The *Vr-PLC3* gene encodes a putative plasma membrane-localized phosphoinositide-specific phospholipase C whose expression is induced by abiotic stress in mung bean (*Vigna radiata* L.)[1]

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Abstract **Phosphoinositide-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-trisphosphate and diacylglycerol, both of which act as secondary messengers in animal cells. In this report, we identified in *Vigna radiata* L. (mung bean) three distinct partial cDNAs (pVr-PLC1, pVr-PLC2, and pVr-PLC3), which encode forms of putative PI-PLC. All three *Vr-PLC* genes were transcriptionally active and displayed unique patterns of expression. The *Vr-PLC1* and *Vr-PLC2* transcripts were constitutively expressed to varying degrees in every tissue of mung bean plants examined. In contrast, the *Vr-PLC3* mRNA level was very low under normal growth conditions and was rapidly induced in an abscisic acid-independent manner under environmental stress conditions (drought and high salinity). An isolated genomic clone, about 8.2 kb in length, showed that *Vr-PLC1* and *Vr-PLC3* are in tandem array in the mung bean genome. The predicted primary sequence of Vr-PLC3 (M_r = 67.4 kDa) is reminiscent of the δ-isoform of animal enzymes which contain core sequences found in typical PI-PLCs, such as the catalytic domain comprising X and Y motifs, a lipid-binding C2 domain, and the less conserved EF-hand domain. Results of in vivo targeting experiment using a green fluorescent protein (GFP) showed that the GFP-Vr-PLC3 fusion protein was localized primarily to the plasma membrane of the *Arabidopsis* protoplast. The C2 domain was essential for Vr-PLC3 to be targeted to the plasma membrane. The possible biological functions of stress-responsive Vr-PLC3 in mung bean plants are discussed.**

Key words: Abiotic stress; Calcium; Differential gene expression; Inositol 1,4,5-trisphosphate; Phospholipase C; Plasma membrane; *Vigna radiata*

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[1] EMBL accession numbers: AY394079 (*Vr-PLC1*), AY461431 (*Vr-PLC2*) and AY394078 (*Vr-PLC3*).

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PCR, polymerase chain reaction; PI, phosphoinositide; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; PLC, phospholipase C; PI-PLC, phosphoinositide-specific phospholipase C; Vr-PLC, *Vigna radiata* phospholipase C

1. Introduction

Abiotic environmental stresses, such as drought, high salinity, extreme temperature, and heavy metals, greatly impair the growth and development of plants grown in soil. Drought and high salinity are responsible for reduction of crop yield on as much as half of the world's irrigated land [1]. A number of genetic and cellular events that occur under such stresses have been widely documented [2,3]. Although a large and increasing number of genes induced by drought, salt stress, or both have been recently identified with the aid of combined molecular and genetic approaches, the physiological roles of these genes in relation to either stress tolerance or sensitivity are largely unknown in higher plants [4–7]. Thus, it is critical to study the functions of stress-inducible genes to understand the molecular mechanisms of stress tolerance of crop plants.

Several lines of evidence implicate cytosolic calcium in signal transduction during drought and salt stress in higher plants [8,9]. In addition, a close association between phosphoinositide (PI) metabolism and cytosolic calcium level has been reported [3,10,11]. Inositol 1,4,5-trisphosphate (IP_3) is generated from hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). IP_3 diffuses rapidly to other parts of the cell to mobilize calcium from the intracellular stores to the cytosol, which results in the regulation of a number of cellular processes in response to drought and salt stress. For example, Knight et al. [12] reported that application of mannitol (simulating drought) to *Arabidopsis* seedlings caused not only influx of extracellular calcium, but also calcium mobilization from vacuoles into the cytosol through IP_3-dependent calcium channels. The IP_3-induced changes in cytosolic calcium level may lead to activation of several drought-induced genes [12]. Hyperosmotic stress rapidly increased IP_3 level in various cell culture systems, including *Galdieria sulphuraria* [13], carrot [14], and *Arabidopsis* [15]. In intact *Arabidopsis* plants, production of PIP_2 and IP_3 and intracellular concentration of calcium rapidly increased in response to salt and osmotic stress [16]. From *Arabidopsis*, Mikami et al. [17] isolated a cDNA encoding phosphatidylinositol 4-phosphate 5-kinase (PIP5K), the enzyme that phosphorylates phosphatidylinositol 4-phosphate to produce PIP_2, the precursor of IP_3 and diacylglycerol (DAG). The PIP5K mRNA was rapidly induced in

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response to drought, salt, and abscisic acid (ABA) [17]. More recently, loss-of-function mutation in the *FRY1* gene was identified in *Arabidopsis* [18]. *FRY1* encodes an inositol polyphosphate 1-phosphatase that is involved in the catabolism of IP_3. The *fry1* mutation conferred super-induction of ABA- and stress-responsive gene expression; this phenotype showed enhanced accumulation of IP_3. All of these results strongly support an association between PI signaling cascades and stress responses in higher plants.

Phosphoinositide-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of PIP_2 to generate IP_3 and DAG (see [19] for a review). The *Arabidopsis* PI-PLC is encoded by a gene family; *AtPLC1* is highly inducible by environmental stimuli including dehydration, salinity, and cold stress, and the *AtPLC2* gene is constitutively expressed [20–22]. In potato leaves, three *StPLC* genes are differentially expressed after wounding or during drought stress [23]. Recently, the function of PLC in vivo was investigated in transgenic *Arabidopsis* plants; transgenic lines expressing the *AtPLC1* antisense gene accumulated lower IP_3 concentrations after being treated with ABA and showed decreased induction of ABA- and stress-responsive genes compared with such induction in control plants [24]. These results all indicate the importance of PLC activity in producing IP_3 and in triggering the expression of stress-related genes.

Because PI-PLC has been shown to be encoded by a gene family, we are interested in elucidating the tissue- and gene-specific expression profiles of individual PLC isogenes in early seedling development. In this report, we examined the differential expression patterns of three isogenes (*Vr-PLC1*, *Vr-PLC2*, and *Vr-PLC3*) which encode putative PLC homologues in different tissues of mung bean (*Vigna radiata* L.) plants in response to drought, high salinity, ABA treatment, and bacterial infection. Our results showed that *Vr-PLC3* is specifically activated by drought and salt stress in an ABA-independent manner, with its induction being faster in roots than in leaf tissue. Results of in vivo targeting experiment suggest that the Vr-PLC3 protein is localized predominantly to the plasma membrane.

2. Materials and methods

2.1. Plant materials and RNA isolation

Dry seeds of mung bean (*V. radiata* L.) were soaked overnight in aerated tap water. Seedlings were grown on 0.8% agar for 4 days in a dark room at 25°C or for 4–8 weeks in an environmentally controlled chamber. Total RNAs of mung bean plants were obtained by a method as described previously [25]. The total RNAs were precipitated overnight at 4°C by the addition of 0.3 volume 10 M LiCl and then precipitated in ethanol.

