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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 embryo 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.24V9G* 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 PxxxDxKxG and [L/M][I/V]D[F/L][A/G][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 ($InsP_3$); Inositol tetrakisphosphate ($InsP_4$); Inositol pentakisphosphate ($InsP_5$); Shoot (S); Inositol hexakisphosphate ($InsP_6$); 1,3,4,5,6 pentakisphosphate 2-kinase (IPK1); Inositol 1,4,5 trisphosphate kinase (IPK2); Inositol tetraphosphate 1-kinase (ITPK1); Leaf (L); 1D-myo-inositol 3phosphate synthase (MIPS); Open reading frame (ORF); Peg (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 ($InsP_4$) and inositol pentakisphosphate ($InsP_5$) are generated by subsequent series of phosphorylation and dephosphorylation [2-5]. Phytic acid pathway proceeded through Ins(3)P, Ins(3,4)P_2, Ins (3,4,6)P_3, Ins(3,4,5,6)P_4, Ins(1,3,4,5,6)P_5, and Ins(1,2,3,4,5,6)P_6 [6,7]. The synthesis of Ins(1,4,5)P_3 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 $InsP_6$. This pathway is called the PLC-dependent or lipid dependent pathway [8].

Myo-inositol tetraphosphate 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 ($InsP_4$) 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)P_4 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-triphosphate 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-tetrakisphosphate 1-kinase', '1D-myo-inositol-trisphosphate 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 $InsP_5$ from $InsP_3$ [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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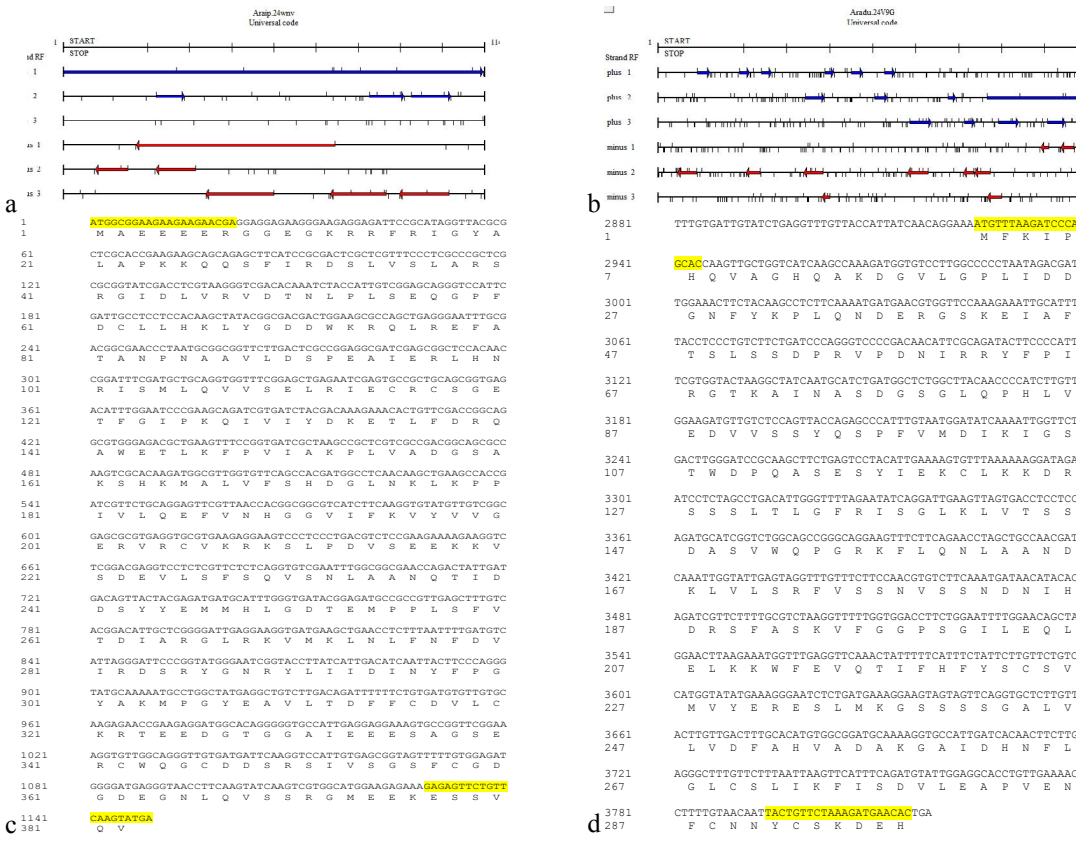

Figure 1: Translation overview of '*Araip.24WNV*' identified for *AhITPK1*.

