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Cloning and characterization of Phospholipase C

Phospholipase C is a well-characterized enzyme and much is known about its regulation and function in mammalian cells (Rhee et al., 1997). In plants, only PLC8 isoforms have been reported (Singer et al., 1997). In order to better understand the function of PLC in plants, the cloning and characterization of PLC from pea was carried out.

4.1 Cloning of PsPLC from Pisum sativum

A cDNA library constructed in λ ZAPII (Stratagene) prepared from leaves of eight-day-old light/dark grown pea seedlings was used for isolation of the PsPLC clone. The probe used for screening was the PstI-XhoI fragment of soybean PLC (Acc. No. U25027). This fragment had the conserved X (partial), Y and C2 domains as well as the 3′ untranslated region (UTR). A total of $1.2 \times 10^4$ pfu. were screened for the isolation of the PsPLC clone and is described in Section 3.13.

In the primary screen, twenty four positive clones were identified. All clones were amplified by PCR using T3 and T7 primers and the PCR products subjected to Southern hybridization as described in Section 3.10. Two clones, 1 and 14, showing fragment sizes of 2-3 Kb and were chosen for further analysis. After secondary and tertiary screening of the selected clones, the plaques were purified to homogeneity (Fig. 1).

The pBluescript SK(+) phagemid containing the cDNA clone was excised from both λZAPII clones. The excision procedure is described in Section 3.15. Restriction analysis of the excised plasmids revealed that the two clones were different and that the approximate size of the cDNA clones was ~2.1 Kb. Upon digestion with BamHI, subcloning and sequencing, Clone 1 (PsPLC) was found to show homology with other reported phosphoinositide-specific Phospholipase C (PI-PLC) sequences and was labelled as PsPLC. This clone was fully sequenced. Clone 14, upon partial sequencing, showed homology with glutamyl–tRNA reductase and was not pursued further.
4.2 Structural organization of PsPLC and homology with other reported PI-PLCs

4.2.1 cDNA Features

Computer analysis of PsPLC cDNA revealed an open reading frame (ORF) of 1775 bp, a 5' untranslated region (UTR) of 109 bp and a 219 bp 3' UTR (Fig. 2). The 3' UTR included the polyadenylation signal (ttatatttg) and a 28 bp poly(A) signal. The ORF encodes a protein consisting of 594 amino acids with a calculated molecular mass of 67441.44 Da and an estimated pI of 5.81. As mentioned earlier, BLAST searches (Altschul et al, 1997) for homologous sequences showed significant homology with other PI-PLCs in the database. The sequence of PsPLC was deposited with EMBL and was assigned the Accession number Y15253.

4.2.2 Sequence alignment

PI-PLCs are ubiquitous and its cloning was first reported from mammalian systems (Rhee et al, 1989). In mammals, different isoforms of PI-PLCs are known and they show variation in structural organization with the δ-isofoms being the simplest as they have only the sequences common to all PI-PLC subfamilies (Irvine, 1996). On the other hand, PI-PLCs cloned from Dictyostelium, yeast, fungi, Chlamydomonas and higher plants are all clearly δ-isofoms. In plants, PLCs have been reported from soybean (Shi et al., 1995a), Arabidopsis (Hirayama et al., 1995), potato (Kopka et al., 1998b), Nicotiana (Pical et al, 1997), Brassica and Vigna. The deduced amino acid sequence of PsPLC was compared with other reported δ-isofoms across species (Fig. 4) using the MacVector v7.0 ClustalW program. PsPLC showed significant homology with soybean PLC that was used for library screening with an overall amino acid identity of 75% and a similarity of 65%. The identity and similarity shown with other sequences that it was aligned with is listed in Table 6.
Table 6: Comparison of PsPLC with other PLCδ reported across species

<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession Number</th>
<th>% Identity</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat PLC-δ</td>
<td>M20637</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>S63468</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td><em>G. max</em></td>
<td>U25027</td>
<td>75</td>
<td>65</td>
</tr>
<tr>
<td><em>A. thaliana</em> 2</td>
<td>D50804</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td><em>A. thaliana</em> 1</td>
<td>U13203</td>
<td>45</td>
<td>13</td>
</tr>
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<td><em>N. rustica</em></td>
<td>X95877</td>
<td>54</td>
<td>11</td>
</tr>
<tr>
<td><em>S. tuberosum</em></td>
<td>X94289</td>
<td>64</td>
<td>13</td>
</tr>
</tbody>
</table>

