DNA chip-based expression profile analysis indicates involvement of the phosphatidylinositol signaling pathway in multiple plant responses to hormone and abiotic treatments

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ABSTRACT

The phosphatidylinositol (PI) metabolic pathway is considered critical in plant responses to many environmental factors, and previous studies have indicated the involvement of multiple PI-related gene families during cellular responses. Through a detailed analysis of the *Arabidopsis thaliana* genome, 82 polypeptides were identified as being involved in PI signaling. These could be grouped into different families including PI synthases (PIS), PI-phosphate kinases (PIPK), phospholipases (PL), inositol polyphosphate phosphatases (IPase), inositol polyphosphate kinases (IPK), PI transfer proteins and putative inositol polyphosphate receptors. The presence of more than 10 isoforms of *PIPK*, *PLC*, *PLD* and *IPase* suggested that these genes might be differentially expressed during plant cellular responses or growth and development. Accordingly, DNA chip technology was employed to study the expression patterns of various isoforms. In total, 79 mRNA clones were amplified and used for DNA chip generation. Expression profile analysis was performed using samples that represented multiple tissues or cellular responses. Tested samples included normal leaf, stem and flower tissues, and leaves from plants treated with various hormones (auxin, cytokinin, gibberellin, abscisic acid and brassinosteroid) or environmental factors (temperature, calcium, sodium, drought, salicylic acid and jasmonic acid). Results showed that many PI pathway-related genes were differentially expressed under these experimental conditions. In particular, the different isoforms of each family were specifically expressed in many cases, suggesting their involvement in tissue specificity and cellular responses to environmental conditions. This work provides a starting point for functional studies of the relevant PI-related proteins and may help shed light onto the role of PI pathways in development and cellular responses.

Keywords: phosphatidylinositol, signal transduction, DNA chip, expression profile.

INTRODUCTION

Although they were first described in the 1950s, it was only recently that researchers recognized the critical importance of phospholipids and the related phosphatidylinositol (PI) signaling pathway in higher plants comparing to the relatively detailed studies in animal cells. In particular, the second messengers inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] and diacylglycerol (DAG) were recently demonstrated to be critical in signal transduction processes such as gene expression, hormone function, cell differentiation, transduction of intracellular

signaling molecules, control of cell responses to environmental factors, regulation of ion-channel gating, energy metabolism and rearrangement of cytoskeleton[1]. In addition, it has been shown that deficiency of these proteins results in exceptional physiological phenomena such as changed cell response to hormone treatments, and some abiotic stresses, which perhaps even involves the interaction with other signaling pathways such as the calcium-related signaling network[2, 3].

To begin the PI metabolic pathway, PI synthase (PIS) synthesizes PI from membrane-bound CDP-diglycerol (CDP-DG) and free cytoplasmic inositol. Sequential phosphorylations at the 4- and 5- positions by PI4-kinase (PI4K) and PI-phosphate 5-kinase (PIPK) result in the production of $\text{PI}(4,5)\text{P}_2$ [4], which is the substrate of phospholipase C (PLC) and the direct precursor of two important second messenger molecules, $\text{Ins}(1,4,5)\text{P}_3$ and diacylglycerol

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(DAG), which can stimulate Ca^{2+} release from internal calcium stores and activate related protein kinases for regulation of downstream pathways or cellular responses. In a tightly regulated manner, these second messengers may be recycled by further phosphorylation of $\text{Ins}(1, 4, 5)\text{P}_3$ to $\text{Ins}(1, 3, 4, 5)\text{P}_4$ by $\text{Ins}(1, 4, 5)\text{P}_3$ 3-kinase (IP3K), or dephosphorylation to $\text{Ins}(1, 4)\text{P}_2$ by inositol polyphosphate 5-phosphatase (IPPase). To date, most molecular characterizations, expression pattern analyses and biochemical studies have focused on key PI pathway-related proteins including PIS [5], PI4K[6-8], PIP5K[9-13], PLC [14,15], PLD [16], PLA_1 [17], PLA_2 [18, 19], PI 3-kinase (PI3K) [20], IPPase [21] and inositol 1, 3, 4-trisphosphate 5/6-kinase (IP_3 5/6K)[22]. In contrast, little attention has been paid to other proteins such as inositol 1, 4, 5-trisphosphate receptor (IP_3R) and PI transfer protein (PITP), and there are many other signal molecules yet to be investigated within this pathway. These signal molecules come from $\text{Ins}(1,4,5)\text{P}_3$ or phosphoinositides and may act as additional second messenger molecules. Examples of these are $\text{PI}(3,4,5)\text{P}_3$ (phosphorylated by PI3K), $\text{Ins}(1,3,4,5)\text{P}_4$ (phosphorylated by IP3K), $\text{Ins}(1,3,4)\text{P}_3$ and the putative IP_3R and PITPs, many or all of which may important for proper signaling.

Many members of the PI pathway are located in or near the membrane, though a few are nuclear[23]. The diversity and possible function of the PI pathway has been extensively studied in mammals, including humans [24, 25], though few studies have investigated the pathway in plants. Little is known about the physiological functions and components of the overall PI pathway in plants, though the genes for PIS, PI4K, PIPK, PLC and PLD have been isolated and characterized from *Arabidopsis thaliana*, *Oryza sativa* and *Glycine max*. Additionally, the identification of multiple isoforms of proteins such as PIPK[26], PLC, PLD [27] and IPPase (Lin *et al.*, in preparation), as well as their differential expression patterns suggests the presence and regulation of a complex PI pathway within plant tissues.

The completed sequencing of the *A. thaliana* genome allowed the first genome-wide screens for relevant genes [28], and the recent development of cDNA chip technology made possible the large-scale analysis of light control-related genes[29], specific gene expression in developing seeds [30], drought- and wound-related genes[31, 32], rice ABA-responsive genes[33], and the relative effects of mutant vs. wild-type gene expression[34].

