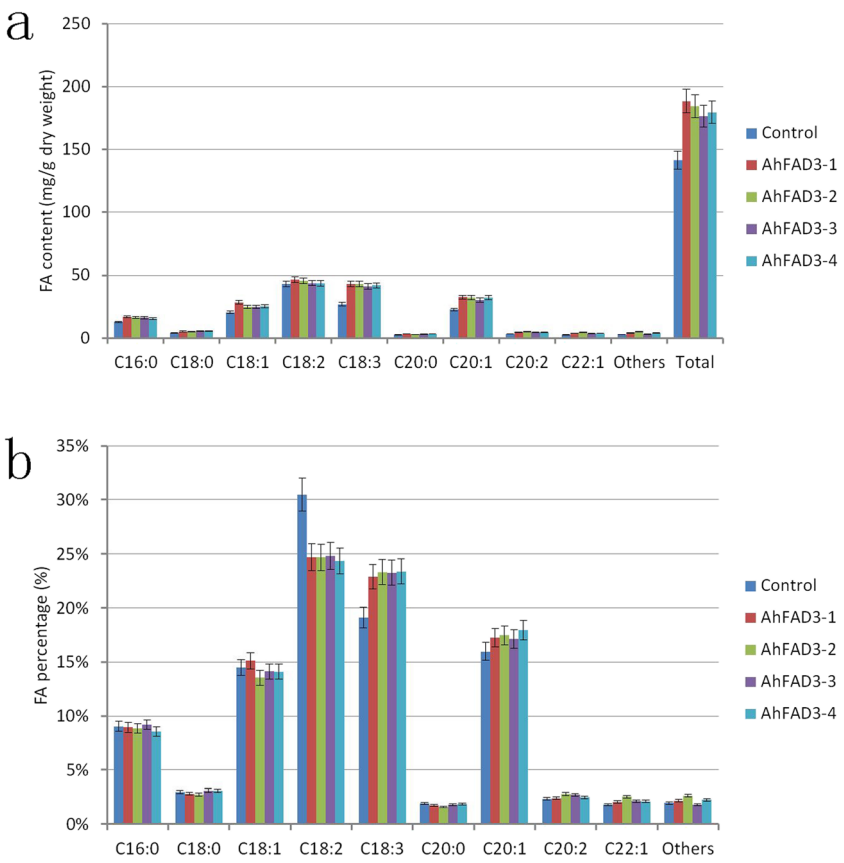

Fig. 7 The FA constitution and content in the oil of seeds set by *A. thaliana* plants harboring an *AhFAD3* transgene. **a** FA content (mg/g dry weight), C18:3 with the greatest increase. **b** Proportion of individual FAs (%), the percentage of C18:3 increased significantly whereas C18:2 decreased compared with the control