(a) translation overview of '*Aradu.24V9G*' identified for *AhIPK2* gene
(b) positions of forward and reverse primers on largest ORF of '*Araip.24WNV*'
(c) and (d) positions of forward and reverse primers on largest ORF of '*Aradu.24V9G*'

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 AtIpk2b 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 some detail in several species such as rice, barley, maize, arabidopsis and soybean, no data is available for genes involved in phytic acid biosynthesis in peanut. Here we report results regarding the isolation and characterization of myo-inositol tetrakisphosphate kinase (ITPK1) and myo-inositol polyphosphate kinase (IPK2) genes in peanut designated as *AhITPK1* and *AhIPK2* respectively. We used bioinformatic tools to identify and map the peanut homologues coding for myo-inositol tetrakisphosphate and myo-inositol polyphosphate kinases.

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 RNease 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 '*Araip.24wnv*' and '*Aradu.24V9G*' which corresponds to gene sequences of inositol tetraphosphate 1-kinase (*ITPK1*) and inositol 1,4,5-tris-phosphate kinase/inositol polyphosphate multikinase (*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 '*Araip.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 '*Araip.24wnv*' and '*Aradu.24V9G*' sequences are presented in (Figures 1a and 1b) respectively. Positions of forward and reverse primers on the largest ORF's of '*Araip.24wnv*' and '*Aradu.24V9G*' are presented in (Figures 1c and 1d) respectively. 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-

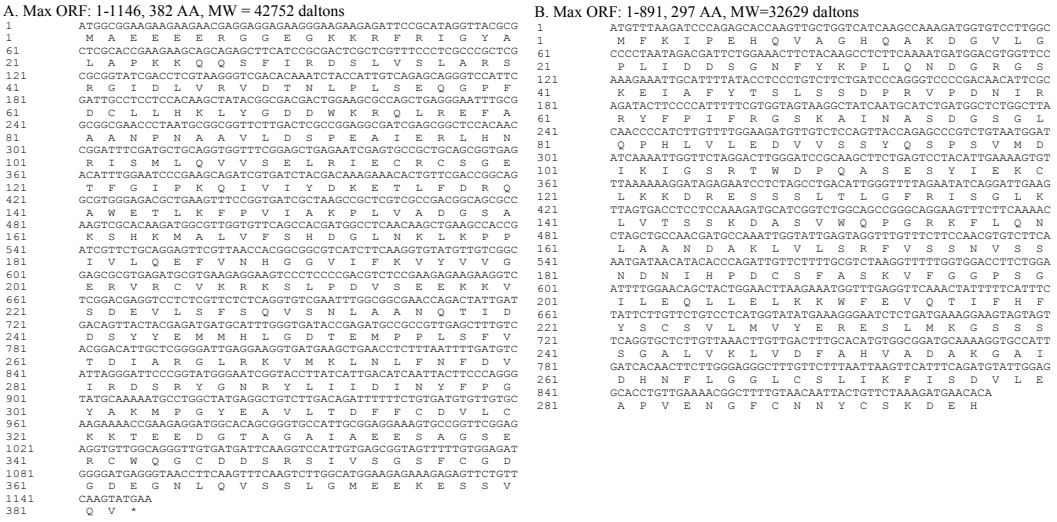

Figure 2: Nucleotide and predicted amino acid sequences of the partial cDNA clone encoding *AhITPK1* (A) and *AhIPK2* (B) identified from *Arachis hypogaea* L. The nucleotide sequence has been submitted to the GenBank with accession number KR778986 and KR778988 respectively.