However, few recent studies have focused on expression profile analysis of a specific metabolic and signaling pathway, although relatively detailed structural analysis has been performed on PLD[27], PIPK and PLC[26] and IPPase (Lin *et al.*, in preparation). Here, we used DNA chip technology to investigate the expression profiles of

A. thaliana genes involved in the PI pathway. In addition, we investigated differential expression of these genes in various tissues and under various stresses or conditions, allowing us to develop a greater understanding of the importance of the PI pathway. This is the first report on the expression profile analysis of a single plant metabolic pathway, and the results will aid researchers by identifying important proteins for further functional studies and hinting at their interrelation in the PI pathway.

MATERIALS AND METHODS

Enzymes and chemicals

PCR primers were synthesized by TibMolBio (Berlin, Germany). 24-epibrassinolide (24-eBL, #E-1641), abscisic acid (ABA, #A1049), indole-3-acetic acid (IAA, #I2886), gibberellic acid (GA_3 , #G7645), kinetin (KT, #K0753) and salicylic acid (SA, #S7401) were obtained from Sigma (USA). Methyl jasmonate (MeJA, #39270-7) and mannitol (#24018-4) were obtained from Aldrich (USA). CaCl_2 and NaCl were obtained from Sangon Company (Shanghai). All other reagents used were standard analytical or electrophoresis grade.

Analysis of PI-related genes in *A. thaliana* genome

To analyze the PI-related genes on a genome-wide level, key words such as phosphatidylinositol, phospholipase, inositol, phosphatase, trisphosphate, kinase and inositol 1, 4, 5-trisphosphate receptor were used to search the *A. thaliana* genome database contained within the National Center of Biotechnological Information (NCBI, <http://www.ncbi.nlm.nih.gov/entrez>). Additionally, key words including inositol, phosphatidylinositol, phospholipase and receptor, as well as isolated cDNA sequences encoding PI synthase, PI kinase, PIP kinase, phospholipase, were used as baits to search the database. The resulting genomic clones and predicted mRNA sequences were further used for sequence analysis and design of PCR primers.

Primer design and amplification of DNA fragments

Primers were designed from the deduced mRNA sequences identified in the above screen or the isolated ones. Sequence comparison analysis and previous studies indicated that most of the PI pathway-related genes were most highly conserved at the C-terminus. Therefore, we designed PCR primers to amplify 600-800 bp N-terminal fragments (spanning the first 1-3 exons), so as to avoid nonspecific amplification (Tab 1). The primers were used for amplification of *A. thaliana* genomic DNA, and the PCR products were checked on agarose gel via electrophoresis. A total of 93 DNA fragments were successfully amplified, purified and spotted on glass-based chips. Because some genes were represented multiple times (with different accession numbers) within the database, some clones were multiply represented on the cDNA chips.

Plant materials and treatment with hormones and compounds

To study the expression profiles of PI-related genes under different conditions, multihormones and stress treatments were performed so as to illustrate their differential expression patterns. Seeds of *A. thaliana* (Columbia) were surface sterilized and germinated on PNS

culture medium with vernalization for 2 days. Germinated seedlings were grown in a dark culture room for 1 day to improve the germination and then further grown under 16 h light (20°C) and 8 h dark (20°C) conditions for another 5 days. After budding, shoots were transferred to pots and grown in the above conditions in a phytotron (70-80% of humidity). After 4 weeks, leaves, stems and flowers of untreated plants, and leaves of treated plants were harvested and used for RNA extraction. For investigation of hormonal and stress responses, plants were given IAA (10 μ M), 24-epi-brassinolide (24-eBL, 10 μ M), ABA (10 μ M), KT (10 μ M), GA₃ (10 μ M), SA (salicylic acid, 200 μ M), MeJA (Methyl Jasmonate, 100 μ M), mannitol (250 μ M), CaCl₂ (5 mM) or NaCl (200 mM) for 4 hs. For investigation of responses to environmental stresses, plants were treated with cold (4°C, for 16 h) or drought (55°C, for 1 h). Selected tissues and treated materials were collected and frozen at -70°C prior to use.

DNA chip preparation

Amplified PCR products were isopropanol precipitated, examined on an agarose gel to ensure quality, and dried (SAVANT SPEEDVAC PLUS, SC 210A, Thermo Life Science). Samples were then dissolved in 3X SSC, and spotted four times each onto silicified slides (Biostar, Shanghai, China) with an Omnigrid Arrayer (Gene Machine, CA) fitted with 16 Major Precision split pins. Slides were hybridized for 2 hours in 70% humidity, dried for 0.5 hour at room temperature and UV cross-linked (65 mJ/cm). This was followed by soaking in room temperature 0.2% sodium dodecyl sulfate (SDS) for 10 min, distilled H₂O for 10 min, and 0.2% sodium borohydride (NaBH₄) for 10 min. The slides were then dried prior to use. To facilitate the evaluation and analysis of chip, mock buffer and 10 house-keeping gene of human and mouse were spotted on the chip as negative controls.

RNA purification, probe labeling and hybridization

Tissue samples and treated *A. thaliana* leaves were ground into a fine powder in a 10 cm ceramic mortar (RNase-free) and homogenized in Trizol solution (Biostar, Shanghai, China). After centrifugation, the supernatant was extracted with an equal volume of chloroform, and the aqueous phase was precipitated with an equal volume of isopropanol at 4°C. The resultant RNA pellet was dissolved in Milli-Q H₂O. The fluorescent cDNA probes were prepared through reverse transcription with Cy3- or Cy5-deoxy UTP (Amersham Pharmacia Biotech, Piscataway, NJ) as follows: 5 μ g of oligo(dT)₁₈ (or antisense primers at concentrations identical to those used for PCR) and 50 μ g of total RNA were incubated at 70°C for 10 min, and then chilled on ice. The reaction was carried out in a mixture containing dNTPs (200 μ M dATP, dCTP, dGTP, 60 μ M dTTP, and 60 μ M Cy3- or Cy5-dUTP), 2 μ L of Superscript II reverse transcriptase (Invitrogen) and 1X reaction buffer for 2 hs at 42°C. Then the RNA was hydrolyzed by addition of 4 μ L of 2.5 M NaOH and incubation at 65°C for 10 min, and neutralized with 4 μ L of 2.5 M HCl. For the most part, the control samples (RNA extracted from untreated materials) were labeled with Cy3-dUTP and treated RNAs were labeled with Cy5-dUTP. After quantitation of fluorescence, the labeled probes were mixed and diluted in 500 μ L TE solution and concentrated to 10 μ L with a Microcon YM-30 filter (Millipore, Bedford, MA).