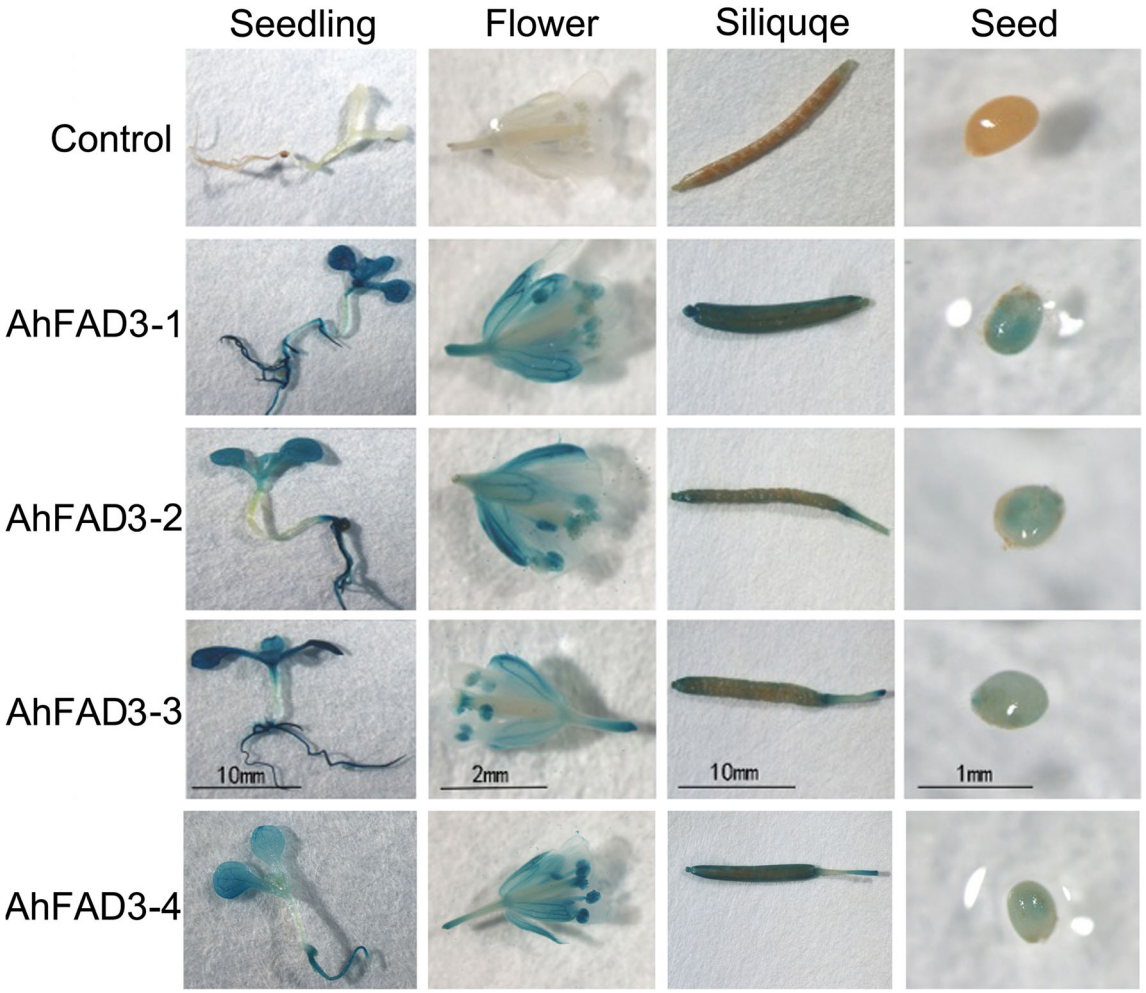

Fig. 8 *AhFAD3* promoter activity assay. *A. thaliana* plants harboring a p*AhFAD3-GUS* fusion transgene stained for GUS activity (blue color). Control: non-transgenic *A. thaliana* seedlings

the other two *FAD6*s, to form a recognizably novel sub-clade (Fig. 1); their absence in both of the two progenitor genomes implies their evolution post the formation of the cultivated peanut alloploid. Curiously, there was no evidence of any transcript from any of them, at least in the four organs surveyed (Fig. 3), raising the possibility that they are pseudogenes. *FAD* genes are known to be readily duplicated: for instance, in cucumber, 12 of the 14 *FAD5*s present appear to cluster closely together, suggestive of a frequent occurrence of gene duplication events following speciation (Dong et al. 2016). Similarly, the cotton ω-3 *FAD* gene family appears to have undergone rapid expansion (Yurchenko et al. 2014).

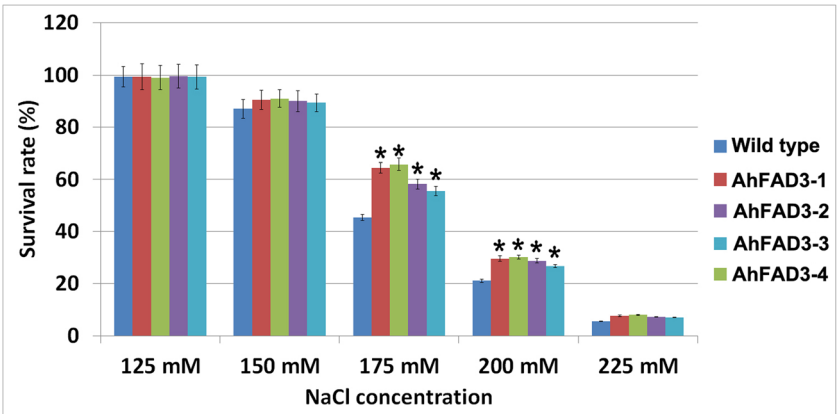

Fig. 9 The survival rate of *A. thaliana* seedlings harboring an *FAD3* transgene challenged by a range of NaCl concentrations. Columns indicate means±SE ($n = 3$). *: mean differs significantly ($P < 0.05$) from that of the non-transgenic seedlings