4.2.3 Dendrogram

As mentioned previously, isoforms of PI-PLCs are known exist in mammalian cells (Rhee et al., 1997). The relationship among the various isozymes is represented in a dendrogram that was prepared using the MacVector v7.0 ClustalW program (Fig. 5). Plant PLCs form a group that is distinct from other PLCs. The β and γ-isoforms also form distinct groups. Interestingly, yeast, *Dictyostelium* and *Botryotinia* (a fungus) PLCs that are all δ-isoforms, do not fall into the same group as plant PLCs.

4.2.4 Structural characteristics

The positions of the X, Y and C2 domains that are characteristically present in all PI-PLCs were also identified in PsPLC using the ProfileScan program at the ISREC server (Switzerland) (Fig 2). The X, Y and C2 domains consist of 145, 84 and 108 amino acids respectively. The spacer region between the X and Y domains consists of 105 amino acids and shows the presence of a 20-amino acid stretch (NNDDDDDEDLNEEEDSEEAE) that is highly negatively charged. The hydrophilicity and antigenicity plots for PsPLC (Fig. 3) were prepared using the MacVector v7.0 program with default parameters. The hydrophilicity plot shows the presence of two
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major hydrophilic segments. Segment I consists of a 40-amino acid stretch that is followed by another 36-amino acid stretch (segment II). Segment II contains the 20-amino acid negatively charged region. Both segments fall within the X/Y linker region. Segment II has two prominent antigenic regions that can be seen in the antigenicity plot. A search for putative post-translational modification sites using PROSITE (ISREC server, Switzerland) revealed the presence of six N-myristoylation sites, six sites for phosphorylation with protein kinase C (PKC) and 17 sites for phosphorylation with casein kinase II (CK2). As already mentioned, rat PLC-δ shares only 23% identity and 12% similarity with PsPLC. The crystal structure of rat PLC-δ is known and all residues that are essential for activity have been identified. A comparison of the PsPLC sequence with rat PLC-δ shows that despite the low degree of conservation across these two species, the amino acid residues required for catalysis are retained (Fig. 4).

4.3 Genomic organization of PsPLC

4.3.1 Gene Structure

The presence of introns in the coding region of the PsPLC gene was determined by amplification of pea genomic DNA and cDNA with the same set of primers and looking for a size difference in the fragments amplified (Fig. 7-A). The details of the conditions used for amplification are given in Section 3.30. The entire coding region for PsPLC could not be amplified from genomic DNA as a single fragment. Therefore, primers corresponding to different regions of the PsPLC cDNA were used to generate overlapping genomic clones which were then subcloned and sequenced (Fig. 7-B). The genomic clone (corresponding to the ORF of PsPLC cDNA) has a total length of 2971 bp, about 1.1 Kb longer than the PsPLC cDNA ORF. Full translation of the ORF in a single frame is only possible when all introns have been removed. The sequence was deposited in Genbank with the Accession Number AF280748 (Fig. 8). A comparison of the ORF of the PsPLC clone with the genomic clone reveals differences in the sequence at eight residues (F14→C, L91→F, A100→P, E148→Q, R149→G, Q390→K, Q535→E and L538→Q). The genomic clone shows the presence of eight introns. The size of the introns
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varies between 80 -289 bp while the size of the exons varies between 82 -320 bp (Fig. 9-A). The introns and exons along with their respective size are listed in Table 7.

Table 7: Intron/ Exon size of the PsPLC clone

<table>
<thead>
<tr>
<th>Intron number</th>
<th>Size (bp.)</th>
<th>Exon number</th>
<th>Size (bp.)</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>184</td>
<td>I</td>
<td>320</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>II</td>
<td>198</td>
</tr>
<tr>
<td>3</td>
<td>151</td>
<td>III</td>
<td>134</td>
</tr>
<tr>
<td>4</td>
<td>80</td>
<td>IV</td>
<td>244</td>
</tr>
<tr>
<td>5</td>
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</tr>
<tr>
<td>-</td>
<td>-</td>
<td>IX</td>
<td>293</td>
</tr>
</tbody>
</table>

The X domain is spread over exons II, III and IV while the Y domain is spread over the exons V, VI and VII. The C2 domain is also spread over three exons (VII, VIII and IX).