2.2. Polymerase chain reaction (PCR)

First-strand cDNA, synthesized from 1 μg of poly(A)$^+$ RNA isolated from etiolated mung bean hypocotyls, was amplified by PCR. The mixed oligonucleotide primers used were derived from conserved amino acid sequences of putative PI-PLCs from *Arabidopsis* and potato plants; upstream primers GG[A/T]GC[A/T/G/C]CA[A/G]ATG-[G/A]T[A/T/G/C]GC and AA[T/C]GG[A/T/G/C]GG[A/T/G/C]TG[T/C]GG[A/T/G/C]TA were derived from Y domain sequences GAQM-(V/I)A and NGGCGY, respectively; and downstream primers TT[A/T]GGIAC[A/T/G/C]CC[A/T/G/C][A/G]C[A/C/T]AT and GGIA[A/C/T][A/G]CA[A/T/G/C]GT[T/C]TG[A/T/G/C]CC were derived from C2 domain sequences I(A/V)GVPD and GQTC(L/I/F)P, respectively [20,23]. The amplified PCR products were subcloned into pGEM-T Easy vector system I (Promega, Madison, WI, USA). PCR was performed in a total volume of 25 μl as described previously [25].

2.3. Isolation of genomic DNA and Southern blot analysis

The mung bean leaf genomic DNA was isolated as described previously [26] with modifications. Each gram of mung bean leaf was pulverized under liquid nitrogen and suspended in 3 ml extraction buffer (8.0 M urea, 50 mM Tris–HCl pH 7.5, 20 mM EDTA, 250 mM NaCl, 2% w/v sarcosyl, 5% v/v phenol and 20 mM 2-mercaptoethanol). After successive extractions with phenol/chloroform/isoamyl alcohol (25:24:1, v/v), the aqueous phase was concentrated by ethanol precipitation. The pellet was resuspended in 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA adjusted to a density of 1.5 g/ml by the addition of CsCl, and the DNA was centrifuged overnight at $200\,000 \times g$. The DNA band was collected, extracted with water-saturated 1-butanol, precipitated by ethanol, and then resuspended in 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA. Mung bean genomic DNA (10 μg per lane) was digested with appropriate enzymes, separated by electrophoresis in a 0.7% agarose gel, and blotted onto a nylon membrane filter (Amersham, Arlington Heights, IL, USA). The filter was hybridized to ^{32}P-labeled gene-specific pVr-PLC1, pVr-PLC2, and pVr-PLC3 under high stringent conditions.

2.4. Application of various stresses and RNA gel blot analysis

To simulate water or salt stress conditions, 4-week-old mung bean plants were soaked in solution containing 250 mM mannitol or 300 mM NaCl, respectively, for various time periods. To simulate drought, 2-week-old intact plants were harvested from agar plates and dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light. The degree of drought was determined by the percentage decrease in the fresh weight (10–40%) of the plants. To test the ABA dependence of the response pathways, the mature leaves of 4-week-old plants were sprayed with 100 μM ABA, or inoculated by vacuum-infiltrating bacterial suspensions (10^8 cfu/ml) into the abaxial side of fully expanded leaves. To test the response to pathogens, two species of bacteria, *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tabaci*, were used as the non-host and avirulent pathogens, respectively, to the mung bean plants. Total RNA (20 μg) isolated from the treated tissues was separated by electrophoresis on a 1% formaldehyde-agarose gel and blotted to Hybond-N nylon membranes (Amersham). To ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. To confirm complete transfer of RNA to the membrane filter, both gel and membrane were viewed under UV light at the end of the transfer. The filter was hybridized to ^{32}P-labeled cDNA probes. The blots were washed as described previously [25] and visualized by autoradiography at −70°C using Kodak XAR-5 film and an intensifying screen.

2.5. Constructing and screening the partial genomic library

A partial genomic library was constructed as described previously [26], with slight modification. Mung bean leaf genomic DNA was fully digested with *Eco*RI and size-fractionated on a 10–40% sucrose gradient. Restriction fragments of about 6–10 kb in length were ligated into *Eco*RI-digested λEMBL4 (Stratagene, La Jolla, CA, USA) arms and packaged in vitro using Gigapack III (Stratagene) extracts according to the manufacturer's protocol. The resulting library comprised 2.0×10^5 individual recombinant plaques. The genomic library was screened using the ^{32}P-labeled pVr-PLC3 as a probe by an established procedure [27].

2.6. Subcellular localization of GFP-Vr-PLC3 protein

The *Vr-PLC* cDNAs corresponding to the full-length coding region (amino acid residues 1–591) or to a truncated mutant which lacked the C2 domain (amino acid residues 1–462) were amplified by PCR using Pfu DNA polymerase to generate *Sac*I sites at both the 5′ and 3′ ends. These amplified cDNAs were cloned into the soluble-modified, green fluorescent protein (smGFP) plasmid to generate GFP-Vr-PLC3, which contained GFP-Vr-PLC3$^{1-591}$ and GFP-Vr-PLC3$^{1-462}$ in-frame fusions under the control of the cauliflower mosaic virus 35S promoter. The GFP-Vr-PLC3 and control smGFP plasmids were introduced into protoplasts prepared from *Arabidopsis* seedlings by polyethylene glycol treatment as described in [28]. After 16 h of incubation, the protoplasts were viewed under a fluorescence microscope. For co-localization experiments, *Arabidopsis* P-type H$^+$-ATPase AHA2 was fused to red fluorescent protein (RFP) and used as a marker protein for the plasma membrane.

3. Results

3.1. Isolation and classification of partial cDNA clones for the putative PI-PLCs in mung bean plants

In animals and yeast, four distinct subfamilies (β, γ, δ, and ε) of PI-PLCs have been identified [29]. These PI-PLC isozymes possess five conserved domains which represent the core sequences of PI-PLCs in animals and yeast: a PH domain, an EF-hand domain, an X and a Y domain (both of which form catalytic domains), and a lipid-binding C2 domain. On the other hand, the structure of all forms of plant PI-PLC is reminiscent of the δ isoform but does not contain the PH domain [19]. Sequence analyses of putative *Arabidopsis* PI-PLCs revealed that three domains, the X, Y, and C2 domains, are significantly conserved [19]. To gain more insight into the differential regulation of the PI-PLC isogenes in response to diverse stresses, we first proceeded to isolate cDNAs encoding putative PI-PLC homologues expressed in mung bean plants. Poly(A)$^+$ RNA was isolated from 4-day-old etiolated mung bean seedlings containing hypocotyls and roots. Following the synthesis of the first-strand cDNA from 1 μg of poly(A)$^+$ RNA, PCR was carried out with mixed oligonucleotides corresponding to the amino acid sequences of GAQM(V/I)A and NGGCGY for the upstream primers, I(A/V)GVPD and GQTC(L/I/F)P for the downstream primers (see Section 2 for sequences), and the first-strand cDNA as the template. These primer amino acid sequences are derived from the Y domain (the upstream primers) and the C2 domain (the downstream primers), respectively, that are conserved in putative PI-PLCs from *Arabidopsis* and potato [20,23]. We obtained PCR products of about 300–500 bp in length. Subsequent subcloning, restriction enzyme mapping, and DNA sequence analyses revealed that these partial cDNA clones (pVr-PLC1, pVr-PLC2, and pVr-PLC3) belonged to three different homology classes and encode amino acid residues between the Y and C2 domains of mung bean PI-PLCs (Fig. 1A). The pVr-PLC1 clone is 318 bp long and encodes 106 amino acids, pVr-PLC2 is 384 bp long and encodes 128 amino acids, and pVr-PLC3 is 459 bp in length and encodes 153 amino acids. The deduced amino acid sequence identity between *Vr-PLC1* and *Vr-PLC2* is 65%, between *Vr-PLC1* and *Vr-PLC3* is 63%, and between *Vr-PLC2* and *Vr-PLC3* is 82%. This result suggests that *Vr-PLC2* and *Vr-PLC3* are the more homologous members of this gene family, while the *Vr-PLC1* gene is more divergent. In addition, *Vr-PLC* genes exhibit a considerable degree of sequence identity when compared with putative *PI-PLC* genes from *Arabidopsis*, potato, and soybean plants (see below).