PCR reaction to amplify full length ORF using primer pairs (AhITPK1F: 5'-ATGGCGGAAGAAGAAGAACGA-3' and AhITPK1R: 5'-TCATACTTGAACAGAACTCTC-3') for *AhITPK1* and (IPK2-1F: 5'- CACCATGTTTAAGATCCCAGAGCAC-3' and IPK2-1R: 5'-GTGTTCATCTTTAGAACAGTA -3') for *AhIPK2*. PCR reaction was carried out using GoTaq Green Master mix (Promega, Madison, WI) in a total volume of 25µl at 95°C for 3 min; 60 cycles of 95°C for 30 sec, 50°C and 52°C respectively for *AhIPK2 and AhITPK1* for 30 sec 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 Nucleo Spin® as per the manufacturer's procedure (Macherey-Nagel, Bethlehem, PA) 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].

Quantitative real time PCR (qRT-PCR): Primers for quantitative real time PCR were designed using the sequence 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 Sso advanced® 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 65 sec; 39 cycles of 95°C for 10 sec, 55°C for 30 sec and meltcurve between 65 and 95°C at 0.5°C increment per cycle for 5 sec. qRT-PCR was performed using primers RTAhITPK1F: 5'-GGGAGACGCTGAAGTTTCCG-3' and RTAhITPK1R:5'-GCCGACAACATACACCTTGAA-3' for *AhITPK1* and RTAhIPK2F-5'-AAAATTGGTTCTAGGACTTGG-3' and RTAhIPK2R: 5'-CCTACTCAATACCAATTTGGC-3' for *AhIPK2*. Peanut actin gene specific markers (AhAct F: 5'-TTGACGGAGCGTGGATACTCC-3' and AhAct R: 5'-CCGTCCGGCAGCTCGTAGCTC-3') were used as reference gene. The qRT-PCR data were analysed using CFX manager software v3.1 (BioRad) Relative quantity of protein was determined by comparing with reference gene, which is an action in the present study [23]. Relative quantity and normalised expression of *AhITPK1* and *AhIPK2* genes were calculated using CFX manager software v3.1 (BioRad).

Results and Discussion

Cloning and characterization of AhITPK1 and AhIPK2 cDNA fragments

Database search using *Arabidopsis thaliana* (AJ404678.2) and *Glycine max* (NC_016103.1) identified *Araip.BR64V* and *Araip.24WNV* loci in *Arachis ipinensis* which corresponded to genes IPK2 and ITPK1 respectively in peanut. The *IPK2* and *ITPK1* genes in peanut were designated as *AhIPK2* and *AhITPK1*. The loci identified from *A. ipinensis* were used as bait to design specific primers for amplification of the longest ORF in the genes. *AhITPK1* cDNA fragment of 1146bp was amplified by PCR using two degenerate primers AhITPK1F and AhITPK1R which represented a putative 382-amino acid polypeptide with a predicted molecular mass of 42.7 kDa Figure 2a, and the sequence was submitted to GenBank (accession no. KR778986). *AhIPK2* cDNA fragment of 891bp was amplified by PCR using two degenerate primers AhIPK2-1F and AhIPK2-1R which represented a putative 297-amino acid polypeptide with a predicted molecular mass of 32.6 kDa Figure 2b, and the sequence was submitted to GenBank (accession no. KR778988).

Sequence analysis revealed that the *AhITPK1* and *AhIPK2* fragments contained a single uninterrupted open reading frame (ORF). Nucleotide BLAST analysis indicated that *AhITPK1* showed high similarity to *ITPK1* gene from *G. max* (XM_003534234.2); whereas *AhIPK2* showed high similarity to *Medicago truncatula* (XM_003627834.1) (Table 1). The nucleotide sequence of *AhITPK1* shared 79% identity with *Glycine max* (XM_003534234.2), 77% identity with *Morus notabilis* (XM_010098662.1), *Frageria vesca* (XM_004300368.2), etc. Whereas the nucleotide sequence of *AhIPK2* shared 71% identity with *M. truncatula* (XM_003627834.1), 71% with *Jatropha curcas* (XM_012235154.1), 70% identity with *Populus euphratica* (XM_011016855.1), etc.

