Isolation and Characterization of Inositol Tetraphosphate 1-Kinase (AhITPK1) and Inositol 1,4,5-Tris-Phosphate Kinase (AhIPK2) Gene in Peanut

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Abstract

In the current study, partial cDNA clones of inositol tetraphosphate 1-kinase (ITPK1) and inositol 1,4,5-tris-phosphate kinase/inositol polyphosphate multikinase (IPK2), were isolated from peanut using RT-PCR and designated as AhITPK1 and AhIPK2 isoforms of the gene. The partial cDNA sequence of AhITPK1 and AhIPK2 genes have an open reading frame (ORF) of 1146 and 891bp respectively and showed high similarity to other plant genes. AhITPK1 shared high homology with Aradu. Q95MC of Arachis duranensis, had a single exon with no introns and belonged to ATP-grasp family of proteins. AhIPK2 shared high similarity with Aradu.24VG of A. duranensis and contained three exons with 5' and 3' UTR's on either side. Unlike other IPK2 genes, AhIPK2 possessed conserved domains such as PxxxDxxGxG and [L/M][I/V][D/F][L/A]10[H/K]. Phylogenetic analysis grouped AhITPK1 with A. duranensis, A. ipinensis and Oryza brachyantha into one cluster, whereas AhIPK2 was grouped along with Cucumis melo and C. sativus. Evolutionarily, AhITPK1 and AhIPK2 were genetically distinct from other plant genera. Furthermore, real-time PCR analysis revealed high expression of AhITPK1 and AhIPK2 genes in the peanut embryo and flower bud. For the first time AhITPK1 (KR778986) and AhIPK2 (KR778988) genes belonging to phytic acid pathway from Arachis hypogaea were identified and characterized the expression pattern of these two isoforms on different tissues. These genes were found to be abundant in flower bud and embryo. Results suggest that embryo development significantly influences the expression of the two AhIPK isoforms in peanut. Evolutionarily they were found to be distinct from their parental species. This study is an important step toward understanding the role of these two AhIPK isoforms in phytic acid synthesis. However, future research involving RNAi-based functional characterization is warranted to establish their link to embryo development in peanut.

Keywords: Arachis hypogaea; Gene model; Multiple sequence alignment; Inositol tetraphosphate 1-kinase (ITPK1); Inositol 1,4,5-tris-phosphate kinase (IPK2); Phylogenetic analysis; Real time PCR

Abbreviations: Arachis hypogaea inositol tetraphosphate 1-kinase (AhITPK1); Arachis hypogaea Inositol 1,4,5-tris-phosphate kinase (AhIPK2); Flower bud (Fb); Fully opened flower (Fo); Glucose 6 phosphate (G6P); Inositol trisphosphate (InsP3); Inositol tetrakisphosphate (InsP4); Inositol pentakisphosphate (InsP5); Shoot (S); Inositol hexakisphosphate (InsP6); 1,3,4,5,6 pentakisphosphate 2-kinase (IPK2); Inositol 1,4,5 trisphosphate kinase (IPK2); Inositol tetraphosphate 1-kinase (ITPK1); Leaf (L); 1D-myo-inositol 3-phosphate synthase (MIPS); Open reading frame (ORF); Peq (P); Phospholipase C (PLC); Phospholipase D (PLD); Quantitative real-time PCR (qRT-PCR)

Introduction

In developing seeds, phytic acid, a major phosphorus storage compound in plant seeds, is mainly synthesized from glucose-6-phosphate (G6P) [1] and 1D-myo-inositol-3-phosphate synthase (MIPS) catalyzes the first step of this pathway. Inositol tetrakisphosphate kinase (InsP6) and inositol pentakisphosphate (InsP5) are generated by subsequent series of phosphorylation and dephosphorylation [2-5]. Phytic acid pathway proceeded through Ins(3)P, Ins(3,4)P2, Ins(3,4,6)P3, Ins(3,4,5,6)P4 [6,7]. The synthesis of Ins(1,4,5)P3 from phosphatidylinositol-4,5-bisphosphate via phospholipase C (PLC) and the subsequent action of two kinases, inositol 1,4,5-tris-phosphate kinase (IPK2) and inositol 1,3,4,5,6-pentakisphosphate 2-kinase (IPK1), produce InsP5. This pathway is called the PLC-dependent or lipid dependent pathway [8].