Labeled probes were purified and dissolved in 20 μ L of hybridization solution (5 x SSC, 0.75 M NaCl and 0.075 M sodium citrate)

with 0.4% SDS and 50% formamide. DNA chips were pre-hybridized with hybridization solution containing 0.5 mg/ml denatured salmon sperm DNA at 42°C for 6h. Fluorescent probes were denatured at 95°C for 5 min and applied to the pre-hybridized chips under a cover glass. Chips were hybridized at 42°C for 15-17 hs. After hybridization, the chips were washed at 60°C for 10 min each in solutions of 2X SSC and 0.2% SDS, 0.1x SSC and 0.2% SDS and 0.1x SSC, then dried at room temperature.

Chip scanning and data analysis

Hybridized chips were scanned with a ScanArray 4000 (GSI Lumonics, Billerica, MA) at 532 and 653 nm to detect emission from Cy3 and Cy5, respectively. The resulting images were analyzed using GenePix Pro 3 software (Axon Instruments Inc., USA). The intensities of each spot at the two wavelengths represented the quantity of Cy3-dUTP and Cy5-dUTP, and the ratios of Cy3 to Cy5 were computed using the GenePix Pro 3 median of ratio method. Overall intensities were normalized using the corresponding GenePix default normalization factor. All spots flagged 'Bad' or 'Not Found' by GenePix software were removed from the final data set. Genes were identified as differentially expressed if the absolute value of the natural logarithm of the ratios was >0.69, which indicated at least one time induction or suppression. Only genes with raw intensity values for both Cy3 and Cy5 of >200 counts were chosen for further analysis.

Cluster analysis was performed with the help of Gene Cluster Program (<http://rana.stanford.edu/software>) to study the expression pattern of PI-related genes in vegetative tissues (flower, root and stem), following hormone treatment and stress conditions.

RESULTS

Multiple PI pathway-related gene families are present in the *A. thaliana* genome

Previous studies have indicated that many proteins including PIS, PI4K, PIP5K, PLC, PLD, IPPase and IP3K are involved in the PI pathway, and that others, such as PI3K, IP3R and PITP, are implicated in the pathway. Searching against the *A. thaliana* genome database using different key words including inositol, phosphatidylinositol, and phospholipase receptor, as well as using isolated cDNA sequences as baits for homologous search, identified 82 PI pathway-related isoforms scattered throughout the genome, including 3 PISs, 4 PI4Ks, 10 PIPKs, 17 PLCs, 10 PLDs, 5 PLA₂s, 8 putative PLs, 4 IPKs, 1 PI3K, 2 IPRs, 15 IPPases, and 3 PITPs (Tab 1). Sequence analysis revealed that within each family, the isoforms were highly homologous at their C-termini, in which contain catalytic regions [37]. The exception to this was the PIPKs and IPPases (structural subfamily I), which contained conserved N-terminal MORN regions and WD-40 repeats, respectively.

DNA amplification, chip preparation, hybridization and data analysis

Tab 1 The *Arabidopsis thaliana* genome contains members of multiple PI pathway-related gene families. Genes were identified by BLAST search against the *Arabidopsis* database. Gene IDs and their chromosomal localizations are indicated. Primers used for DNA or cDNA amplification are shown. PIS, PI synthase; PI4K, PI 4-kinase; PIPK, PI phosphate kinase; PL, phospholipase; IPK, inositol polyphosphate kinase; PI3K, PI 3-kinase; IPR, IP₃ receptor; IPPase, inositol polyphosphate phosphatase.