	Max score	Total score	Query cover	Evalue	Ident	Accession
ITPK1						
Glycine max	767	815	84%	0.00E+00	79%	XM_003534234.2
Morus notabilis	713	713	79%	0.00E+00	77%	XM_010098662.1
Fragaria vesca	697	697	79%	0.00E+00	77%	XM_004300368.2
Prunus mume	690	690	79%	0.00E+00	77%	XM_008239992.1
malus x domestica	652	652	80%	0.00E+00	75%	XM_008375369.1
Pyrus x bretschneideri	636	636	80%	3.00E-178	75%	XM_009348119.1
Cicer arietinum	601	601	79%	7.00E-168	74%	XM_004509680.1
Eucalyptus grandis	587	587	79%	2.00E-163	75%	XM_010055323.1
Phoenix dactylifera	511	511	81%	9.00E-141	73%	XM_008788125.1
Medicago truncatula	509	509	79%	3.00E-140	72%	XM_003628739.1
Elaeis guineensis	484	484	81%	1.00E-132	72%	XM_010935149.1
Theobroma cacao	443	443	79%	4.00E-120	71%	XM_007040574.1
Populus euphratica	434	434	79%	2.00E-117	71%	XM_011029855.1
Cucumis melo	407	407	80%	3.00E-109	70%	XM_008450445.1
Cucumis sativus	390	390	79%	2.00E-104	70%	XM_004148716.2
Beta vulgaris	387	387	80%	3.00E-103	69%	XM_010697247.1
Musa acuminata	387	387	79%	3.00E-103	70%	XM_009409561.1
Nicotiana sylvestris	289	289	80%	6.00E-74	68%	XM_009782918.1
Hordeum vulgare	277	277	78%	4.00E-70	68%	AM404177.1
Sesamum indicum	275	275	68%	1.00E-69	68%	XM_011084055.1
Zea mays	246	316	63%	6.00E-61	70%	EU965009.1
Oryza brachyantha	237	323	64%	3.00E-58	69%	XM_006662571.1
Nicotiana tomentosiformis	233	233	79%	4.00E-57	66%	XM_009624351.1
Setaria italica	232	310	67%	1.00E-56	69%	XM_004983491.1
Oryza sativa	232	323	63%	1.00E-56	69%	AM410634.1
IPK2						
Medicago truncatula	412	412	93%	5.00E-111	71%	XM_003627834.1
Jatropha curcas	399	399	93%	3.00E-107	71%	XM_012235154.1
Populus euphratica	354	354	93%	1.00E-93	70%	XM_011016855.1
Prunus mume	351	351	93%	2.00E-92	70%	XM_008241075.1
Nelumbo nucifera	329	329	93%	5.00E-86	69%	XM_010274410.1
Cicer arietinum	315	315	93%	1.00E-81	68%	XM_004510783.1
Cucumis melo	298	298	93%	9.00E-77	68%	XM_008455890.1
Vitis vinifera	298	298	93%	9.00E-77	68%	KF752483.1
Cucumis sativus	297	297	93%	3.00E-76	68%	XM_011654717.1
Pyrus x bretschneideri	297	297	93%	3.00E-76	69%	XM_009375399.1
Malus x domestica	297	297	93%	3.00E-76	69%	XM_008394459.1
Citrus sinensis	289	289	93%	5.00E-74	68%	XM_006484990.1
Solanum lycopersicum	284	284	93%	2.00E-72	68%	XM_004252242.2
Fragaria vesca	279	279	92%	8.00E-71	68%	XM_004309442.2
Gossypium raimondii	271	271	93%	1.00E-68	68%	XM_012583629.1
Solanum tuberosum	271	271	86%	1.00E-68	68%	NM_001288223.1
Morus notabilis	253	253	94%	3.00E-63	67%	XM_010092920.1
Nicotiana sylvestris	250	250	93%	4.00E-62	67%	XM_009803100.1
Nicotiana tomentosiformis	248	248	93%	1.00E-61	67%	XM_009600164.1
Beta vulgaris	215	215	93%	8.00E-52	67%	XM_010672527.1
Sesamum indicum	185	185	93%	1.00E-42	66%	XM_011074962.1
Glycine max	145	145	92%	1.00E-30	65%	KF297702.1

Table 1: Nucleotide BLAST analysis indicating similarity between *AhITPK1* gene and ITPK1 (Inositol tetraphosphate 1-kinase 1-like) and between *AhIPK2* and *IPK2* (Inositol polyphosphate multikinase) gene from other known species.