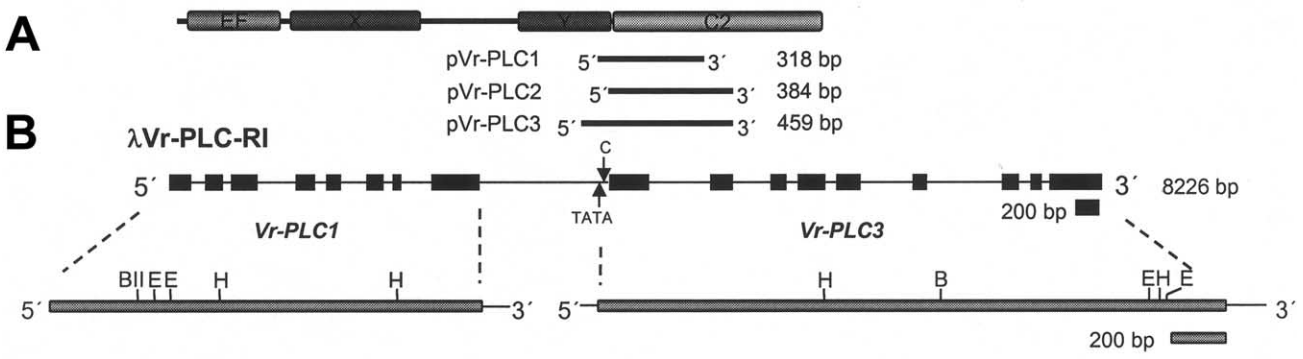
3.2. Genomic DNA and RNA gel blot analyses of Vr-PLCs

To confirm that the partial pVr-PLC clones were not cloning artifacts generated during the course of PCR and to ascertain their genomic organization, DNA gel blot analyses were conducted with mung bean genomic DNA digested with *Eco*RI, *Hin*dIII, or *Xba*I. The probes were the ^{32}P-labeled PCR fragments which corresponded to each homology class. With each probe, all three genes had different restriction patterns, and only one DNA hybridization band was detected for each restriction enzyme reaction (Fig. 2A). No additional fragments were visible in any of the gel blots after longer exposure of the blots to X-ray film. The mung bean plant is diploid, therefore these results indicate that the mung bean genome contains a single copy of the *Vr-PLC1*, *Vr-PLC2*, and *Vr-PLC3* genes.

To investigate the spatial and temporal expression profile of *Vr-PLC* gene members, we used RNA gel blot analysis to monitor the level of their corresponding mRNAs in different mung bean vegetative and reproductive tissues. Total RNAs isolated from apical hooks, hypocotyls, and roots of dark-grown 4-day-old seedlings, or from leaves, stems, and flowers of light-grown 4- or 8-week-old plants, were hybridized with ^{32}P-labeled probes. As shown in Fig. 2B, substantial steady-state levels of *Vr-PLC1* and *Vr-PLC2* transcripts (each about 2.2 kb long) were detected in various tissues of both dark- and light-grown plants (Fig. 2B). However, relative expression profiles of the two different mRNAs varied significantly; *Vr-PLC2* transcripts were predominant. The *Vr-PLC2* mRNA was most highly expressed in hypocotyls of etiolated plants and stems of light-grown plants (Fig. 2B). In contrast, the level of *Vr-PLC3* transcript was very low in every tissue examined, and background level was hardly detectable in leaves, apical hooks, and flowers (Fig. 2B). These combined results strongly suggest that the homologous *Vr-PLC1*, *Vr-PLC2*, and *Vr-PLC3* genes we isolated from the mung bean plant are not cloning artifacts, but are three different *Vr-PLC* genes which are differentially regulated in different parts of the plant.