	Names	Gene ID	Chr.	Forward primer (5'-3')	Reverse primer (5'-3')
PIS	PIS1	AT1G68000	1	AAGGAGAGACCTAGACCTGAGAAG AGGAGAGACCTAGACCTGAGAA	GCTTCCTACATATTCTAGTCATGGC GGCTTCTGCTGCTTCTCTATA
	PIS2	AT4G38570	4	CGACGTTGTCAGTCTACCTCTACATTC GACGTTGTCTAGTCTACCTCTACATTC	CTGTGCTGACTCTGTAACCATGC GAGTTGTGTGTTACGTTCAATCG
	PIS3	AT1G51040	1	AGGCTAAGTCCATCAACAAAGCAC	ATCGAGTACATCATCCATCACCTG
PI4K	PI4Kβ1	AT5G64070	5	CTTTCTATCTTTGGTCCGAGGAGAC ATGCCGATGGGACGCTTTCTA	GCATCTCTGACTTTAGGACTTGGG CCTCTGAGTTAGGTATTGGTTCA
	PI4Kα1	AT1G49340	1	CATGTTACGGATGTGTCTGCATAG	TACCACCGTATCTGTTTCATCCAG
	PI4Kα2	AT1G51040	1	GAGAGATTGAATGAGCTTTGCGAC	TATTTCTCAACAGACTCACCTG
	PI4Kβ2	AT5G09350	5	GATTCGATTGAGTCTCCTCGTGAG	ACATTCCTCGTCTTCTTTGTGTCAGC
PIPK	PIP5K1	AT1G21980	1	GTACGAAGGAGATTGGAACGAG ACTTGGAGTGATGGGAGTTCTTG	GCCCTTTAGAAATAGTCTGTCCAGG CTTTCCAGGTGAAGATAGTTCGC
	PIP5K2	AT1G77740	1	AAGGATGGGTGTATGTACGAAGG	CTCTCCAGGTTTCTTAGCCTCAC
	PIP5K3	AT2G26420	2	GGCTGCCGAGATTAGAATAGTAGA	TACATGATCACACACGCCAGTATC
	PIP5K4	AT3G56960	3	GTTAATGGCCACTGATCAACCAC	GTTGAGAAGCTATTGCTGCTGAG
	PIP5K5	AT2G41210	2	TTTGGAACAGTATCGGTAGCTCC	CATTTCTCTGGATCCTTAGACC
	PIP5K6	AT3G07960	3	GATGAATCTGAGACATGGGCAAG	CCAAGCTGCAAGTTAAGCATGAG
	PIP5K8	AT1G60890	1	GACCGTAAGAAAGACCCAACAAG	ACCAACGTAGAACTCCCATCAG
	PIP5K9	AT3G09920	3	CTGGTCAACTCAAAGGAACACTTC	CTCAGAAAGACTTCGTGATACAGC
	PIP5K10	AT1G01460	1	TCCTCTGGAACCATTAAGT	GTCTGGCCAAAGAATCCACATAG
	PIP5K11	P5K2	2	CGCGTTTCATGTGTCTGACTATC	AAGATCCGTTCTCTGGTCTCTAA
PLC	PLC1	AT4G38690	4	CGCGTTTCATGTGTCTGACTATC CGCGTTTCATGTGTCTGACTATC TCCATCACCAGAAGAACTTAAGGG	AAGATCCGTTCTCTGGTCTCTAA AAGATCCGTTCTCTGGTCTCTAA CAACATGAGGATCGTAGTTTGACG
	PLC2	AT3G08510	3	CGCGTTTCATGTGTCTGACTATC	AAGATCCGTTCTCTGGTCTCTAA
	PLC3	AT5G58670	5	TCCATCACCAGAAGAACTTAAGGG CAGAGAATGGAGTTATGACCGTTG CCATGATTACTCTCAAGACGACAGG	CAACATGAGGATCGTAGTTTGACG TGCGATATAGGAGCATCCATGTC AGCACAACAGGATAAGCAGACACC
	PLC4	AT5G58680	5	CTTTGGCTTGAAGATCTGGAGTC	TTGTAGAGTATACCGAGGGCAGG
	PLC5	AT5G58690	5	AAGGAAGTTCAGGATGACGGAGT	CAGCTGCAGAGTACACACCAAGA
	PLC6	AT1G64050	1	GGCTAAGCTTATGGGATCTGAAGG	ATCTCTCTGTGTCCTACCGTTTC
	PLC7	AT4G38690	4	GGCTCTCACCTTTCCAAACAAC	CCTTGAAGTAGCTGGTTGCTGC
	PLC8	T11111.160	4	ACCAACGATATCGGAATACCTCTG	ATCTGCAACTTATCTCCACATCCC
	PLC9	T11111.170	4	CCCATCTCTCTGTGGATAAATAACC	CGATCAGTAACCTGTTTAACCCAC
	PLC10	AT4G17050	4	GTATACGGCGAAAGAAAGCCAT	TCGTCTTCTCATCCGATTCAAC
	PLC11	AT4G34920	4	GGCTGGTCTTACGTTGGAGAAAC	ATCCGATCAGTCACCGGTTTAAC
	PLC12	AT4G34930	4	GAAGTGGTAAATCCAACGGTGTC	TCTCTGATACCGGCTTGTTGAAC
	PLC13	AT4G38530	4	TGTGAGTGAAGTCCAAGGAGAAC	GGATAGTCAGACACATGAAACGC
	PLC14	AT4G38690	4	GCTCTCACCTTTCCAAACAAC	TTCTTGAAGTAGCTGGTTGCTG
	PLC15	AT3G55940	3	GTCGAAGCAAACATACAAAGTCTG	GCTCCACAGGTGATGTAAGAGTC
	PLC16	DL4555W	4	CTCAGATTACGTGTAGAAGACGG	GTGAAGGAGAGAGTGTGGATTTC
	PLC17	AT2G26870	2	TGATTCAACTACTATCCGTCACG	CTTGATAGACAGGAACCTTATCAGG
PLD	PLDα-1	AT3G15730	3	AAGCTAGAGTTGGGAGAACCAGG	CTTTGGACTATGCTACCACCGTC
	PLDα-2	AT1G52570	1	AAGTGCTAAGTTTCCAGGAGTTCC	AAGCAATAGGTCCTTCAAGACGAC
	PLDα-4	AT1G55180	1	CTATCACCATCACGCTCAAGACTC	CACCCGTGTATCCAGTGTTATTG
	PLDβ-1	AT2G42010	2	ACCCTTATTACCCTTACAGGCCTC	GAATCGACAATGATAGAGTTGCCC
	PLDβ-2	AT4G00240	4	GGGTGATATGTTTGGAAGATTGC	GATGCAGGACCTAATTTATCACG
	PLDγ-1	AT4G11850	4	ACCCTTATTACCCTTACAGGCCTC	GAATCGACAATGATAGAGTTGCCC