Myo-inositol tetrakisphosphate kinase (ITPK1) belongs to a larger family of ATP-grasp fold proteins. They show some functional and structural similarity with IPK2. Inositol 1,3,4,5-tetrakisphosphate (InsP4) was first identified by Batty in stimulated rat cerebral cortical slices and it is likely to be a second messenger, and act as a precursor of inositol 1,3,4-trisphosphate and possibly of inositol 1,4,5-trisphosphate [9]. Ins(1,3,4,5)P4 can function in animal cells as a second messenger to control the entry of calcium from the extracellular space [10]. It is conceivable that these mechanisms of signal transduction may be involved in seed maturation and/or seedling growth and may be regulated to some extent by the synthesis of InsP6 during germination [11,12].

Previous studies suggested that the ITPK gene was identified in other organisms as an inositol 1,3,4,5-tetrasphosphate 5/6-kinase/inositol 3,4,5,6-tetraphosphate 1-kinase [13-15]. Inositol-tetrakisphosphate 1-kinase is also known by several synonyms such as '1D-myo-inositol-tetrakisphosphate 1-kinase', 'inositol 3,4,5,6-tetraphosphate 1-kinase', '1D-myo-inositol-tetrasphosphate 5-kinase', '1D-myo-inositol-trisphosphate 6-kinase', 'inositol-trisphosphate 5-kinase' and 'inositol-trisphosphate 6-kinase'.

Inositol 1,4,5-tris-phosphate kinase, or more appropriately inositol polyphosphate multikinase (IPK2), is a dual specificity IP3/IP4 6/3-kinase that sequentially generates InsP3 from InsP4 [16-18]. IPK2 is among the enzymes central to the production of IP species downstream of phospholipase C activation. IPK2 was first designated as ArgrIII and

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later renamed as IPK2 based on the discovery that it functioned as an inositol phosphate kinase [8,19]. Involvement of this enzyme activity in phytic acid biosynthesis has been demonstrated by Stevenson-Paulik et al. [20]. Whereby it was showed that the loss-of-function obtained by T-DNA insertion in the Arabidopsis Atipk2 gene resulted in an accumulation of intermediate inositol phosphorylated forms and a seed phytic acid reduction of about 35%.

Infact, although the phytic acid pathway has been studied in several species such as rice, barley, maize, arabidopsis phytic acid reduction of about 35%. Accumulation of intermediate inositol phosphorylated forms and a seed

**Material and Methods**

**Plant material**

Peanut variety 'Georgia green' was grown in five gallon pots at the Centre for Viticulture and Small Fruit Research, Florida A&M University, USA. Different plant tissues such as leaf (L), shoot (S), flower bud (Fb), fully opened flower (Fo) and peg (P) were collected from 60 day old plants. Kernel (K) and embryo (E) were collected from matured plants.

**Total RNA isolation and amplification of AhITPK1 and AhIPK2:**

Total RNA was extracted from peanut embryo and cotyledons using RNase plant mini kit (Qiagen, CA) as described in the manufacturer's instructions. All the RNA samples were quality checked on 1% agarose gel and quantified by Nanodrop spectrophotometer (ThermoScientific, Rochester, USA). First-strand cDNA synthesis was performed using iScript cDNA synthesis kit (BioRad Laboratories, Hercules, CA) using 1 µg of total RNA isolated from embryo as template. The locus 'Aradu.24wnv' and 'Aradu.24V9G' which corresponds to gene sequences of inositol tetrakispase 1-kinase (ITPK1) and inositol 1,4,5-tris-phosphate kinase (IPK2) respectively from wild progenitors of *Arachis hypogaea* were used for designing primer pairs and to clone full length open reading frame (ORF) regions of *AhITPK1* and *AhIPK2* in cultivated peanut.