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*,

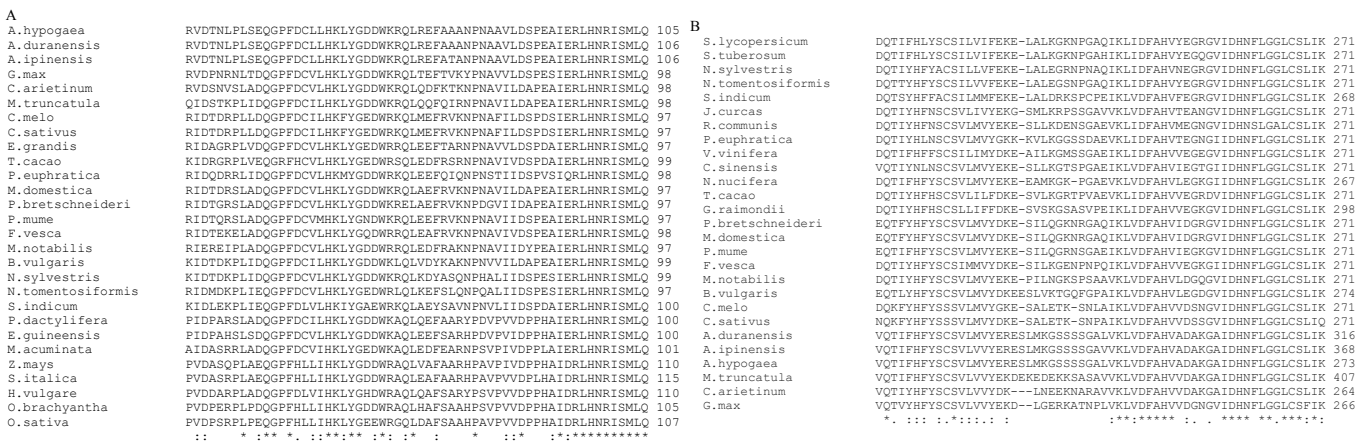

Figure 3: Alignment of conserved domains of *AhITPK1* (KR778986) (A) and *AhIPK2* (KR778988) (B) protein sequences. Identical amino acids are denoted by asterisk (*), ':' indicates conserved substitutions and '.' indicates semi-conserved substitutions. . Alignment is shown for deduced amino acid sequence.

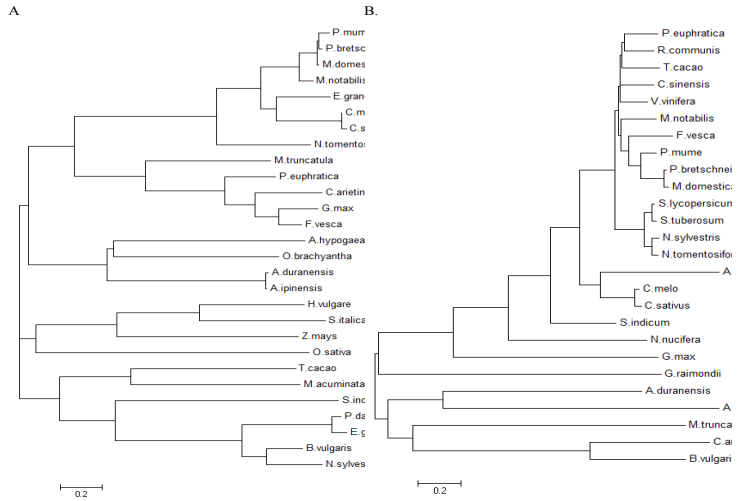

Figure 4: Phylogenetic relationship of *AhITPK1* (A) and *AhIPK2* (B) of peanut (*Arachis hypogaea* L.). Unrooted neighbour joining tree was constructed using plant ITPK1 and IPK2 protein sequences from GenBank.