Although the peanut genome harbors more than 30 *FAD*s, but under non-stress conditions the transcripts of every one of them could not be detected, with a few of the genes being transcribed very strongly in one or more organs of the plant (Fig. 3), as also revealed by an earlier study (Yurchenko et al. 2014). In the olive fruit, *FAD3A* appears to be the key gene responsible for accumulation of C18:3 in the seed, while *FAD7* contributes most materially in the mesocarp (Hernández et al. 2016). C18:1 and C18:2 represent the major components of peanut seed oil, accounting for about 80% of its total fatty acid content. AhSAD and AhFAD2 are largely responsible for the synthesis of these components, so it was not unexpected to find that the two *AhSAD* genes *Arahy.7G971V* and *Arahy.H654N0* and the *AhFAD2* gene *Arahy.H654N0* were all strongly transcribed in the developing seed. So far, four separate *AhFAD2* genes have been identified as present in the peanut genome, and the inactivation of one of them is known to change the FA constitution of the seed oil (Chi et al. 2011; Wang et al. 2015). The synthesis of ALA, a very minor component of the oil (accounting for <0.1%) but an essential fatty acid in the human daily diet, is catalyzed by ω-3 FADs (*FAD3/4 s* and *7/8 s*), genes which are transcribed in the seed at a very low level; the implication is that an attempt to boost the content of C18:3 through the manipulation of *FAD* genes will require increasing the intensity with which one or more of the *ω-3 FAD*s are transcribed. The four *AhFAD3* genes which have been functionally characterized here were shown to be active largely in the ER (Fig. 6), but their constitutive expression *A. thaliana* did succeed in increasing both the representation of C18:3 in, and the total fatty acid content of the seed oil (Fig. 7).

The importance of ALA for the plant's metabolism is that represents a precursor of the phytohormone jasmonic acid, which contributes in many ways to both development and the response to abiotic stress (Weber 2002). The desaturation of FAs in the membrane is used by some plants as a strategy for surviving low temperature stress. In *A. thaliana,* the *ads2* mutant produces a reduced level of unsaturated fatty acids and a higher one of saturated fatty acids; the mutant is dwarfed and sterile when exposed to a sub-optimal temperature (Chen and Thelen 2013). FAD2 has been shown to be required by *A. thaliana* to combat salinity stress, because it helps to maintain the level of desaturation of vacuolar and plasma membrane fatty acids necessary for the functioning of Na^+/H^+ exchangers (Zhang et al. 2012). In cucumber, some *FAD* genes can be induced by low temperature and repressed by high temperature, which has been taken to imply that FADs form part of the species' machinery to cope with temperature stress (Dong et al. 2016). In sunflower, in contrast, a *FAD2* gene has been reported as being repressible by both low temperature and salinity (Zhou et al. 2017). Meanwhile, in cotton, one *FAD7/8* responds positively to low temperature, whereas a second one scarcely responds to either of these stresses (Yurchenko et al. 2014). It has earlier been demonstrated that in peanut, some – but not all – Ah*FADs* respond to a variety of stress agents (Chi et al. 2017). Here, it has been shown that the heterologous expression in *A. thaliana* of each of four *AhFAD3s* had a positive impact on the survival of NaCl-challenged seedlings (Fig. 9), implying that their products may in some way contribute to the ability of peanut plants to combat salinity stress.

In conclusion, a scan of the recently completed peanut genome has revealed the presence of 36 likely *AhFAD* genes, most of which were represented by transcript in some, or all parts of the plant. Four members of *AhFAD3* subfamily were functionally characterized in some detail: their expression was concentrated largely in the ER, and their transcript abundance was typically higher in seed than in either root or leaf tissue. When heterologously expressed in *A. thaliana*, each had the effect of increasing both the representation of ALA and the total FA content of the seed oil. They also acted to enhance the survival of seedlings grown in a medium containing >150 mM NaCl.

Authors' Contributions All authors contributed to the study conception and design. PCR amplification, sequencing and RT-PCR were performed by Jian Ruan and Haiying Tian. Vector construction and sub-cellular localization were performed by Lei Shan and Jingjing Meng. *A. thaliana* transformation, screening of positive strains and salinity resistance test were performed by Feng Guo. Fatty acid composition analysis was performed by Zhimeng Zhang. Promoter cloning and GUS-staining were performed by Hong Ding. Design and drafting of the manuscript, revised the manuscript and gave final approval of the version to be published were performed by Zhenying Peng, Shubo Wan and Xinguo Li. All authors read and approved the final manuscript.

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Data Availability The sequences of the four ω-3 *AhFAD3* genes have been submitted to GenBank under accession numbers MH085086 through MH085088, MK757485. All the supporting data are included as additional files.

Compliance with Ethical Standards

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.

Conflict of Interest The authors declare that they have no conflict of interest.

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