3.3. Vr-PLC3 mRNA accumulation is strongly stimulated by drought or salt stress in an ABA-independent manner

Among the seven putative *Arabidopsis* PI-PLC gene family members, *AtPLC1* is rapidly induced by drought (within 1 h), high salinity (within 2 h), and cold stress (within 10 h), whereas the *AtPLC2* gene is constitutively expressed [19–22]. The expression of the other five genes has not been published. At least three *StPLC* genes exist in potato plants, and they have different patterns of expression [23]. In response to wounding and wilting, *StPLC1* exhibited strong reduction of its mRNA level, whereas the *StPLC2* transcript was markedly induced by those stresses. Both treatments resulted in only minor alterations in expression of *StPLC3*, indicating that *StPLC3* is a constitutive gene. Thus, we considered the possibility that the expression of the *Vr-PLC* gene family is also differentially regulated by different growth and environmental conditions. To investigate this possibility, we analyzed accumulation of transcripts of the three *Vr-PLC* isoforms in various tissues in response to diverse biotic and abiotic stresses. As a first step, 4-week-old light-grown intact mung bean plants were subjected to 250 mM mannitol (drought treatment) or 300 mM NaCl (salt treatment). Total RNAs were then prepared at different time points of incubation from leaf and root tissues, and the expression profile of individual *Vr-PLC* genes was determined. Fig. 3A,B shows that the levels of mRNAs for *Vr-PLC1* and *Vr-PLC2* remain unchanged in leaves and roots during the entire 24-h incubation period, with the level of *Vr-PLC2* transcript being more abundant. In contrast, induction of the *Vr-PLC3* transcript was clearly detected with different induction kinetics in two different tissues in response to mannitol or NaCl treatment (Fig. 3A). In leaves, *Vr-PLC3* mRNA began to accumulate by the fourth hour of stress treatment, and this induction was maintained for at least 24 h after treatment. In roots, the *Vr-PLC3* mRNA level rapidly increased in response to water or salt stress, attained a maximum level at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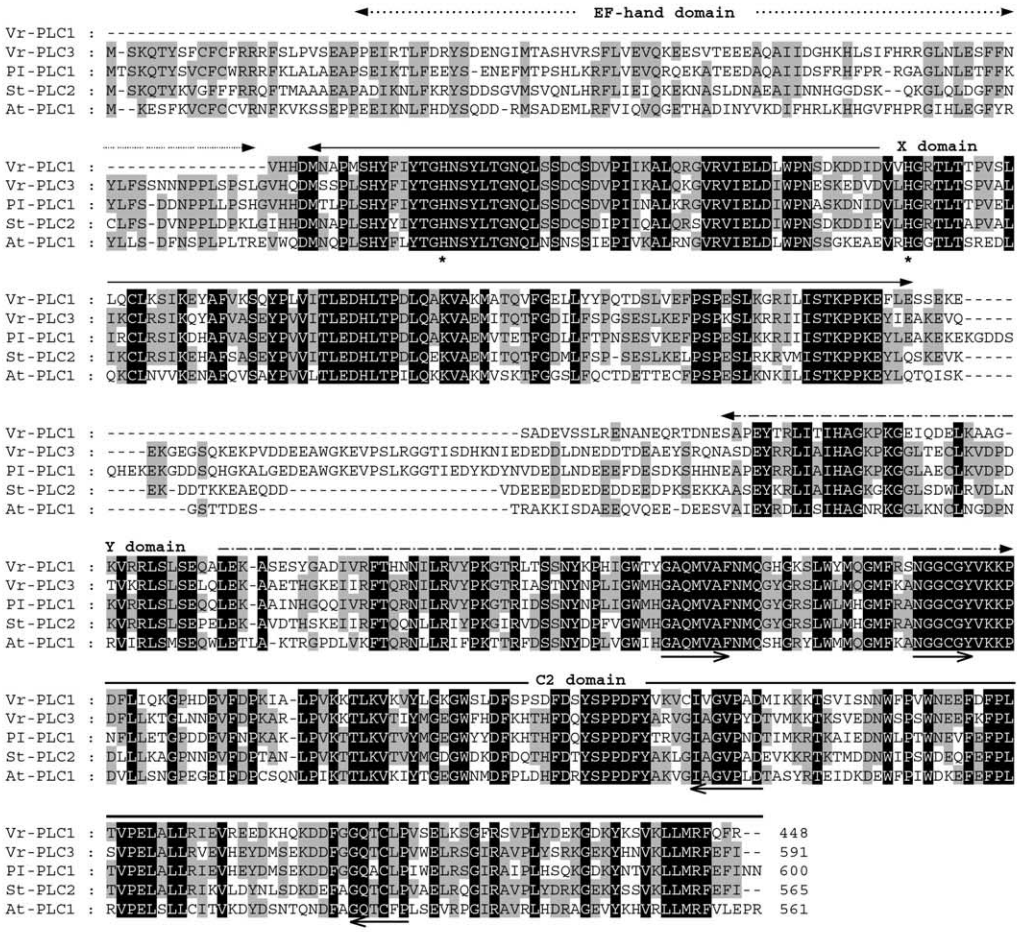
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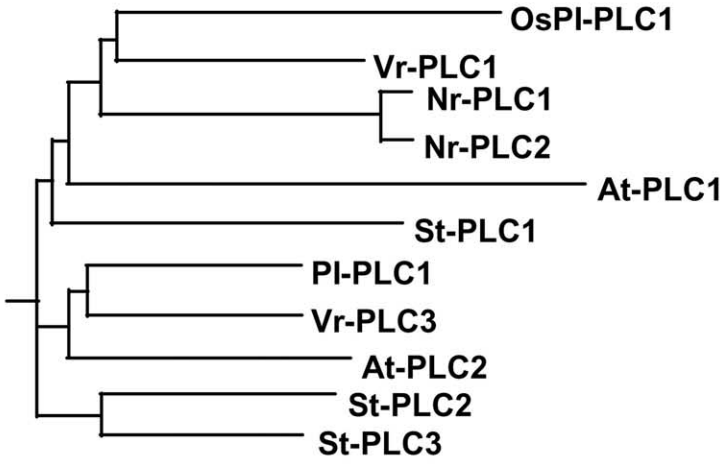
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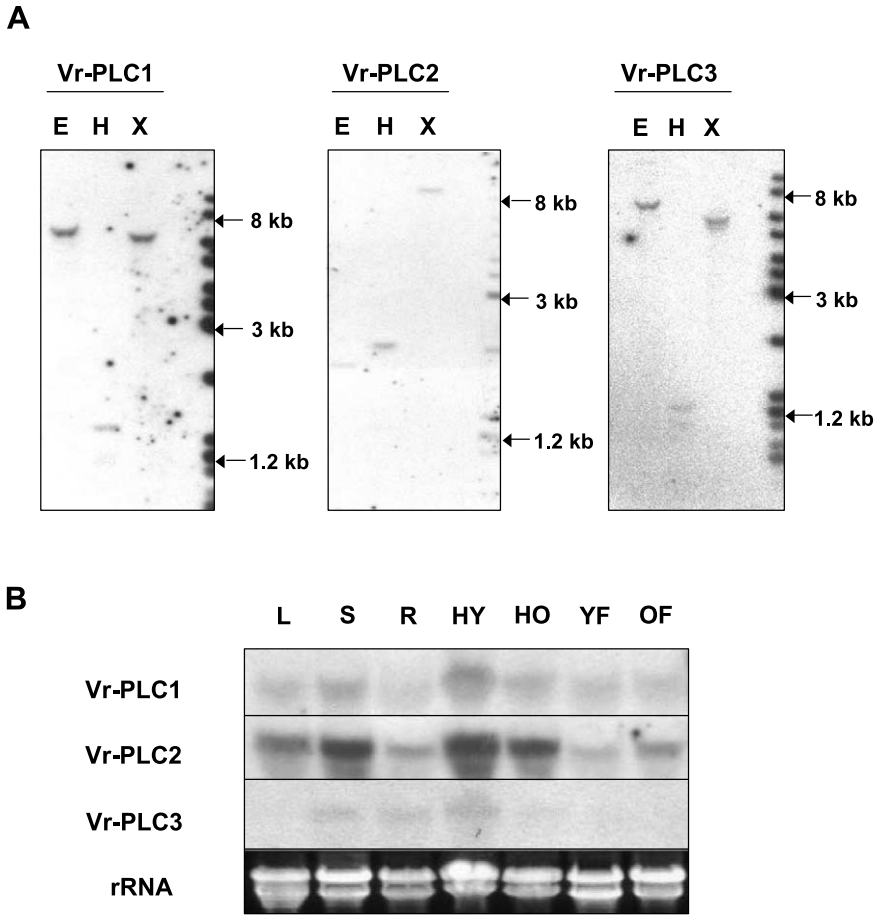

Fig. 2. Organization and expression of the mung bean *Vr-PLC* genes. A: Genomic Southern blot analysis of the *Vr-PLC* genes. The mung bean genomic DNA (10 μg per lane) was isolated from leaf tissue, digested with *Eco*RI (R), *Hin*dIII (H), or *Xba*I (X), and resolved on a 0.7% (w/v) agarose gel. DNA on the gel was transferred to a nylon membrane filter. The filter was hybridized to the ^{32}P-labeled pVr-PLC1, pVr-PLC2, or pVr-PLC3 PCR fragments under high stringent conditions. B: RNA gel blot analysis of the *Vr-PLC* genes. Total RNAs (20 μg per lane) were isolated from apical hooks (HO), hypocotyls (HY), and roots (R) of 4-day-old etiolated seedlings, or leaves (L), stems (S), young flowers (YF), and old flowers (OF) of 4–8-week-old light-grown mung bean plants and were resolved on a 1.0% formaldehyde-agarose gel. The gel was blotted onto a membrane filter and the blot was hybridized to the ^{32}P-labeled probes under high stringent conditions. The blots were visualized by autoradiography. Equivalence RNA loading among lanes of the agarose gel was confirmed by ethidium bromide staining of rRNA on the gel.

2 h, and thereafter slowly declined through the remainder of the 24-h incubation.