M. truncatula forming separate cluster. *AhIPK2* gene was grouped in one cluster with *Cucumis 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]. Josefsen 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 tissues 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 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

Sample	Mean Cq	Normalized Expression	Relative Normalized Expression	Regulation	Compared to Regulation Threshold
AhIPK2					
E	29.39	3.33	29.50	29.50	Up regulated
Fb	27.82	0.01	0.09	-11.14	Down regulated
Fo	29.54	0.00	0.03	-33.09	Down 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.

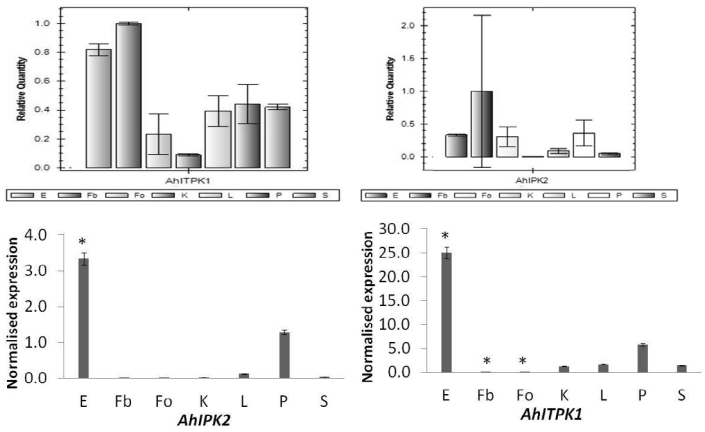

Figure 5: Relative quantity and normalised gene expression of *AhITPK1* and *AhIPK2* in peanut (*Arachis hypogaea* L.*)*. Error bars represent values with 5% value. T test analysis showed significant difference (*P < 0.01). Tissues characterized for expression patterns of the two *AhIPK* isoforms included 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).

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

I Peanut Blast	II E-value	III Peanut chromosome	IV Gene position on peanut genome
AhITPK1			
Aradu.Q95MC	0	Aradu.A05	1172401 1173549
Araip.24WNV	0	Araip.B05	1151045....1152193
AhIPK2			
Aradu.24V9G	0	Aradu.A08	43384964....43388780
Araip.HXN3H	0	Araip.B08	121202848…121207248

Table 3: Position of *AhITPK1* and *AhIPK2* genes on peanut chromosome.