	Names	Gene ID	Chr.	Forward primer (5'-3')	Reverse primer (5'-3')
	PLD γ -2	AT4G11830	4	CTTGGGACTTTGATCTTAGGAGC CATCCGGCTTATACTGAGACTATG	GCTGGTGCTGTCATAGGAAGAAC GATACAGAGTAACCCCTACCGCC
	PLD γ -3	AT4G11840	4	CATCCGGCTTATACTGAGACTATG GACCAGCAACTCGTTCCGTTAG	TCCTGATACAGAGTAACCCCTACCG TCGGAAGAGTACCGTCATCGAC
	PLD δ	AT4G35790	4	AGTTAGGTCTGGAGGATTCGTAAAG GACTATGTCTATGGGAGGAGGG	CCAACACTCTAACACCTTCTTGAG AAGAGTAACCCCTACCGCCTTTC
	PLD ξ -2	AT3G05630	3	GACTATGTCTATGGGAGGAGGGTC CATGGATCGTCCCTCTGGAATTATC	CTGATAAAGAGTAACCCCTACCGCC AGGCTCTTTCAACATGTGGGTATC
PLA2	PLA2-1	AT1G61850	1	GGAGAAAGTATCGGAGGACGTTA AGAATGACCAGAGCTGATGCTG	TAACATGAGCGTCTCTGGTAAAGC TGTTCTTGCTTGTCGTGAAGTT
	PLA2-2	AT3G45880	3	TCTACATGTTCTTCTCCATCCGC GGAGAGAAGTGAGAGAATTGAGC	TAAGATGCGATAACGGTCACCTG ATAGAGACGGTGAACATCAGTCG
	PLA2-3	AT4G29460	4	TGCTTTGTACAGTGTTGCTTTC	ACCTGCGCAGAGATTATTCAACA
	PLA2-4	AT4G29470	4	CCTATCTCTGTTTCAGTTCTCGG	GCACTTGCTGGCTTCTTAGGTA
	PLA2-5	AT4G29780	4	TCAGGACCGAGTCATAGGAGGTTA	CTATCCAGCTGTCACGTAACATCC
PL	PL1	AT2G39400	2	TCATTACTCTGCAGCTGGTGAAAC	ATCGAGTACGACGTCTTCTGATTC
	PL2	AT2G39410	2	GTTGAACTCACGAGGCATGAAGT	TCTGGCTAAGAGCAACACAACCTG
	PL3	AT2G39420	2	GTTGTTCACATGCAGATGGCTAC TGCAGGCAAGTGAAACAGAGAAC	CTCCTCCCATTGACTCTCCTAAC TTTGGGACATAAGCACTAAGCCC
	PL4	AT2G47630	2	TTTAGAATGCGGAATCAGGCTG	TTGACGAGGTCTTGAAGCAG
	PL5	AT3G03540	3	AAAGGCTCAGAATCGTATCCGA	AACCAATTCTTGTAACCCGGC
	PL6	F27M3_29	1	GATGAAGAATCTCCTCCAACCTC	TTCTGTATAGCAGTTGTGCGAC
	PL7	AT4G38550	4	CGCAGAGACCACTACGACATGTA	GGAGAGCTGTTTCGTGGTGTTAC
	PL8	AT4G38560	4	GACGTTGTCAGTCTACCTCTACATTC	GAGTTGTGTGTTACGTTCAATCG
IPK	IPK1	AT5G07370	5	TGTAGCAAGGAAACGCCTCTTAC	GTTACCACCGATGTTGAAGGATG
	IPK2	AT4G08170	4	GGTTGCAGGACACATTGCTAAAG TGAAGCTAACGGACAACGAAGAG	GGTCTAGCATCATCATATTCCC ACAATATCAAACGGACCTGCTC
	IPK3	AT5G16760	5	TACTTAGTTGGATACGCACTCGC	TGTTCTTGCTCCTCTTGAGCAGTC
	IPK4	AT4G33770	4	CTTTGGAGGTAGCTCAGCAACAC	GACAATCGATGCTTCAGGGTATG
PI3K	PI3K1	AT1G60490	1	AGTTTCGTTTCTTCCTCTCCTGC	CCAGGAGTAGAGGTTGGAAATGA
IPR	IPR1	AT3G10380	3	GTCAAAGTCCGCGTGTAGTCAGG	TGTAGCCTCCAGTAGCTTGGGAT
	IPR2	AT5G27230	5	TTGATTTGGAACAGAAGGCAGAG	GTCATTCTTCATATTGGCTTTAGC
IPPase	IPPase1	AT1G65880	1	AATGGAAGATCGTCAAAACG	ATACAAACAACAGCAGCAGACCC
	IPPase2	AT4G18010	4	ATGGAAGATCGTCAAAACGATCA	CATAAGCCGAAATGGCAATGGA
	IPPase3	AT2G43900	2	AGAAGTGGTTCCATTAAACGCTG	ATCTTGTCACCATCCTCTTCGTC
	IPPase4	AT2G32010	2	AACAACCACCGTGACGAAAA GCTGGTAATGTTCTTGAGC	TCATCACCATCATCAAGCACA GACGTGTTCTCGTAACTCG
	IPPase5	AT2G31830	2	CGTTGGCATCGCTTGTCCTGCG CGCAAATATAGCCTTGATGAGGG	CATCTGCAGTAGTCGTATTGGG CCATGAGAGTCGTTCTTGAAAG
	IPPase6	AT1G05630	1	CGAAGAAGAAGACGAAGAGGCG GCATTCTATAGCCTACAATTTCG	GGAGACACGCTCCTTGAAAGG AGAGCTCTGATCCATCTTCCAAG
	IPPase8	AT2G01900	2	GCCGATTTCCACCTAATACTGA	AGATCCTCCATGTCAAGTCCATC
	IPPase9	AT1G71710	1	GAGTTATAGTGATCCACCATCTCC GAGCTGAAGATGATCGTCTGTT	CAACACCAACAGTAGAGACTCGT TCCTCTCCATTAGCAACTTCAGG
	IPPase10	AT1G34120	1	GAAATCGTTCCCTTAAATGCTGG	ACAAAGGTTGCTTATGTGCTTGC
	IPPase11	AT1G71710	1	TTCTGGAGGAAGACTTAGCCCAA	ACATCTAAGTTTCTGCGGCGTTC
	IPPase12	AT5G65090	5	TAGGCCTATGGCACCAACTACAG	TGGAATCAACTCTCTTCTAGCCC

Tab. 2 The correlation coefficient of ratios from two replicates in each experiment. /WT: treated seedling versus untreated seedling. KT: 6-furfurylamino-purin (kinetin), one type of synthesized cytokinin. BR: 24-eBL. Drought^a: mixed reverse PCR primers were used in reverse transcription; Drought^b: oligo-dT primers were used for comparison of antisense primer data as an additional reproducibility control.