We further analyzed the induction kinetics of *Vr-PLC3* in response to drought. Two-week-old intact mung bean plants were harvested from agar plates and dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light. The degree of water stress was determined by the percentage decrease in the fresh weight of the plants. As shown in Fig. 4A, the low basal level of *Vr-PLC3* transcripts began to increase in leaf tissue in response to a 40% water loss. On the other hand, induction of *Vr-PLC3* mRNA was first observed in roots after a 20% water

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Fig. 1. Structure and translated product of the mung bean *Vr-PLC3* gene. A: A schematic model of the structure of plant PI-PLC homologues and restriction enzyme map analysis of pVr-PLC1, pVr-PLC2, and pVr-PLC3 partial cDNA fragments obtained by RT-PCR. The conserved sequences, including the EF-hand domain, the X and Y domains, and C2 domain, are indicated. B: Overall structure of the genomic clone λVr-PLC-RI that includes a full-length *Vr-PLC3* and truncated *Vr-PLC1* genes. Solid bars represent exons, whereas solid lines designate the 5′ and 3′ untranslated regions and introns. The coding regions of the full-length *Vr-PLC3* and partial *Vr-PLC1* are shown as open boxes. Arrows indicate the putative TATA box and transcriptional start site as determined by the primer extension experiment. The sequences of *Vr-PLC1*, *Vr-PLC2* and *Vr-PLC3* have been deposited in the GenBank database under accession numbers AY394079, AY461431 and AY394078, respectively. C: Comparison of the derived amino acid sequences of mung bean Vr-PLC1 and Vr-PLC3 with PI-PLC enzymes from *Arabidopsis* (AtPLC1) [20], potato (StPLC2) [23], and soybean (PI-PLC1) [32]. Amino acid residues that are identical in all five proteins are shown in black, and amino acid residues that are conserved in at least three of the five sequences are shaded. The four conserved motifs, including the EF-hand domain, the X and Y domains (which together constitute the catalytic domain), and the C2 domain, are indicated. Two histidine residues at positions 126 and 169 have been identified within the active site of rat PLCδ1 and are marked here by asterisks. The arrows represent the primer amino acid sequences for RT-PCR. Dashes show gaps in the amino acid sequences introduced to optimize alignment. D: Phylogenetic alignment of PI-PLC homologues from mung bean (Vr-PLC1 and Vr-PLC3), *Arabidopsis* (AtPLC1 and AtPLC2) [20,21], potato (StPLC1, StPLC2, and StPLC3) [23], soybean (PI-PLC1) [32], rice (OsPI-PLC1, accession number AAK01711), and *Nicotiana rustica* (wild tobacco NrPLC1 and NrPLC2, accession numbers X95877 and Y11931, respectively).

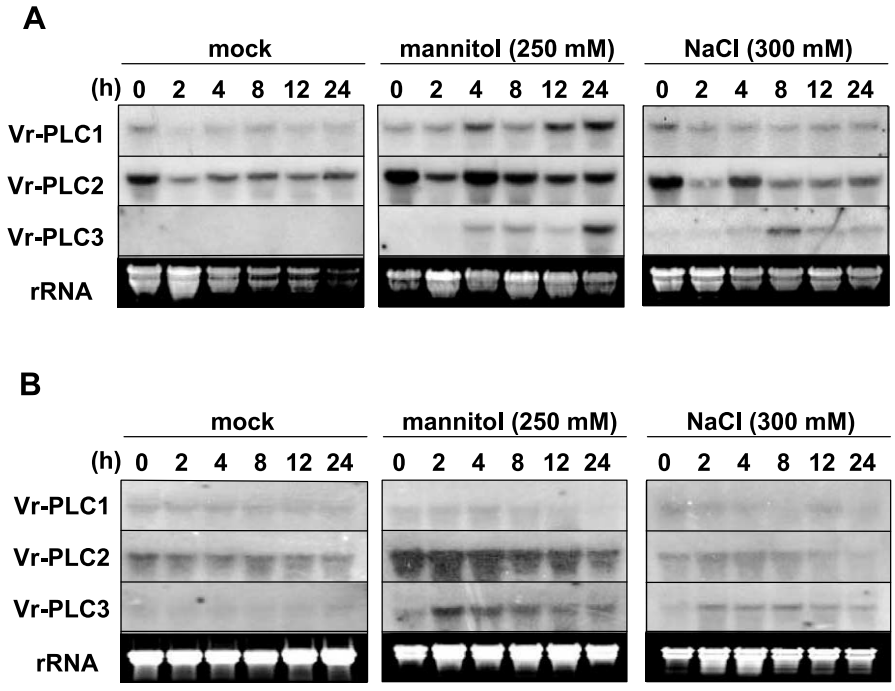

Fig. 3. Induction kinetics of *Vr-PLCs* in response to conditions of environmental stress in leaf and root tissues of mung bean plants. Four-week-old, light-grown, intact plants were subjected to 250 mM mannitol (drought treatment) or 300 mM NaCl (salt treatment). The stress-treated leaves (A) and roots (B) were harvested at indicated time points and total RNAs were isolated. Total RNAs (20 μg) were separated by electrophoresis on a 1% formaldehyde-agarose gel and blotted to a Hybond-N nylon membrane. To ensure equal loading of RNA, the gel was stained with ethidium bromide after electrophoresis. In order to confirm complete transfer of RNA to membrane filter, both gel and membrane were viewed under UV light at the end of the transfer. The filter was hybridized to ^{32}P-labeled pVr-PLC1, pVr-PLC2, or pVr-PLC3 probes, washed and visualized by autoradiography at −70°C with an intensifying screen.

loss. The expression of *Vr-PLC3* transcript in roots was further enhanced as the plants were exposed to more severe water loss (30%), and thereafter declined (Fig. 4A). The discrepancy between the patterns of induction in roots and leaves under conditions of drought and high salinity, as shown in Figs. 3 and 4A, indicates that different parts of the mung bean plant respond differently to environmental stress with regard to *Vr-PLC3* gene activation; roots are more sensitive than leaves. As previously mentioned, the levels of *Vr-PLC1* and *Vr-PLC2* mRNAs were constant until plants underwent a 40% water loss, a result which strongly suggests that both genes are constitutively expressed in mung bean plants.

Responses to drought and salt stress in plants are transduced via two separate signaling pathways: the ABA-dependent and -independent pathways [3,11]. To assess the possible role of ABA on expression of *Vr-PLC3*, 4-week-old mature mung bean plants were subjected to 100 μM ABA for different time periods and the levels of *Vr-PLC3* transcript monitored. Northern blot analyses revealed that the expression of *Vr-PLC3* mRNA was unaffected by treatment with exogenous ABA for 24 h (Fig. 4B). This raises the possibility that water and salt stress induction of *Vr-PLC3* is mediated by an ABA-independent pathway. The gene that encodes the mung bean ethylene biosynthetic ACC synthase 1, *Vr-ACS1*, was included in the mRNA expression analysis as a positive control for ABA-dependent gene induction [30]. As previously reported [30], its mRNA accumulated to significant levels in both leaf and root tissues during ABA treatment, which indicates that exogenously supplied ABA worked properly and therefore the ABA-independent expression pattern of *Vr-PLC3* was not an experimental artifact.