Sequences of 'Aradu.24wnv' and 'Aradu.24V9G' were downloaded from peanutbase. Translation overview of these sequences was observed using DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada). Largest open reading frame (ORF) from translation overview was selected for designing primer pairs. Translation overview of 'Aradu.24wnv' and 'Aradu.24V9G' sequences are presented (Figures 1a and 1b) respectively. Positions of forward and reverse primers on the largest ORFs of 'Aradu.24wnv' and 'Aradu.24V9G' are presented in (Figures 1c and 1d). For designing forward primer sequence from 5' end was used; whereas for designing reverse primer reverse complementary of sequence from 3' end was used.

The total cDNA obtained was used as a template in RT-
PCR reaction to amplify full length ORF using primer pairs (AhITPK1F: 5'-ATGCGGGAAGAAGAAGAAGC-3' and AhITPK1R: 5'-TCACTATGGAAGAAGAAGACG-3') and AhITPK2: 5'-GACATGGTTCTAGGACTTGGG-3' and AhITPK2R: 5'-AAAATTGGTTCTAGGACTTGGG-3').

**Results and Discussion**

Cloning and characterization of AhITPK1 and AhITPK2 cDNA fragments

Database search using Arabidopsis thaliana (AJ404678.2) and Glycine max (NC_016103.1) identified Araip.Rb64V and Araip.244WV loci in Arachis hypogaea L. The loci identified from NCBI GenBank and peanut base databases. Phylogenetic analysis was performed using MEGA 6 software [21]. Evolutionary divergence analysis was based on the number of amino acid substitutions per site between different sequences and was conducted using the Poisson correction model [22] on MEGA 6 software [21].

**Quantitative real time PCR (qRT-PCR):** Primers for quantitative real-time PCR were designed using the sequences obtained above. Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicate in 96-well optical plates using a BioRad CFX96 real-time PCR system. Each reaction (20 µl) included 10 µl of 5× SYBR green super mix (BioRad), 0.4 µM each of forward and reverse primers and 12.5 ng of cDNA. PCR amplification program included 95°C for 3 min; 60 cycles of 95°C for 30 sec, 60°C and 72°C for 30 sec followed by final extension of 72°C for 15 min. PCR fragment (~1kb) was eluted from the gel using QIAquick gel extraction kit (Qiagen) as per the manufacturer's protocol. The eluted PCR fragment was cloned into pGEM-T vector according to manufacturer's procedure (Promega) and transformed to JM109 E. coli competent cells. Plasmid DNA was extracted from transformed white colonies using NucleoSpin® as per the manufacturer's procedure (Macherey-Nagel, Dassel, Germany) and confirmed by PCR. Plasmid DNA sequencing was performed by MWG operon using T7 and SP6 primers. Sequence obtained was trimmed to remove vector sequences and used to search NCBI GenBank and peanutbase for homologous sequences.

**Sequence analysis and phylogenetic tree construction:** Database search for similarity was performed using the BLASTN algorithm against NCBI GenBank and peanutbase databases. Phylogenetic analysis was performed using MEGA 6 software [21]. Evolutionary divergence analysis was based on the number of amino acid substitutions per site between different sequences and was conducted using the Poisson correction model [22] on MEGA 6 software [21].

PCR reaction to amplify full length ORF using primer pairs (AhITPK1F: 5'-ATGCGGGAAGAAGAAGAAGC-3' and AhITPK1R: 5'-TCACTATGGAAGAAGAAGACG-3') and AhITPK2: 5'-GACATGGTTCTAGGACTTGGG-3' and AhITPK2R: 5'-AAAATTGGTTCTAGGACTTGGG-3').

**Figure 2:** Nucleotide and predicted amino acid sequences of the partial cDNA clone encoding AhITPK1 (A) and AhIPK2 (B) identified from Arachis hypogaea L. The nucleotide sequence has been submitted to the GenBank with accession number KR778986 and KR778988 respectively.
The sequences that showed high similarity to AhITPK1 and AhIPK2 genes as identified by NCBI BLAST were downloaded from the database, and their deduced amino acid sequences were used for ClustalW multiple sequence alignment using MEGA6 program. Multiple sequence alignment Figure 3 identified several conserved regions between 45 and 107 amino acid residues for AhITPK1 and from 211 to 271 amino acid residues for AhIPK2 (Figure 2b).