	Correlation Coefficient
Leaf-Cy5 versus flower-Cy3	0.93
Leaf-Cy5 versus stem-Cy3	0.92
KT / WT	0.98
IAA / WT	0.95
BR / WT	0.78
ABA / WT	0.97
GA ₃ / WT	0.81
Drought ^a / WT	0.97
Drought ^b / WT	0.89
SA / WT	0.94
Cold / WT	0.99
MeJA / WT	0.84
CaCl ₂ / WT	0.89
Mannitol / WT	0.86
NaCl / WT	0.83

PCR amplified DNA products were used for DNA chip preparation. Primers were based on the isolated and annotated mRNA sequences and were designed to amplify N-terminal fragments within the first few exons, so as to avoid nonspecific amplification. 93 PCR fragments, representing 79 independent clones, were amplified and purified for chip preparation. Repetition of the clones was due to repetition within the database, i.e. the genomic and mRNA sequences of one gene being assigned different accession numbers. These were carefully analyzed and compared after hybridization, to increase data veracity.

DNA fragments were spotted four times on each chip to increase the power of the experiment by showing reproducibility. *A. thaliana* total RNA was extracted from different treated and untreated tissues, fluorescently labeled via reverse transcription and then used for hybridization. The resulting images were scanned and analyzed. To confirm the reproducibility of the results, each hybridization was repeated at least four times, once with the Cy3/Cy5 probe labeling reversed. As shown in Fig 1A and Tab 2, the signals were very similar across the replicates; the correlation coefficient for most of the experiments was around 0.9, indicating that the results were reproducible. As an additional control, reverse transcription was performed with both oligo-dT primers and a mixture of the reverse primers used for PCR amplification in the drought treatment experiments, revealing the reproducibility of the results in a second fashion (Tab 2). And finally, we note

Fig 1. Analysis of PI pathway-related genes via DNA chip. (A) A typical image after hybridization using drought-treated materials. DNA samples were spotted on the glass slide in quadruplicate to assess the reproducibility of results. (B) Cluster analysis of the hybridizations.

that our experiments were in good agreement with the differential expression of previously studied genes including PI4Kβ1 (AB008266) treated with auxin (IAA) and NaCl [7] and AtPLC1 (D38544) and AtIP5PII (IPPase2, AF289634) treated with ABA and drought[35,36].

Differential expression patterns of PI-involved genes

Expression patterns of PI-involved genes in the vegetative tissues including leaf, stem and roots, and hormone-treated and stressed tissues were studied. Cluster analysis (<http://rana.stanford.edu/software>) showed that expression profilings of PI pathway genes was similar following treatments with ABA and IAA, Ca²⁺ and mannitol (Fig 1B).

PIS, using CDP-DG and inositol as substrates, generates PI which is not only the starting point of PI pathway but also the major component of membrane. Three members of the PIS gene family (one cloned and two putative ones) were identified and all of them were expressed in all tissues tested including leaf, root stem and flowers. Nearly constitutive expression was seen following hormone treat-

Tab 3. Differential expressions of PI involved genes. Numbers show the ratios sample vs. control after hybridization. Clones with ratios >2 or <0.5 were selected, which indicated at least one time induction or suppressions. To expand the evaluations, clones with ratios >1.5 or <0.75 were supplemented. Le, leaf; Fl, flower; St, stem; Man, mannitol [for detailed information please see “materials and methods”, plants were treated with IAA (10 μ M), 24-epi-brassinolide (24-eBL, 10 μ M), ABA (10 μ M), KT (10 μ M), GA3 (10 μ M), SA (salicylic acid, 200 μ M), MeJA (Methyl Jasmonate, 100 μ M), mannitol (250 μ M), CaCl₂ (5 mM) or NaCl (200 mM) for 4 h, and with cold (4°C, for 16 h) or drought (55°C, for 1 h)].

Names	1 Le/Fl.	2 Le/St.	3 IAA	4 BR	5 KT	6 ABA	7 GA ₃	8 Dry	9 Man.	10 NaCl	11 Ca ²⁺	12 SA	13 MeJA	14 Cold
IP3K1		4.161	1.938			2.078	1.518					1.738		
IP3K3	2.309	0.287	1.518		0.579	1.627		1.599	1.868				1.650	
IP3K4		1.875										2.295		
PI3K1									2.020				0.747	
IPR1	0.650		2.573	2.160		1.518	0.445			1.625				1.808
IPR2		2.942		1.749										
PIS1	1.625	10.552	1.540	0.544		1.918	1.562					4.396		
PIS2							1.612				0.653	1.583		0.585
PIS3		2.525	1.664	1.521										
PI4K β 1			4.301				0.720	0.673		1.891		0.400		
PI4K α 2					0.470									0.460
PI4K β 2		2.061			0.731			3.563						0.663
PIPK1	0.704										0.687			
PIPK2										0.647		1.591		
PIPK4			1.525				2.776	0.638				2.770		1.882
PIPK5	3.771	2.468												
PIPK6		0.514			3.983		1.817			0.651	0.730			
PIPK7		5.307	0.500	0.717		0.734						1.996		0.325
PIPK8								0.532		1.611				
PIPK9							2.265							0.671
PIPK10	1.602							2.133	1.587		0.617			
PLA2-1														0.345
PLA2-2					0.744		1.642					0.592		
PLA2-4		1.747		0.689	1.935							0.534		
PLA2-5	0.058		0.243			0.586		0.265	0.029	0.318	0.065	2.044	0.574	16.846
PL1	0.333	0.107		0.680	0.199	0.656	0.424	1.636	1.655			0.220	0.565	
PL2		3.072	0.368		1.526		2.080					1.919		0.576
PL3	0.635		0.748					6.437	1.645				2.371	0.696
PL4		1.797		1.896				1.886			0.531		1.527	
PL5	2.124	2.251												
PL6				1.732	0.685			0.609	1.531					0.489
PL7	0.447		1.500		3.008		0.706	0.673				0.483	0.709	
PL8	0.368								0.680		0.524	0.665	0.430	