We next examined changes in the *Vr-PLC3* mRNA levels in response to pathogen infection. Mung bean leaves infected with the non-host strain Ds1 of *X. campestris* pv. *vesicatoria* did not cause disease symptoms within 5–6 days after inoculation. In contrast, when mung bean leaves were infected with the avirulent bacterium *P. syringae* pv. *tabaci*, they displayed a distinct hypersensitive response (HR) at 16–18 h after inoculation (data not shown). Although infection of two different bacterial pathogens resulted in clearly different phenotypic responses in leaves, neither the non-host *X. campestris* nor avirulent *P. syringae* stimulated an increase in levels of *Vr-PLC3* mRNA during the 24-h infection period (Fig. 4C). As was the case for other types of stress, the levels of *Vr-PLC1* and *Vr-PLC2* mRNAs remained the same after pathogen inoculation (data not shown). Thus, pathogen infection may not function as a trigger for induction of the *Vr-PLC* genes.

3.4. *Structural analysis of stress-induced* Vr-PLC3

From our combined results, it appeared that, among the three homologous *Vr-PLC* genes, *Vr-PLC3* was expressed at a very low level under normal growth conditions and was specifically induced under environmental stress, such as drought and high salinity, in an ABA-independent fashion. We therefore wanted to characterize the *Vr-PLC3* gene in more detail. To help determine the structural properties of the stress-responsive Vr-PLC3, we isolated the genomic clone. The mung bean genomic DNA was fully digested with *Eco*RI and size-fractionated on a 10–40% sucrose gradient (data not shown). Restriction fragments of about 6–10 kb in length were collected, ligated into *Eco*RI-digested λEMBL4 arms

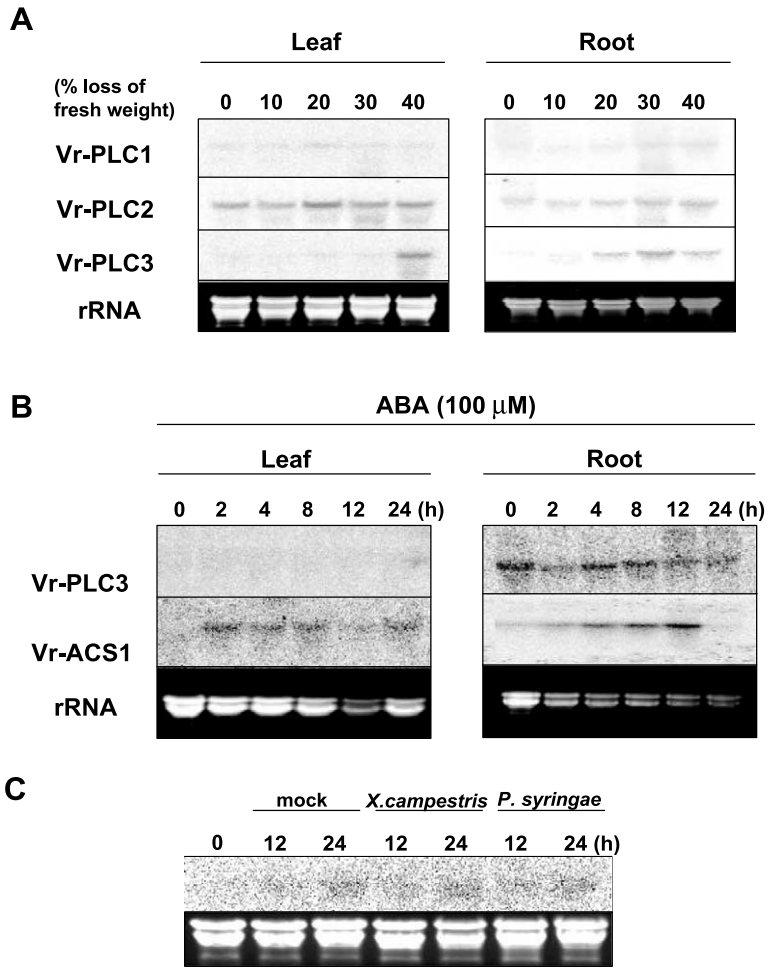

Fig. 4. Time course of accumulation of *Vr-PLC3* mRNA in mung bean plants in response to wilting (A), ABA application (B), and bacterial infection (C). A: Two-week-old intact mung bean plants were harvested from agar plates and dehydrated on Whatman 3MM filter paper at room temperature and approximately 60% humidity under dim light. The degree of water stress was determined by the percentage decrease in the fresh weight (10–40%) of the plants. The mature leaves of 4-week-old plants were sprayed with 100 μM ABA (B), or inoculated by vacuum-infiltrating bacterial suspensions (10^8 cfu/ml) into the abaxial side of fully expended leaves (C). The two bacterial species, *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tabaci*, were used as the non-host and avirulent pathogens, respectively, to the mung bean plants. Infection of leaves by *P. syringae* resulted in the hypersensitive response (HR) at 16–18 h after inoculation. The induction patterns of *Vr-PLC* genes were investigated by RNA gel blot analysis with ^{32}P-labeled pVr-PLC probes as described in Fig. 3. The gene which encodes a mung bean ethylene biosynthetic ACC synthase 1 (*Vr-ACS1*) was included in the RNA expression experiment as a positive control for ABA-dependent induction.

and packaged in vitro. Genomic Southern blot analysis (Fig. 2A) and PCR (data not shown) confirmed that these fragments contained *Vr-PLC3*. The resulting partial genomic library was screened using the ^{32}P-labeled pVr-PLC3 fragment as a probe; one clone, λVr-PLC-RI, was obtained. Restriction enzyme digests of DNA isolated from this clone showed that it contained an insert of approximately 8.0 kb (Fig. 1B). Interestingly, subsequent restriction enzyme mapping and DNA sequence analysis revealed that the genomic clone encodes a full-length *Vr-PLC3* and a truncated *Vr-PLC1*. Thus, the *Vr-PLC1* and *Vr-PLC3* genes are juxtaposed in the mung bean genome with an identical transcriptional direction (Fig. 1B). The λVr-PLC-RI genomic clone (λVr-PLC1 and λVr-PLC3) was sequenced for a total of 8226 bp. The λVr-PLC3 clone (GenBank accession number AY394078) consists of nine exons interrupted by eight introns whose junctions are in agreement with the consensus intron/exon borders of plant genes [31]. Individual sizes of the eight introns are, in order, 573, 352, 80, 104, 464, 666, 105, and 96 bp. The start site of transcription for *Vr-PLC3* was determined by primer extension analysis. A single primer extension product was obtained, and the major start site is located 39 nucleotides upstream of the 5′ end of the coding region of the *Vr-PLC3* gene (Fig. 1B). The putative TATA box was found 34 nucleotides upstream of the transcriptional start site. The primer extension data thus define the 5′ untranslated region of the *Vr-PLC3* mRNA as 39 nucleotides in length (Fig. 1B). The coding region of *Vr-PLC3* comprises 1773 bp which encode a polypeptide of 591 amino acid residues with a predicted molecular mass of 67.4 kDa, which is slightly larger than those of *Arabidopsis* AtPLC1 (64.3 kDa) [20] and of potato StPLC2 (64.2 kDa) [23], but is similar to that of soybean PI-PLC1 (68.8 kDa) [32] (Fig. 1C). On the other hand, λVr-PLC1 is a partial clone comprising a 1344-bp-long coding region (encoding 448 amino acids) with eight exons (GenBank accession number AY394079), indicating that λVr-PLC1 lacks the first exon and intron (Fig. 1B,C). Although the expression patterns of *Vr-PLC1* and *Vr-PLC3* are quite different (Figs. 3 and 4), the

deduced amino acid sequences of their gene products show 66% sequence identity.