Phylogenetic analysis revealed that ITPK1 genes were grouped into five different clusters whereby AhITPK1 isoform from A. hypogaea was grouped along with A. duranensis, A. ipinensis, and O. brachyantha (Figure 4a). Classification of ITPK1 genes was based on either monocot-dicot specificity or botanical classification with cluster 1-3 having dicots, cluster 4 having monocots and cluster 5 having both dicots and monocots. Phylogenetic analysis revealed that IPK2 genes were grouped into 10 clusters (Figure 4b) with IPK2 genes from Sesamum indicum, N. nucifera, G. max, Gossypium raimondii, A. duranensis, A. ipinensis,
5. truncatula forming separate cluster. AhIPK2 gene was grouped in one cluster with Cacumis melo and C. sativus, Cicer arietinum and Beta vulgaris and all other genotypes were grouped in one other cluster.

Quantitative real time PCR analysis of AhITPK1 and AhIPK2 cDNA fragment

Quantitative real-time PCR analysis showed that AhITPK1 and AhIPK2 genes were expressed in embryo, flower bud, fully opened flower, kernel, leaf, shoot and peg indicating that in mature plants AhITPK1 and AhIPK2 genes are not differentially expressed in these tissues and is in agreement with earlier studies [16,17]. Josefen et al. [15] also reported that IPK genes in rice and barley are constitutively expressed in all the tissues studied. This indicates that AhITPK1 and AhIPK2 genes are expressed in all the tissue examined at different developmental stages or the activity of gene product in embryo could be regulated at another level than the mRNA level [15]. Mean threshold cycle values (Cq values) were obtained for both the genes from different tissues of peanut (Table 2). In the current study, there was no correlation between gene expression and corresponding relative quantities of protein for AhITPK1 and AhIPK2 genes. This difference may have been induced by post-translational modification [24] or low supply of substrate, because cyclization of glucose-6-P to Ins (3) P is irreversible [25]. The highest level of gene expression for both genes was observed in the embryo followed by the peg, whereas highest relative quantity was observed in flower bud followed by embryo (Figure 5) which confirms its role in phytic acid biosynthesis in the tissue. Results are in agreement with the findings of Fileppi. [26] wherein highest expression of PvIPK2 was in developing cotyledons. Zhang observed are in agreement with the findings of Fileppi. [26] wherein highest expression of PvIPK2 was in developing cotyledons. Zhang observed high levels of AhCHI gene expression in the pegs as compared to leaf, stem, seed and flower [27]. Related results were observed in the present study indicating that the expression of AhITPK1 and AhIPK2 genes in the embryo was higher compared to leaf, stem, seed and flower. AhIPK2 gene expression was upregulated in embryo and peg, down regulated in flower bud, fully opened flower and kernel and there was no change in leaf and shoot. Similarly AhITPK1 gene expression was up regulated in embryo, down regulated in flower bud and fully opened flower and there was no change in other tissues.

In silico southern hybridization of AhITPK1 and AhIPK2 genes of phytic acid pathway

Peanut (Arachis hypogaea), is an autogamous allotetraploid legume (2n = 4x = 40) harboring homologous A and B genomes derived from
two diploids which are most likely *Arachis duranensis* (A genome) and *Arachis ipaensis* (B genome) [28,29]. Taking the advantage of consensus map of these two wild parents of peanut available in the peanut base database, the newly identified nucleotide sequence of *AhITPK1* gene from *A. hypogaea* was used to search against peanut draft genome sequence and four chromosome regions were identified with different degrees of similarity. Among four regions, *Aradu.Q95MC* (*Arachis duranensis*) and *Araip.24WNV* (*Arachis ipinensis*) showed highest similarity to *AhITPK1* gene. Our cDNA sequence was 99.8 and 98.9% identical to the region from *A. duranensis* and *A. ipinensis* respectively, whereas other two regions had lowest similarity. The high similarity between amplified cDNA sequence and *Aradu.Q95MC* and *Araip.24WNV* positioned the *AhITPK1* gene on chromosome A05 and B05 respectively (Table 3). High similarity of the *AhITPK1* gene of *A. hypogaea* with *Aradu.Q95MC* contributed to the study of its complete gene model. The *AhITPK1* gene had one single exonic region without any introns (Figure 6). Derived amino acid sequences of *AhITPK1* were searched in 'Motif scan' (http://myhits.isb-sib.ch/cgi-bin/motif_scan#GRAPHIC) which revealed the presence of ATP_GRASP fold profile between 113-323 amino acid sequence. This indicates that the newly identified *AhITPK1* from *A. hypogaea* also belong to a larger family of ATP-grasp fold proteins.