Names	1 Le/FI.	2 Le/St.	3 IAA	4 BR	5 KT	6 ABA	7 GA ₃	8 Dry	9 Man.	10 NaCl	11 Ca ²⁺	12 SA	13 MeJA	14 Cold
AtPLC1		2.684	1.713	2.154	1.942	1.562		2.623					1.628	0.714
PLC2		0.252		1.641	0.395		0.365	2.009				0.474		0.638
PLC3			1.601				2.253	2.556			0.661		2.184	
PLC4	2.217	0.391					0.510					0.410		
PLC5		2.588	0.004			0.003								
PLC6		3.370			2.038		0.526	0.656		1.514				
PLC7	0.476	2.246	1.647	0.630	2.056	1.503	1.552	0.499					0.748	
PLC8	1.913		1.940			1.774		0.524	2.781	2.657	2.980		1.989	
PLC9	2.195	0.734	1.501		0.631			0.500	1.976	2.021			1.712	
PLC10					0.578							1.724		
PLC11	2.198	0.563							1.610	1.555		0.467		
PLC12		2.038										2.712		
PLC13	3.090	2.678	0.665	1.556			0.749				0.728	0.669		0.622
PLC14	0.738	0.255			0.444		0.722	1.724	2.045		1.761	0.618		
PLC15		0.174	0.341	0.679	0.334		0.464					0.453		0.391
PLC16					0.559									0.621
PLC17	0.687						0.717	0.626				0.430	0.671	
PLD α	0.665		1.815				0.647						0.742	
PLD- α 2						0.625	2.620	0.616						
PLD- α 4								0.734		0.584	0.386			0.347
PLD- β 1			0.754	0.455		0.455						0.545		
PLD- β 2		4.043		0.584	3.299		1.671				0.628		0.632	
PLD- γ 1	0.402	2.317		2.636	0.664									1.761
PLD- γ 2	0.632	1.807		1.789			0.704							
PLD- γ 3		1.966	1.673	1.588										
PLD- δ		0.410	2.000		0.556		0.668			1.548		0.712		
PLD ξ -2	1.581	0.450			0.445		0.406	1.783				0.516		
IPPase1	0.231	2.183	0.644						0.384	0.625	0.612	0.732		
IPPase2	1.700	2.038	1.551	0.614		1.578		1.755	0.709		0.712			
IPPase3							4.175	1.687		1.653				
IPPase4	1.776		1.990			1.592		0.322	1.605					2.397
IPPase5		3.865		0.534	1.818					1.882				
IPPase6									0.466		0.545		0.583	
IPPase8		0.207	0.451		0.301		0.559					0.444		0.439
IPPase9	1.540				2.220					0.740	0.507		0.691	2.214
IPPase10					2.230					0.655				
IPPase11							2.268		2.038		0.609			0.562
IPPase12			0.739							1.686				

ment and environmental stresses except for the expression of PIS1 following SA treatment (Tab 3), which increased significantly.

Phosphorylation of PI at 4-position, by PI4K, generates PI4P involving in reorganization of cell skeletons, and so on. Analysis on *A. thaliana* genome identified four members of the family (two known, two putative) and studies via DNA chips indicated that expression of PI4K α 2 was relatively high in leaves though it did not change following hormone treatment or stress, while PI4K β 1 and PI4K β 2 were present in the normal tissues and up-regulated by auxin and down-regulated by cytokinin, respectively. Additionally, expressions of both were regulated following SA treatment and cold stress (Tab 3), in a manner similar to that seen in rice *OsPI4K1* [8], which was induced by SA, suggesting the involvement of PI4K in plant wounding responses.

The presence of more than 10 isoforms of PIPK in the *A. thaliana* genome suggested that it could be differentially expressed in different tissues, or during cellular responses to environmental factors such as, which has been demonstrated, NaCl, drought or ABA. As shown in Tab 3 besides the constitutive expression in various tissues, some PIPKs were differentially expressed, i.e. relatively highly expressed in leaves (PIPK5, 7), up-regulated by cytokinin (PIPK6), GA₃ (PIPK4, 9), mannitol (PIPK10) and SA treatment (PIPK4, 7), or down-regulated following auxin treatment or cold stress (PIPK7) (Tab 3).

Phospholipases, including PLC, PLD and PLA₂, are believed to play significant roles in multiple developmental stages and constitute the largest gene family of PI pathway identified in the *A. thaliana* genome. The identified phospholipases belonged to the PLC (17 members), PLD (12 members), PLA₂ (4 members), or putative phospholipase (8 members) families. Most PLCs were highly expressed in leaves (4-9, 11-13), but fewer were expressed in flowers (PLC 7) and stems (PLC2, 4, 14, 15). Most PLDs were constitutively expressed in all tested tissues, except for PLD β -2 (higher in leaves) and PLD ξ -2 (higher in leaves and stems) (Fig 3, Tab 3). Putative PL2 and 5 were highly expressed in leaves, while PL1 was strongly expressed in flowers (PL7 and 8) and stems (Fig 3, Tab 3).

With the focus on the expression profiling under hormone and stresses conditions, as revealed by DNA chips, PLCs were differentially expressed in response to auxin (PLC8 was up- and PLC5 and 15 were down-regulated),

► **Fig 2.** Cluster analysis of PI-involved genes in flowers (A) and stems (B), and following hormone treatment (C) or environmental stresses (D).

Fig. 3 Draft model of involvement of PI pathway-related genes in multiple plant developmental processes. Multi-gene family of PI pathway, as well as individual isoform, is multi-functionally (with distinguished alternative trend) involved in plant developmental processes and cell responses to environmental factors, making up of a complex network.

cytokinin (AtPLC1, PLC6 and 7 were up- and PLC2, 14 and 15 were down-regulated), ABA (AtPLC1 was up- and PLC5 was down-regulated) and GA₃ (PLC2-4 and 15 were down-regulated) (Fig 3), as well as cell response to drought (PLC1), mannitol (PLC8, 9 and 14), NaCl (PLC8 and 9), Ca²⁺ (PLC8), SA (PLC12 up, PLC2, 4, 11, 15 and 17 down), MeJA (PLC8) and cold treatment (PLC15 down). PLDs were altered in response to plant hormones including brassinosteroid (PLDγ-1), and GA₃ (PLDα-2). Only PLDα-4 was suppressed by cold treatment. PLA₂ has been reported to be regulated by auxin. In addition, PLA₂-1 and 4 were suppressed by cold and SA treatment, and PLA₂-5 was suppressed by drought, mannitol, NaCl and Ca²⁺, and up regulated by SA and cold. Several putative phospholipases were transcribed in leaves, flowers and stems, and were suppressed by cytokinin (PL1), GA₃ (PL1), SA (PL1 and 7), MeJA (PL8) and cold (PL6), or up-regulated by cytokinin (PL7) or SA (PL2).