The genomic clone for an Arabidopsis PLC cDNA, AtPLC1S, (Acc. No. D38544) is reported (Hartweck et al., 1997). This clone is 2.5 Kb. in length and shows the presence of seven introns. Thus PsPLC has an additional intron in the Y domain. A comparison between the genomic clones of PsPLC and AtPLC suggests that there is a fair degree of conservation in the position of the introns relative to the cDNA sequences (Fig. 9-B).

4.3.2 Gene Family

Genomic DNA (30μg) was digested with restriction enzymes (NcoI, EcoRV and Xbal) whose recognition sites were not present in the PsPLC cDNA sequence (Fig. 6-B). Hybridization of digested genomic DNA with radio-labeled EcoRI fragment of PsPLC cDNA revealed multiple bands in all lanes suggesting that PsPLC was probably a member of a multigene family in pea. Southern hybridization of pea genomic DNA
digested with enzymes that cut the PsPLC cDNA sequence once/more than once with the same probe gave the following bands: XbaI (5.5 Kb., 2.3 Kb.), SacI (6.5 Kb. and 12.0 Kb.), BglII (4.3 Kb., 5.5 Kb., 8.0 Kb. and 11.0 Kb.), BamHI (1.5 Kb. and 0.90 Kb.) and HindIII (10.0 Kb., 3.5 Kb. and 0.70 Kb.). The restriction fragments detected in the Southern differ in size from the restriction fragments expected from the PsPLC genomic clone (Fig. 6-A). This again suggests that PsPLC is a member of a multigene family in pea.

4.4 Over-expression of full length PsPLC in E. coli

In order to study the function of PsPLC and to demonstrate that it indeed codes for a functional PI-PLC, the entire coding region for the same was over-expressed in E. coli. Three different expression vectors (pET14b, pET28a and pET32b) were used.

Fig. 10-A is a schematic representation of the PsPLC gene cloned in pET14b. The expression of PsPLC in the total cell extract was monitored by SDS-PAGE. Fig. 10-B shows a Commassie blue stained gel of total cell extracts from E. coli transformed with pET14b-PLC and pET14b plasmids after induction with IPTG for various time intervals. Maximum level of expression was found after 3 hours of induction. A comparison of the soluble fraction of cells transformed with pET14b-PLC before and after three hours of induction did not show any visible difference in the level of the PsPLC protein. About 98% of the PsPLC protein was in the inclusion body fraction and therefore solubilized in SDS-PAGE sample buffer and purified by elution of the PsPLC protein after extended electrophoresis of the sample on a 8.5%T preparative SDS-PAGE gel (Fig. 11-A and B). The preparative SDS-PAGE gel allows milligram quantities of protein to be loaded and separation is based on molecular weight as the gel is both denaturing and reducing. Commassie blue stained analytical gels in which the eluted fractions were loaded suggest the presence of a single PsPLC band in the eluted fractions. Details of the expression and purification of PsPLC is given in Sections 2.20 and 2.21. Western blot analysis (Fig. 12-A and B) with antibodies raised against the PsPLC protein show that the fractions containing the eluted PsPLC band is only 85% pure.
PsPLC was expressed in pET28a with a His tag at the N-terminus. The expression levels of PsPLC seen in pET28a was comparable to that seen in pET14b (Fig. 13-B). Again, there was no detectable difference between soluble fraction of cells transformed with pET28a-PLC before and after three hours of induction. Western blot analysis of cell extracts expressing PsPLC with anti-PsPLC antibodies suggested that there was a small amount of PsPLC protein in the soluble fraction (about 2% of total expression). Since the PsPLC protein had a His tag at its N-terminus, purification of the protein was attempted using Ni-NTA agarose. A partially purified band (~68 KDa) was detected on a Commassie stained gel that was recognized by anti-PsPLC antibodies (Fig 13-C and D).