The complete sequence for Vr-PLC3 allowed us to compare it to PI-PLCs from other organisms and to analyze their structural relationships. Mung bean Vr-PLC3 shares 53–76% identity at the amino acid level with the *Arabidopsis* AtPLC1 and AtPLC2 [20,21]; potato StPLC1, StPLC2, and StPLC3 [23]; soybean PI-PLC1 [32]; wild tobacco (*Nicotiana rustica*) NrPLC1 and NrPLC2 (GenBank accession number X95877 and Y11931, respectively); and rice OsPI-PLC1 (GenBank accession number AAK01711) (Fig. 1C and D). Phylogenetic alignment revealed that the Vr-PLC3 protein is most closely related to soybean PI-PLC1 (76%) and most distantly related to *Arabidopsis* AtPLC1 (53%) (Fig. 1D). As found in other putative PI-PLC homologues, the Vr-PLC3 protein possesses significantly conserved X and Y domains, which together constitute the catalytic core of the enzyme (Fig. 1C) [19,33]. In addition, the Vr-PLC3 polypeptide has a carboxy-terminal C2 domain that is known to be a lipid-binding site. A putative EF-hand domain is near the N-terminus, but it is much less conserved among plant PI-PLCs. Finally, Vr-PLC3 contains invariable histidine residues at positions 126 and 169; both are within the X domains essential for the enzymatic function of PI-PLCs (Fig. 1C, indicated by asterisks) [23,34]. Overall, these architectural conservations suggest that the stress-induced Vr-PLC3 protein is indeed biochemically active and may play a role in the production of IP_3 in mung bean plants.

3.5. *The* Vr-PLC3 *protein is predominantly localized in plasma membrane*

On the basis of results from in vitro enzyme activity assays, two kinds of PI-PLC have been identified in cells of higher plants; one is predominantly present in the cytosolic fraction, and the other localized to the plasma membrane [19,35,36]. Shi et al. [32] isolated a cDNA clone which encoded soybean PI-PLC by screening an expression library using serum with anti-plasma membrane antibodies raised against proteins from purified plasma membrane. Immunoblot analysis of the cell fractions prepared from transgenic tobacco plants over-expressing the FLAG epitope-tagged PI-PLC fusion protein showed that PI-PLC was present in the cytosol as well as in the plasma membrane. Otterhag et al. [37] found that a polyclonal antibody raised against a synthetic polypeptide specific for the *Arabidopsis* AtPLC2 isoform cross-reacted with a 66-kDa protein that was significantly enriched in the plasma membrane fraction as compared with the intracellular membrane and microsomal fractions of *Arabidopsis* cells. These results suggest that the plasma membrane is a major cellular site for the presence of AtPLC2 enzyme. To obtain more direct evidence for the cellular localization of PI-PLC, we conducted an in vivo targeting experiment using Vr-PLC3-fused GFP as a fluorescent marker in a transient transfection assay. The *GFP* gene was fused to the 5′ end of the *Vr-PLC3* coding region in-frame under the control of the cauliflower mosaic virus 35S promoter (Fig. 5A). As we previously established the protein localization method using protoplasts of *Arabidopsis* seedlings [38], the resulting construct was introduced into *Arabidopsis* protoplasts by polyethylene glycol treatment [28]. Localization of the fusion protein was visualized with a fluorescence microscope. As shown in Fig. 5B, the control GFP was uniformly distributed throughout the cytosolic fraction of protoplast (panel a), while the GFP-Vr-PLC3 fusion protein was localized predominantly in the plasma membrane (panel b). In contrast, the GFP-Vr-PLC3$^{1-462}$ protein, which lacked the C-terminal 129 amino acid residues corresponding to the C2 domain, exhibited uniform accumulation in the cytosol (panel c), a result which indicates that the C2 domain is critical for the Vr-PLC3 protein to be targeted to the plasma membrane. To further confirm the plasma membrane-localized staining pattern, we attempted co-localization of GFP-Vr-PLC3 with *Arabidopsis* P-type H^+-ATPase AHA2, a marker protein for the plasma membrane [39]. Previously, it was shown that H^+-ATPase-GFP was targeted to the plasma membrane in *Arabidopsis* protoplasts [38]. In this study, we replaced GFP with RFP. As shown in panel d, the green fluorescent signal of GFP-Vr-PLC3 closely overlapped the red fluorescent signal of H^+-ATPase-RFP. On the basis of these in vivo targeting results, we concluded that Vr-PLC3 is primarily present in the plasma membrane.

4. Discussion

During their entire life cycle, higher plants are constantly faced with diverse environmental stresses. To survive under such unfavorable growth conditions, plants have developed a number of unique defense mechanisms and processes for acclimation that enhance tolerance to the detrimental conditions. Cytosolic calcium has been generally regarded as a secondary messenger that transmits the extracellular stimuli into the cells to turn on the defense response [8,9,40,41]. In addition, IP_3, produced from the membrane phospholipid PIP_2 by the action of PI-PLC, is also an important secondary messenger that triggers Ca^{2+} release from intracellular reservoirs, such as vacuole and endoplasmic reticulum [3,10,11,42].

The mung bean plant is a commercially important crop and widely cultured in Korea. The physiological responses of mung bean seedlings to various hormones, including auxin, ABA and ethylene, and to environmental factors have been extensively studied [25,30,43–45]. However, far less has been done to investigate the secondary messengers involved in the signaling pathway in response to environmental and developmental cues. In the present study, we examined the expression profiles of three homologous *Vr-PLC* genes, and found that *Vr-PLC3* is specifically activated by drought and high salinity in an ABA-independent manner in intact mung bean plants (Figs. 3 and 4). The deduced primary sequence of stress-responsive Vr-PLC3 displays a high degree of sequence identity (53–76%) with other plant putative PI-PLC homologues, and contains core sequences found in typical PI-PLC enzymes, including the catalytic domains comprising X and Y domains, a lipid-binding C2 domain, and less conserved EF-hand domain (Fig. 1). Two histidine residues identified within the active site of rat PLCδ1 are also well conserved in Vr-PLC3 [34]. Thus, as is the case for *Arabidopsis*, potato, and soybean PI-PLCs [20,23,32], the architectural features of Vr-PLC3 are reminiscent of the δ isoform of the animal enzyme. In addition, employing an in vivo targeting experiment, we demonstrated that Vr-PLC3 is predominantly present in the plasma membrane of *Arabidopsis* protoplasts (Fig. 5). Our results clearly show that the C2 domain is essential for Vr-PLC3 to be localized to the plasma membrane. This result is consistent with a recent finding that the C2 domain, which contains distinct membrane-binding properties, controls the subcellular localization of the mammalian PLC-δ isoform [46]. By anal-