The *AhIPK2* gene sequence from *A. hypogaea* was used to search against peanut draft genome sequence database i.e., peanutbase (peanutbase.org) and two chromosome regions i.e., *Aradu.24V9G* and *Araip.BR64V* were identified with high degrees of similarity. Our cDNA sequence was 99.3 and 98.2% identical to the region from *A. duranensis* and *A. ipinensis* respectively. The high similarity between amplified cDNA sequence and *Aradu.24V9G* and *Araip.BR64V* positioned the *AhIPK2* gene on chromosome A08 and B08 respectively (Table 3). High similarity of the *AhIPK2* gene with *Aradu.24V9G* contributed to the study of its complete gene model. Structural differences were observed for the *AhIPK2* gene between chromosome A and B. The *AhIPK2* gene on chromosome A represented by *Aradu.24V9G* had three exons with

| Sample | Mean Cq | Normalized Expression | Relative Normalized Expression | Regulation | Compared to Regulation Threshold |
|--------|---------|-----------------------|--------------------------------|------------|----------------------------------|
| AhIPK2 |         |                       |                                |            |                                  |
| E      | 29.39   | 3.33                  | 29.50                          | -11.14     | Down regulated                   |
| Fb     | 27.82   | 0.01                  | 0.09                           | -33.09     | Down regulated                   |
| Fo     | 29.54   | 0.00                  | 0.35                           | -11.39     | Up regulated                     |
| K      | 35.22   | 0.02                  | 0.22                           | -4.53      | Down regulated                   |
| L      | 31.28   | 0.11                  | 1.00                           | 1.00       | No change                        |
| P      | 29.27   | 1.29                  | 11.39                          | 11.39      | Up regulated                     |
| S      | 32.28   | 0.04                  | 0.35                           | -2.83      | No change                        |
| AhITPK1|         |                       |                                |            |                                  |
| E      | 26.28   | 24.96                 | 15.13                          | -15.13     | Up regulated                     |
| Fb     | 25.99   | 0.04                  | 0.03                           | -39.94     | Down regulated                   |
| Fo     | 28.09   | 0.02                  | 0.01                           | -96.18     | Down regulated                   |
| K      | 29.45   | 1.20                  | 0.73                           | -1.37      | No change                        |
| L      | 27.33   | 1.65                  | 1.00                           | 1.00       | No change                        |
| P      | 27.16   | 5.76                  | 3.49                           | 3.49       | No change                        |
| S      | 27.24   | 1.37                  | 0.83                           | -1.20      | No change                        |

Table 2: Mean threshold cycle values (Cq) normalised expression and regulation of *AhIPK2* and *AhITPK1* genes among different tissues of peanut using peanut actin as a control.

Figure 5: Relative quantity and normalised gene expression of *AhITPK1* and AhITPK2 in peanut (*Arachis hypogaea* L.). Error bars represent values with 5% value. T test analysis showed significant difference (*P < 0.01). Tissues characterized for expression patterns of the two *AhIPK* isoforms included embryo (E), flower bud (Fb), fully opened flower (Fo), kernel (K), leaf (L), peg (P), and shoot (S).