PsPLC was also expressed in pET32b as a fusion protein with thioredoxin. This construct also had a His tag at the N-terminus, between the thioredoxin tag and PsPLC, to facilitate purification using Ni-NTA agarose (Fig. 14-A). The expression levels of the fusion product in the total cell extracts were substantially higher (about 4-fold higher) than those seen for the pET14b-PLC and pET28a-PLC constructs (Fig. 14-B). The amount of fusion product detected in the soluble fraction was also examined at various time points after induction (Fig. 15-A). It was seen that the induced band was detectable in the soluble extract half an hour after induction with the levels increasing till three hours after induction. The difference in the amount of protein in the uninduced and induced soluble extracts was only visible upon allowing extended electrophoresis of the gel for 40 minutes after the bromophenol dye had run out of the gel. The amount of fusion product that was detected in the soluble fraction was also substantially higher than that seen in the other two cases (~10% of total expression). Purification of Thioredoxin-PsPLC from the soluble fraction was attempted using Ni-NTA agarose. A partially purified band was obtained (Fig. 15-B). Again, since a substantial portion of the fusion product was seen to occur in the insoluble fraction (~90% of total expression), purification of the protein from the inclusion body fraction was carried out. Solubilized inclusion body fractions were loaded onto Ni-NTA agarose. The partially purified eluted fractions were cleaved with thrombin to separate the fusion product (Fig 14-B).
4.5 Over-expression of the C2 domain of PsPLC

The C2 domain of PsPLC was expressed in pET28a with a His tag at the N-terminus. The C2 domain was also expressed in pET32b as a fusion protein with Thioredoxin (Fig. 16). Details of the primers used and the sites used for cloning are given in Section 3.24. Expression levels of the C2 domain (total cell extract) seen in pET28a was comparable to that seen in pET32b. (Fig. 16-A and B). There was no detectable difference between soluble fraction of cells transformed with pET28a-PLC before and after three hours of induction. In contrast, 30% of the total induced protein was found to occur in the soluble fraction after three hours of induction for the C2 domain cloned in pET32b (Fig. 17-A and B). Since the Thioredoxin-C2 domain had a His tag at its N-terminus, purification of the protein was attempted using Ni-NTA agarose. A partially purified band (~33 KDa.) was detected on a Commassie stained gel. Upon cleavage with thrombin, the band corresponding to the C2 domain was indistinguishable from low molecular weight contaminants present in the purified fractions. Therefore, purification of the C2 domain from the inclusion body fraction of the induced sample (pET28a) was carried out (Fig. 18-A and B). The procedure followed for purification is given in Section 3.25. The C2 domain was found in the wash fraction and was also present in the eluted fraction. The protein was refolded following the protocol given in Section 3.26.

4.6 Functional characterization of full length PsPLC and the C2 Domain

4.6.1 Phospholipase activity of PsPLC

Characterization of the phospholipase C activity is currently underway.

4.6.2 Calcium binding activity of full length PsPLC and the C2 domain

PsPLC purified by preparative SDS-PAGE was checked for calcium binding activity using calcium shift and calcium overlay assays. Both are described in Sections 3.27 and 3.28. For the calcium shift assay, the purified PsPLC protein (in the SDS-PAGE running
buffer) was incubated with 0.5 and 1.0 mM CaCl₂ (Fig. 19). Samples incubated with EGTA served as control. The samples incubated with CaCl₂ showed a slight downward shift as compared to samples incubated with EGTA. This pattern was seen to repeat with two concentrations of PsPLC protein tested. In the calcium overlay assay, PsPLC protein electro-blotted onto nitrocellulose was incubated with ⁴⁵CaCl₂. The lane containing PsPLC protein was found to bind radio-labeled calcium. Entamoeba histolytica calcium binding protein (EhCaBP) that was loaded onto the gel approximately 20 minutes after the other samples were loaded served as positive control. BSA was used as negative control (Fig. 20).