	Ah	Ad	Ai	Gm	Mn	Fv	Pm	Md	Pb	Ca	Eg	Pd	Mt	Eg	Tc	Pe	Cm	Cs	Bv	Ma	Ns	Hv	Si	Zm	Ob	Nt	Sit
Ah																											
Ad	1.7																										
Ai	1.7	0.0																									
Gm	2.6	2.6	2.6																								
Mn	2.4	2.9	2.9	2.5																							
Fv	2.5	2.4	2.4	0.3	2.4																						
Pm	2.7	3.0	3.0	2.6	0.2	2.5																					
Md	2.6	2.8	2.8	2.6	0.2	2.6	0.1																				
Pb	2.6	2.8	2.8	2.6	0.2	2.5	0.1	0.0																			
Ca	2.3	2.7	2.7	0.6	2.4	0.6	2.4	2.5	2.5																		
Egr	2.7	2.5	2.6	2.9	0.6	2.7	0.6	0.6	0.6	3.0																	
Pd	2.7	3.0	2.9	3.0	2.9	2.8	2.8	2.8	2.8	3.0	2.7																
Mt	2.5	1.4	1.5	1.3	2.6	1.3	2.7	2.7	2.7	1.2	2.4	3.0															
Egu	2.9	3.0	3.0	3.0	2.9	2.9	2.9	2.8	2.8	3.1	2.7	0.1	3.2														
Tc	2.8	2.5	2.4	1.2	3.0	1.1	3.0	3.0	3.1	2.3	2.8	2.5	2.8	2.6													
Pe	2.6	2.9	2.9	0.7	1.2	0.6	1.2	1.2	1.2	0.7	2.9	2.8	1.4	2.8	2.7												
Cm	2.6	2.7	2.7	3.0	0.6	2.9	0.6	0.6	0.6	2.6	0.6	3.0	2.6	3.0	2.9	2.9											
Cs	2.6	2.8	2.8	3.0	0.6	3.0	0.6	0.6	0.7	2.6	0.6	3.0	2.6	3.0	2.9	2.9	0.0										
Bv	2.7	2.6	2.6	2.5	2.6	2.3	2.7	2.6	2.7	2.7	2.5	0.8	2.6	0.8	1.3	2.8	2.4	2.5									
Ma	2.7	2.5	2.5	1.6	2.7	1.6	2.9	2.9	2.9	2.8	2.8	2.6	2.9	2.4	1.6	2.9	3.2	3.3	2.7								
Ns	2.6	2.6	2.6	2.9	2.8	2.9	2.8	2.7	2.8	2.8	2.7	0.9	2.8	0.9	1.3	2.9	2.5	2.5	0.4	2.7							
Hv	2.9	2.6	2.6	2.7	2.6	2.7	2.8	2.6	2.7	2.6	2.6	2.9	2.9	2.9	2.7	2.7	2.6	2.6	2.6	2.6	2.6						
Si	2.9	2.7	2.7	3.2	2.5	3.2	2.6	2.6	2.6	2.8	2.6	1.5	2.7	1.5	2.8	2.8	2.6	2.8	2.8	2.4	2.5	2.8					
Zm	2.4	2.6	2.6	2.7	2.6	2.7	2.7	2.6	2.6	2.7	2.7	2.9	2.6	2.9	2.6	2.8	2.8	2.8	2.8	2.7	2.8	1.2	2.8				
Ob	1.7	1.6	1.6	2.8	2.7	2.8	3.0	2.9	2.9	2.6	2.7	2.8	1.6	2.8	2.7	2.8	2.7	2.7	2.8	2.6	2.9	2.5	2.5	2.6			
Nt	2.7	2.7	2.7	1.4	0.7	1.4	0.7	0.7	0.7	1.4	1.3	2.7	2.6	2.8	2.8	0.8	1.2	1.3	2.7	2.7	2.8	2.6	2.7	2.8	3.1		
Sit	3.3	2.8	2.8	2.8	2.6	2.7	2.6	2.6	2.6	2.6	2.6	2.7	2.6	2.8	2.8	2.9	2.4	2.5	2.6	2.8	2.8	1.1	2.7	2.4	2.9	2.8	
Os	2.8	2.5	2.4	3.0	3.0	2.9	3.0	3.0	3.0	2.9	2.9	2.8	2.4	2.7	2.7	2.8	2.8	2.8	3.0	2.6	2.9	2.6	3.2	2.6	2.4	2.8	2.7

Ad = *Arachis duranensis*; Ah = *Arachis hypogaea*; Ai = *Arachis ipinensis*; Bv = *Beta vulgaris*; Ca = *Cicer arietinum*; Cm = *Cucumis melo*; Cs = *Camelina sativa*; Egr = Eucalyptus grandis; Egu = Elaeis guineensis; Fv = *Frageria vesca*; Gm = *Glycine max*; Hv = H. Vulgare; Ma = Musa acuminate; Md = *Malus x domestica*; Mn = *Morus notabilis*; Mt = *Medicago truncatula*; Ns = *Nicotiana sylvestris*; Nt = *Nicotiana tomentosiformis*; Pb = *Pyrus x bretschneideri*; Pd = Phoenix dactylifera; Pe = *Populous euphratica*; Pm = *Prunus mume*; Si = *Sesamum indicum*; Sit = *Setaria italica*; Ob = *Oryza brachyantha*; Os = *Oryza sativa*; Tc = *Theobroma cacao*; Zm = *Zea mays*

885, 38 and 100 bp long respectively and one 5'UTR with 351 bp long. The *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 (*A. hypogaea*) was genetically distinct from other plant genera (Tables 4 and 5). *AhITPK1* and *AhIPK2* from *A. hypogaea* were genetically diverse from their parental species such as *A. duranensis* and *A. ipinensis* as well as other plant species. The *ITPK1* gene from *Pyrus bretschneideri* was closely related to *Prunus mume* and shared close similarly with *ITPK1* genes from *Cucumis melo* and *Camelina sativa*. The *IPK2* gene from *Malus domestica* was closely related to *Pyrus bretschneideri*. Results indicated that *ITPK1* and *IPK2* genes from cultivated types were genetically different from