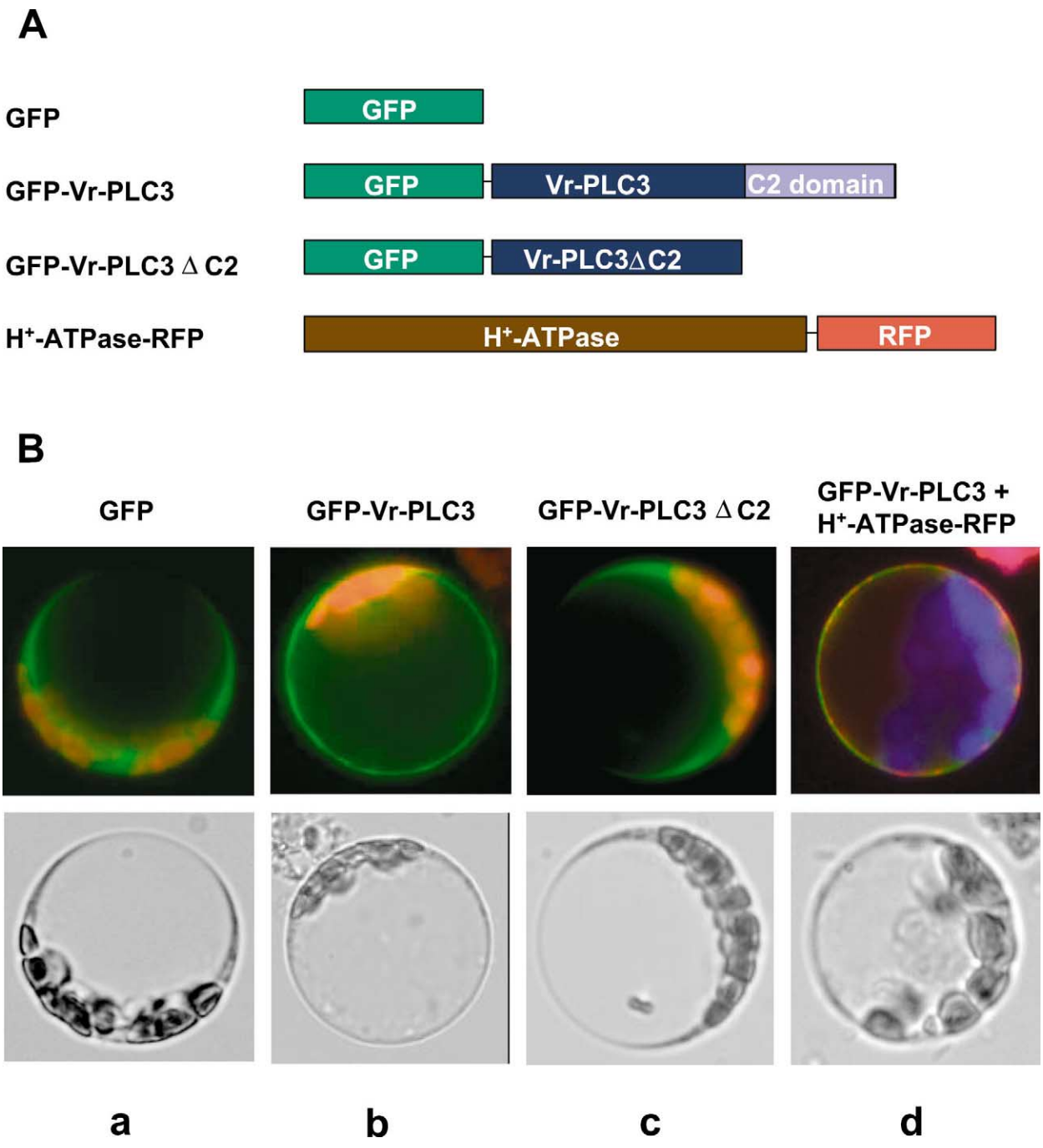

Fig. 5. Subcellular localization of the *Vr-PLC3* gene products. A: The *GFP* coding region was fused in-frame to the full-length pVr-PLC3 coding region or to the truncated pVr-PLC3 mutant. H^+-ATPase-RFP was used as a marker for the plasma membrane protein. Constructs were introduced into protoplasts prepared from *Arabidopsis* seedlings by polyethylene glycol treatment and expressed under the control of the cauliflower mosaic virus 35S promoter. B: Expression of the introduced genes as viewed after 16 h by fluorescence microscopy under dark field or light field.

ysis of the crystallized structure of mammalian PI-PLC-δ, Essen et al. [34] suggested that the C2 domain regulates PI-PLC enzyme activity by modulating membrane interaction and helps with access to the PI substrate. On the basis of these structural properties and the subcellular localization pattern, we presume that Vr-PLC3 is indeed biochemically active and plays a role in the stress response of mung bean plants.

The *Vr-PLC3* gene is composed of nine exons interrupted by eight introns (Fig. 1B). This number of introns and exons is identical to those of *Arabidopsis AtPLC2*, *AtPLC4*, *AtPLC6*, and *AtPLC7* genes, but is different from those of *AtPLC1* and *AtPLC5* with eight exons and *AtPLC3* with seven exons [19]. Because the expression patterns of most *Arabidopsis PI-PLC* genes, except for *AtPLC1* and *AtPLC2*, are unknown, it would be intriguing to investigate if the number of exons/introns is correlated with the induction pattern of *PI-PLC* genes. Interestingly, mung bean *Vr-PLC1* and *Vr-PLC3* exist as a tandem array with the same transcriptional orientation within an 8.2-kb *Eco*RI DNA fragment (Figs. 1B and 2A). The *Vr-PLC1* gene, along with *Vr-PLC2*, is consti-

tutively expressed in all tissues examined in mung bean plants (Figs. 2–4). Thus, *Vr-PLCs* are not expressed in a strict tissue-specific fashion. This result indicates that PI metabolism actively occurs under normal growth conditions and may help ensure the optimal growth and development of mung bean plants. Conversely, *Vr-PLC3* is expressed at very low levels and is rapidly induced by water or salt stress (Figs. 3 and 4). We speculate that the accumulation of Vr-PLC3, in addition to Vr-PLC1 and Vr-PLC2, may contribute to enhance tolerance or may help plants to cope with such stresses. At this moment, however, we do not know the exact physiological relevance of differential expression of *Vr-PLC* gene family members. Plant PI-PLC enzymes, as found with animal PI-PLCs, are activated by Ca^{2+} ion. Using wheat plasma membrane system, Pical et al. [47] showed that Mg^{2+} is an activator of PI-PLC in the presence of Ca^{2+}, while Al^{3+} ion markedly inhibits PI-PLC enzyme activity [48]. Although we do not provide biochemical data, we are tempted to assume that individual Vr-PLC isoforms may have different catalytic properties (e.g. sensitivity to Ca^{2+}, Mg^{2+}, and Al^{3+} ions, and specificity or affinity toward PIP_2, PIP, and PI substrates). It would also be possible that each Vr-PLC exhibits a different mode of interaction with the plasma membrane or a different subcellular localization pattern. Overall, these distinct features of Vr-PLCs would permit the plant to fine-tune its response to different developmental and environmental cues. Further experiments are required to define the detailed biological roles of Vr-PLCs in the stress responses of higher plants.

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