C2 domains are known to have a calcium-mediated lipid-binding function (Essen et al., 1996). For the calcium shift assay, the refolded C2 domain of PsPLC was incubated with 2.0 and 5.0mM CaCl₂ (Fig. 21). Samples incubated with EGTA served as control. The samples incubated with CaCl₂ showed a slight upward shift as compared to samples incubated with EGTA. This pattern was seen to repeat with two concentrations of the C2 domain protein tested. In the calcium overlay assay, total cell extracts (uninduced and induced) along with purified protein was electro-blotted onto PVDF and was incubated with ⁴⁵CaCl₂. The lane containing C2 domain protein (total induced extract) was found to bind radio-labeled calcium. Weaker signals were detectable in lanes containing purified the C2 domain protein. (Fig. 22-A and B) Calnexin, a calcium binding protein served as positive control. BSA was used as negative control.

4.7 Isolation of the PsPLC promoter

Pea genomic DNA was digested with several enzymes (NheI, XbaI, SpeI, BamHI, BglII, BclI and Sau3AI), end-filled and ligated with primer-adapters (Fig. 23; details are given in Section 3.31). The adapter-ligated DNA was amplified with T7 and PLCPR primers as mentioned in Section 3.31. The PCR amplified DNA was transferred to nylon membrane and probed with the EcoRI fragment of PsPLC cDNA. A band of approximate size 1.1 Kb was detected in the XbaI digested DNA (Fig. 24-I and II). In the NheI and SpeI digested DNA bands of ~0.25 Kb. and 0.39 Kb were detected. A band of approximately 0.35 Kb was seen in the Sau3AI digested DNA. The genomic clone for PsPLC has only one site for XbaI and is expected to give two fragments of 2.3 Kb and 0.4 Kb size upon
digestion. Since the size of the band detected in the XbaI digested fragments did not correspond with the expected size of genomic fragments, it was presumed that the 1.1 Kb band detected in the Southern blot contained the promoter sequence for PsPLC. The XbaI digested DNA was thus taken for further analysis. In the second round of amplification, T7 and PLCPR01 primers were used. The PLCPR01 primer was 120 bp upstream of the PLCPR primer and was expected to give a fragment of 0.92 Kb upon second amplification (Fig. 24-III). The amplified band obtained was cloned in pGEM-T, subcloned and sequenced.

The amplified fragment was 916 bp in length and showed the presence of a 124 bp overlap with the PsPLC cDNA sequence at its 5' end. The PsPLC promoter sequence (minus the segment overlapping with the PsPLC cDNA) was deposited in Genbank with the Accession number AF354752. The putative cis-acting elements present in the PsPLC promoter were identified using PLACE (Higo et al., 1999; http://www.dna.affrc.go.jp; Fig. 25). The cis-acting elements seen in the PsPLC promoter are listed in Fig. 26. The cis elements identified include the CAAT Box (-336 to -333, -322 to -319 and -301 to -298), CCA1 Box (-529 to -522), Dof-binding sites (-535 to -532, -492 to -489 and -360 to -356), GATA Box (-652 to -649, -599 to -596 and -400 to -397), GT-1 Box (-631 to -625, -532 to -536 and -496 to -490) LTREs (-73 to -67, -116 to -112 and -72 to -68) and pollen and root-specific motifs (-545 to -541, -292 to -288, -576 to -572 and -497 to -491).

Primer extension analysis to identify the start site of transcription is described in section 3.32. Primer extension revealed the presence of two transcription start sites (Fig. 27). The major transcript was found to initiate from “C”, 198 nucleotides upstream of the translation initiation site (ATG) while the minor transcript was found to start from “A”, 190 nucleotides upstream of ATG. The minor transcription start site corresponds with the first nucleotide of the PsPLC cDNA.

4.8 Regulation of PsPLC gene expression

PLCs, being components of the signal transduction cascade are expected to be induced in response to various stimuli such as abiotic and biotic stress, light and hormones (Hirayama et al., 1995; Ettlinger and Lehle, 1988; Blatt et al., 1990; Young et al., 1997;
Coursol et al., 2000; DeWald et al., 2001) Upregulation of PI-PLC in response to dehydration, salt and ABA has been reported in *Arabidopsis* (Hirayama et al., 1995) and wounding in potato (Kopka et al., 1998b). We have examined the effect of light on PsPLC expression in various tissues in pea seedlings on different days of growth. Total RNA was isolated from leaves, roots and stem of 5-10-day old pea seedlings grown either in total darkness or in dark/light conditions and probed with radio-labeled PsPLC under high stringency conditions. A transcript of ~2.1 Kb. was detected which corresponds to size of PsPLC.