	Ah	Ad	Ai	Mt	Pe	Pm	Rc	Tc	Nn	Ca	Cm	Vv	Cs	Pb	Md	cs	Sl	Fv	Gr	St	Mt	Ns	Nt	Bv	Si
Ah																									
Ad	2.4																								
Ai	2.5	2.2																							
Mt	2.5	2.5	2.7																						
Pe	1.0	2.5	2.9	2.9																					
Pm	1.0	2.5	2.8	2.9	0.4																				
Rc	1.0	2.5	2.7	2.9	0.3	0.3																			
Tc	1.1	2.4	2.7	3.1	0.3	0.4	0.3																		
Nn	1.4	2.6	2.6	2.6	1.3	1.3	1.2	1.4																	
Ca	2.3	3.0	3.1	2.4	2.9	3.1	2.9	3.1	2.5																
Cm	0.7	2.6	3.0	2.5	0.6	0.6	0.6	0.7	1.4	2.7															
Vv	0.9	2.4	3.0	2.7	0.3	0.3	0.3	0.3	1.2	2.9	0.6														
Cs	0.7	2.5	3.1	2.5	0.6	0.6	0.6	0.7	1.5	2.7	0.1	0.6													
Pb	1.1	2.5	2.9	3.1	0.4	0.2	0.4	0.5	1.3	3.3	0.6	0.4	0.6												
Md	1.1	2.5	2.8	3.1	0.4	0.2	0.4	0.5	1.3	3.2	0.6	0.4	0.6	0.0											
cs	1.0	2.5	2.7	2.8	0.3	0.3	0.3	0.3	1.3	3.0	0.6	0.3	0.6	0.4	0.4										
Sl	1.0	2.5	3.0	2.9	0.4	0.4	0.4	0.4	1.4	2.7	0.6	0.3	0.6	0.4	0.4	0.4									
Fv	1.1	2.7	3.1	2.8	0.4	0.4	0.5	0.5	1.5	2.7	0.7	0.4	0.7	0.4	0.4	0.4	0.5								
Gr	3.0	2.6	2.7	2.7	2.5	2.6	2.5	2.7	2.9	3.2	2.7	2.7	2.8	2.5	2.5	2.6	2.4	2.5							
St	1.0	2.6	3.1	2.7	0.4	0.4	0.4	0.4	1.5	2.7	0.6	0.3	0.6	0.4	0.4	0.4	0.0	0.5	2.4						
Mt	1.0	2.5	3.1	2.8	0.4	0.4	0.3	0.4	1.4	2.6	0.6	0.3	0.6	0.4	0.4	0.4	0.4	0.4	2.6	0.4					
Ns	1.0	2.6	3.1	2.5	0.4	0.4	0.4	0.4	1.4	2.7	0.6	0.4	0.7	0.4	0.4	0.4	0.1	0.5	2.5	0.1	0.4				
Nt	1.0	2.5	3.1	2.6	0.4	0.4	0.4	0.4	1.4	2.7	0.6	0.4	0.6	0.4	0.4	0.4	0.1	0.5	2.5	0.1	0.4	0.1			
Bv	2.7	2.7	2.8	2.7	2.7	2.7	2.7	2.6	3.1	1.0	2.6	2.5	2.6	2.9	2.8	2.6	2.6	3.0	2.9	2.6	2.7	3.0	2.7		
Si	1.0	2.6	3.4	2.5	0.8	0.8	0.8	0.8	1.0	2.8	0.8	0.8	0.9	0.9	0.9	0.8	0.7	0.9	2.5	0.7	0.8	0.8	0.7	2.7	
Gm	1.9	2.9	2.9	2.5	1.9	1.9	1.8	1.9	1.7	3.0	1.9	1.8	1.9	2.0	2.0	1.8	1.9	1.9	2.6	1.9	1.9	1.9	1.9	2.7	1.9

Table 5: Estimates of evolutionary divergence between plant species based on IPK 2 sequences.

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)P_3 and Ins(1,3,4,5)P_4 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* (KR778988) 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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