Figure 6: Schematic representation of gene model of *AhIPK2* (A) and *AhITPK1* (B) of peanut as revealed by nucleotide blast in peanutbase (www.peanutbase.org).
AhIPK2 had a another common motif [L/M][I/V][D/F/L][A/G][H/K], which is considered a putative ATP/Mg\(^{2+}\) binding site \([25,31]\). The IPK2 has a another common motif [L/M][I/V][D/F/L][A/G][H/K], which is a catalytic site for phosphate transfer from ATP to the inositol ring of the motif ‘PxxxDxKxG’ common to a protein family of IPK2 and isb-sib.ch/cgi-bin/motif\_scan#GRAPHIC) which revealed the presence were searched in ‘Motif scan’ (http://myhits.isb-sib.ch/cgi-bin/motif\_scan). AhIPK2 gene on chromosome B represented by Araip.BR64V had one 3’ UTR with 571 bp long, 5’ UTR with 299 bp long and four exons with 898, 80, 38 and 163 bp long respectively (Figure 6). Derived amino acid sequences of AhIPK2 were searched in ‘Motif scan’ (http://myhits.isb-sib.ch/cgi-bin/motif\_scan#GRAPHIC) which revealed the presence of the motif ‘PxxxDxKxG’ common to a protein family of IPK2 and is a catalytic site for phosphate transfer from ATP to the inositol ring \([30]\). The IPK2 has a another common motif [L/M][I/V][D/F/L][A/G][H/K], which is considered a putative ATP/Mg\(^{2+}\) binding site \([25,31]\). The deduced protein sequence of AhIPK2 in the present study also contained the motifs ‘PSVMDIKIG’ and ‘LVDFAH’ similar to other IPK2 genes.

### Evolutionary divergence

The evolutionary divergence analysis involved 31 amino acid sequences whereby all positions with gaps and missing data were eliminated from the final dataset. Results of divergence analysis showed that cultivated peanut (\textit{A. hypogaea}) was genetically distinct from other plant genera (Tables 4 and 5). AhITPK1 and AhIPK2 from \textit{A. hypogaea}) was genetically distinct from other plant species. The \textit{ITPK1} gene from \textit{Prunus bretschneideri} was closely related to \textit{Pyrus bretschneideri} as well as other plant species. The \textit{ITPK1} gene from \textit{Pyrus bretschneideri} was closely related to \textit{Pyrus bretschneideri} and shared close similarly with \textit{IPK2} from \textit{Cucumis melo} and \textit{Camelina sativa}. The \textit{IPK2} gene from \textit{Malus domestica} was genetically distinct from their parental species such as \textit{A. duranensis} and \textit{A. ipinensis} and shared close similarly with \textit{IPK2} from \textit{Camelina sativa}.
AhIPK2 consisted of one exon whereas linkage group A08 and B08 gene was located on linkage group A05 and B05, whereas gene expression pattern on different tissues in this study. AhITPK1 (KR778986) from A. hypogaea was cloned and characterized their 3'UTR. Evolutionarily based functional characterization is warranted to establish their link to peanut embryo and flower bud. Embryo development and maturity in AhIPK2 and AhITPK1 and their wild relatives as observed in the case of Oryza sativa with O. brachyantha and A. hypogaea with A. duranensis and A. ipinensis. This may be attributed to the evolution of new combinations of genes when hybridization and introgression occur between wild relatives [32-35]. Similar to AhITPK1, AhIPK2 from A. hypogaea was genetically diverse from its parental species.

Our work advances understanding of the set of genes which are important to phytic acid synthesis in peanut. The identification of kinases that phosphorylate Ins(1,3,4)P3 and Ins(1,3,4,5)P4 raises the possibility of their involvement in phytic acid synthesis in peanut kernels.

Conclusion

For the first time, the cDNA of AhITPK1 (KR778986) and AhIPK2 (KR779888) from A. hypogaea was cloned and characterized their expression pattern on different tissues in this study. AhITPK1 gene was located on linkage group A05 and B05, whereas AhIPK2 gene was linkage group A08 and B08. AhITPK1 consisted of one exon whereas AhIPK2 gene on chromosome A had 3 exons and one 5' UTR and AhIPK2 gene on chromosome B consisted of four exons 5'UTR and 3'UTR. Evolutionarily AhITPK1 and AhIPK2 genes from A. hypogaea are distinct from their parental species and other plant species. Expression profiling among different tissues and developmental stages suggest that AhITPK1 and AhIPK2 isoforms are more abundant in the peanut embryo and flower bud. Embryo development and maturity significantly influence the expression of AhITPK1 and AhIPK2 in peanut (A. hypogaea). However, future research involving RNAi-based functional characterization is warranted to establish their link to embryo development.

Author Contribution Statement

AC and AA conceived, designed and conducted the experiments, DK and JO helped in data analysis, AC and AA wrote the paper with the inputs from DK, JO and VT.

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Conflict of Interest

Authors declare that they have no conflict of interest.

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