**Fig. 28** shows the effect of light treatment on PsPLC expression in leaves of pea. The level of PsPLC transcript was consistently higher in dark grown tissue as compared to leaves taken from dark/light grown pea seedlings on all days examined. This was difference 1.7-fold on day 8 and 1.1-fold on Day 7. On day 10, the PsPLC transcript was 1.1 fold lesser in dark grown tissues. **Fig. 29** shows the effect of light treatment on PsPLC expression in stem segments of pea. PsPLC transcript was consistently lower on any given day in dark grown stem tissue as compared to dark/ light grown tissue. PsPLC transcript was 2.3-fold higher in stem segments of dark/ light grown tissues on day 10 and this difference was only 1.42-fold on day 5 In root tissues, expression in dark/ light grown samples was consistently higher on any given day as compared to dark grown tissue (**Fig. 30**). This difference was maximum on day 8 (2.1-fold) and minimum on day 10.

The kinetics of PsPLC expression under short term light exposure was also studied (**Fig. 33**). Etiolated pea seedlings on the fifth day of growth were transferred to light for 24 hours and total RNA isolated from leaves and roots at defined time intervals. The expression of PsPLC in roots was higher or equal (12 hours) to that seen in leaf tissue. There was a 1.28-fold increase in PsPLC transcript in roots at 2 hours of light exposure which dropped to levels equal to the dark control (0 hours) at 8 hours and then remained steady till 24 hours. This pattern was seen to repeat for the leaf tissue. The abnormally high value obtained for leaf tissue after 4 hours of light exposure is attributable to non-specific binding in the lane and has therefore not been taken into consideration. To check if light mediated changes in root tissue were due to direct effect of light on roots total RNA was isolated from leaves and roots of 10-day old pea
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Seedlings grown either in dark or dark/light conditions in pots covered with/without aluminum foil. Northern blot analysis shows that the PsPLC transcript level was marginally higher in dark grown leaves as compared to dark/light grown leaves (Fig. 34). This pattern was reversed in leaves of pea seedlings grown in pots covered with aluminum foil. PsPLC expression levels in dark grown roots was slightly higher than that in dark/light grown roots. The elevated levels of PsPLC expression seen in dark grown roots (minus aluminum foil) seems to be due to a three fold increase in RNA loading for that sample. For roots covered with aluminum foil, the increase in PsPLC transcript was marginal in dark grown tissue as compared to dark/light grown roots.

PsPLC expression in different stages of flower development was examined. The flowering stages examined are listed in Fig. 32. Flowering stages F2, F3 and F4 are comparable with respect to total RNA in the lanes (Fig. 31). PsPLC expression in these samples was found to increase from stage F2 to F3. The expression at stage F4 was almost the same as that at stage F3. The elevated levels of the PsPLC transcript seen in stages F1 and F5 in part due to increased loading of RNA. In F6, RNA loading was low. Therefore the decreased PsPLC expression observed is due to differences in loading. In summary, there is not much variation in the PsPLC transcript levels across the flowering stages examined except for slightly elevated levels at stage F1 and F5.

The effect of salt stress on PsPLC expression in roots on the fifth and tenth day of growth was also examined (Figs. 35 and 36). The data was normalized relative to control (water). For the fifth day, with 50 mM NaCl, PsPLC was found to increase till 24 hours and then fall after 48 hours whereas with 100 mM NaCl there was a steady increase in the PsPLC transcript till 48 hours. For 200 mM NaCl treatment, the PsPLC transcript dropped drastically at 12 hours and again after 24 hours. At 300 mM NaCl, PsPLC transcript peaked at 24 hours and dropped at 48 hours. For 10-day old pea seedlings, all concentrations of salt tested showed a steady increase in the PsPLC transcript. For all the samples, the 48 hour sample appear to show a drastic increase in transcript level for PsPLC. This is attributable to under-loading in the water control at 48 hours.