Abscisic Acid Stimulates a Calcium-Dependent Protein Kinase in Grape Berry1[W]

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It has been demonstrated that calcium plays a central role in mediating abscisic acid (ABA) signaling, but many of the Ca\(^{2+}\)-binding sensory proteins as the components of the ABA-signaling pathway remain to be elucidated. Here we identified, characterized, and purified a 58-kD ABA-stimulated calcium-dependent protein kinase from the mesocarp of grape berries (Vitis vinifera × Vitis labrusca), designated ACPK1 (for ABA-stimulated calcium-dependent protein kinase1). ABA stimulates ACPK1 in a dose-dependent manner, and the ACPK1 expression and enzyme activities alter accordantly with the endogenous ABA concentrations during fruit development. The ABA-induced ACPK1 stimulation appears to be transient with a rapid effect in 15 min but also with a slow and steady state of induction after 60 min. ABA acts on ACPK1 indirectly and dependently on in vivo state of the tissues. Two inactive ABA isomers, (−)-2-cis, 4-trans-ABA and 2-trans, 4-trans-(±)-ABA, are ineffective for inducing ACPK1 stimulation, revealing that the ABA-induced effect is stereo specific to physiological active (+)-2-cis, 4-trans-ABA. The other phytohormones such as auxin indoleacetic acid, gibberellic acid, synthetic cytokinin N-benzyl-6-aminopurine, and brassinolide are also ineffective in this ACPK1 stimulation. Based on sequencing of the two-dimensional electrophoresis-purified ACPK1, we cloned the ACPK1 gene. The ACPK1 is expressed specifically in grape berry covering a fleshy portion and seeds, and in a developmental stage-dependent manner. We further showed that ACPK1 is localized in both plasma membranes and chloroplasts/plastids and positively regulates plasma membrane H\(^+-\)ATPase in vitro, suggesting that ACPK1 may be involved in the ABA-signaling pathway.

The phytohormone abscisic acid (ABA) regulates many important events during both vegetative and reproductive growth of plants as well as in plants' adaptation to their environment. During vegetative growth, ABA is a central signal of plant response to various environmental challenges including drought, salt, and cold stresses (for review, see Koomruee et al., 1998; Leung and Giraudat, 1998; Finkelstein and Rock, 2002). In reproductive organ seeds, ABA is responsible for the seed storage reserve synthesis, acquisition of desiccation tolerance and dormancy, and induction of stress tolerance (for review, see Finkelstein et al., 2002). Fleshy fruits are also the essential portions of reproductive organs and economically important harvest organs, as are crop seeds. In fleshy fruits as in seeds, ABA regulates various processes concerning assimilate uptake and metabolism to enhance reserve accumulation in these economic sinks (Yamaki and Asakura, 1991; Rock and Quatrano, 1995; Wayne and John, 1996; Opaskornkul et al., 1999; Peng et al., 2003; Pan et al., 2005). Grape berry (Vitis vinifera and also Vitis labrusca) is one of the most widely cultivated fruit trees in the world and also one of the typical non-climacteric fruits. The ripening of grape berry is considered to be independent of the hormone ethylene but to be triggered essentially by ABA (for review, see Coombe, 1992). However, the ABA-signaling pathway in regulating development of the fleshy fruits remains essentially elusive.

ABA signal transduction has been extensively studied in the past years. Numerous cellular components that modulate ABA responses have been identified, leading to considerable progress in understanding the ABA-signaling pathway (for review, see Finkelstein et al., 2002; Himmelbach et al., 2003; Fan et al., 2004). Reversible protein phosphorylation, catalyzed by protein kinases and phosphatases, has been believed to play central roles in ABA signal transduction. Calcium-modulated protein phosphatases (PPs) 2C ABI1 and ABI2 are the two most characterized, homologous negative regulators of ABA signaling (Leung et al., 1994, 1997; Meyer et al., 1994; Sheen, 1998; Gosti et al., 1999; Pan et al., 2003; Merlot et al., 2001). They interact with multiple cellular targets such as a calcineurin B-like (CBL) protein kinase CIPK15 and a CBL Ca\(^{2+}\)-binding protein ScaBP5. CIPK15 and one of its homologs CIPK3 and ScaBP5 are all involved in ABA signaling as negative

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Recently, three homologs of AAPK and OST1 in rice of stomatal response to ABA (Mustilli et al., 2002). A nuclear-localized transcription factor homeodomain protein ATHB6 has also been identified as a more downstream component of the ABI1 and ABI2 (Himmelbach et al., 2002). In contrast to PP2Cs ABI1 and ABI2, a PP 2A, RCN1, has been identified as a positive regulator of ABA response involved in early events of ABA signaling (Kwak et al., 2002).