A few members of the IPK, PI3K and IPR families were identified. These were constitutively transcribed in many of the normal and treated tissues, though some were regulated by auxin (IPK1, IPR1), brassinosteroid (IPR1), ABA (IPK1), SA (IPK4) or mannitol (PI3K1). Fifteen isoforms were classified as IPPases based on homologous analysis,

and 12 of these were analyzed by DNA chip technology. IPPase1, 2 and 5 were highly expressed, while expression of IPPase8 was lower in stems, and IPPase2 was lower in flowers. Further analysis revealed that some IPPases were up regulated by plant hormones including auxin (IPPase4), cytokinin (IPPase9, 10), GA₃ (IPPase3 and 11), while IPPase8 was down regulated by both auxin and cytokinin. IPPase1 and 6 were suppressed by mannitol treatment while IPPase11 was up regulated. IPPase4 was up regulated under cold treatment, and IPPase6 was suppressed by mannitol treatment (Fig 3, Tab 3).

DISCUSSION

The PI metabolic pathway plays significant roles in many developmental processes, as well as in cell responses to environmental factors. Analysis of the expression patterns of genes involved in the pathway, particularly the individual isoforms, will aid in focusing various functional studies. Accordingly, we employed DNA chip technology for high-throughput analysis of the pathway, particularly the individual isoforms. A detailed analysis of the *A. thaliana* genome revealed the presence of 82 PI-involved clones, including 10 or more members of the PIPK, phospholipase and IPPase families. To ensure reliability and to

minimize the inherent variability of microarray assays, several strategies were used. As replication is very important for array study [38], two double-dotted microarray slides (four replicates) were used to analyze the mRNA abundance of each sample pair in the studies. Also, the labeling of the sample in each pair was reversed on the second slide to overcome potential artifacts caused by dye-related differences in labeling efficiency, different laser settings, and nonlinearity of photomultiplier tubes in the scanner. In addition, in one experiment analyzing the expression profiles of drought-stressed seedlings, chips were labeled by probes reverse transcribed with different primers (commonly used oligo-dT vs. specific reverse PCR primers), which showed that the results were consistent across both methods. As illustrated in Tab 2, the correlation coefficient of the ratio from each replicate pair was ~0.9, confirming reproducibility among individual arrays in the same experiment. Therefore, signals from the replicates were reproducible and the conclusions derived from this analysis are reliable.

The presence of multiple members of PI pathway gene families within the genome suggests that there may be differential expression of individual isoforms in various tissues, during different developmental stages, or in response to environmental factors. Our results indeed showed differential expression of PI-involved genes in different tissues including stem, root and flowers (Fig 2A, B) and following treatment with various compounds or changes of temperature (Fig 2C, D). Differential expression patterns in stem, root and flowers suggest involvements of PI-related genes in the development of relevant tissues or cell types, which has been confirmed in root or root hairs (PIPK), filaments (PIPK and PLC), anthers (PLC and IPK), pollen grains and pollen tubes (IPK and IPPase).

As summarized in Fig 3, following hormone treatments and environmental stresses, both phospholipase and inositol polyphosphate phosphatase, both of which are capable of generating $\text{Ins}(1,4,5)\text{P}_3$ via hydrolysis or degrading $\text{Ins}(1,4,5)\text{P}_3$ by dephosphorylation, were altered in almost all experimental cases. It has been shown that $\text{Ins}(1,4,5)\text{P}_3$ is capable of stimulating Ca^{2+} release from internal stores, resulting in the regulation of downstream Ca^{2+} -dependent signaling pathways to involve the cell response to hormone, especially to stresses treatment including drought, NaCl and etc. However, involvement of different isoforms in various developmental processes indicates complicated regulating mechanisms. For example, IAA treatment, associated with plant growth, triggered up-regulation of members of the PLC and PIPK families. In other cases, PLC8 was up-regulated following IAA, ABA, mannitol, NaCl, Ca^{2+} and MeJA treatments, while PLC5 was suppressed by IAA and ABA treatments, and isoforms of PLA_2

were down-regulated in other situations, and different PLC isoforms were also altered. This was similar to the behavior of IPPase following KT and SA treatments, when IPPase8 was suppressed whereas IPPase9 and 10 were up-regulated, or the case of PIPK family members following treatment with GA_3 and SA, when PIPK4, PIPK7 and 9 were up-regulated (Fig 2). Our detailed studies on the individual isoforms of PIPK and IPPase confirmed that these genes are differentially expressed under various conditions, suggesting that they have specific functions in plant hormone and stress responses.

Overall, chip technology facilitates the studies of expression profiles and physiological roles of the pathway. Differential expressions in different tissues or under treatment of environmental factors provide informative clues on functional characterization of relevant isoform. As many members of the pathway are not only precursors but also messenger molecules themselves, knowledge of their expressions will certainly help us to study the relevant signaling pathways. We believe the detailed analysis of the expression patterns presented herein is a good basis for further expression study and functional analyses of the differentially expressed genes.

ACKNOWLEDGEMENTS

This study was supported by grants from the National Natural Science Foundation of China (No. 30100101) and the State Key Project of Basic Research (No. G19990-11604). We thank Dr. Yao Li for the preparation of the DNA chips and Ke Duan for help with data analysis.

Received, May 23, 2003

Revised, Oct 25, 2003

Accepted, Nov 23, 2003

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