Two members of the family of SNF1-related protein kinase, PKABA1 from wheat (Triticum aestivum) and AAPK from broad bean (Vicia faba), were characterized as the ABA-stimulated protein kinases and positive regulators of ABA response (Anderberg and Walker-Simmons, 1992; Li and Assmann, 1996; Gomez-Cadenas et al., 1999, 2001; Li et al., 2000, 2002; Johnson et al., 2002). PKABA1 mediates ABA-suppressed gene expression (Gomez-Cadenas et al., 1999), and phosphorylates and activates the ABA-responsive basic-domain Leu-zipper (bZIP) transcription factor TaABF, a homolog of ABI5 of Arabidopsis (Arabidopsis thaliana; Johnson et al., 2002). AAPK mediates ABA-induced stomatal closure and anion channels (Li et al., 2000; Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005). A nuclear-localized transcription factor AtETR1 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al., 2005).

It has been well accepted that calcium is a central regulator of plant growth and development (Hepler, 2005). Specific calcium signatures are recognized by different calcium sensors to transduce specific calcium-mediating signal into downstream events (Sanders et al., 1999; Harmon et al., 2000; Rudd and Franklin-Tong, 2001). In plants, there are several known classes of Ca\(^{2+}\)-binding sensory proteins, including calmodulin (CaM) and CaM-related proteins (Zielinski, 1998; Luan et al., 2002), CBL proteins (Luan et al., 2002), and calcium-dependent protein kinases (CDPKs; Harmon et al., 2001; Cheng et al., 2002). Among them, CDPKs, a novel class of Ca\(^{2+}\) sensors having both kinase and CaM-like domain, are the best characterized and involved in most of calcium-stimulated protein kinase activities identified in plants (Harmon et al., 2001; Cheng et al., 2002). Encoded by a large multigene family with possible redundancy and/or diversity in their functions (Harmon et al., 2001; Cheng et al., 2002), CDPKs are believed to be important components in plant hormone signaling (Cheng et al., 2002; Ludwig et al., 2004). Using a protoplast transient expression system, two Arabidopsis CDPKs, AtCPK10 and AtCPK30, have been demonstrated to activate a stress- and ABA-inducible promoter, showing the connection of CDPKs to ABA-signaling pathway (Sheen, 1996). However, a previous report showed that ABA had no effect on the induction of AtCPK10 mRNA (Urao et al., 1994), and it has been unclear whether AtCPK30 is stimulated by ABA. This suggests complexity of CDPK signaling in mediating ABA signal. In addition, calcium-dependent histone-phosphorylating activity was reported to be activated by ABA in rice seedling, but the CDPK gene responsible for this activity was not identified (Li and Komatsu, 2000). In tobacco (Nicotiana tabacum), the transcripts of a CDPK gene, NtCDPK1, were enhanced unspecifically by various phytohormones ABA, indole-3-acetic acid, IAA, GA\(_3\), and synthetic cytokinin benzyladenine and other growth substances such as jasmonic acid, but it is unknown whether the NtCDPK1 is activated by ABA in its kinase activities (Yoon et al., 1999). Thus, information is currently lacking about ABA-activated CDPKs identified by combined biochemical and molecular approaches as potential ABA signal transducers. In this report, we identified, characterized, and purified a 58-kd ABA specifically stimulated CDPK from grape berry, designated ACPK1. We isolated the cDNA coding for the ACPK1 based on sequencing of the purified ACPK1 protein. Also, we showed that ACPK1 positively regulates plasma membrane (PM) H\(^{-}\)-ATPase in vitro, suggesting that ACPK1 may be involved in ABA-signaling pathway in grape berry.

**RESULTS**

**Characterization of a 58-kd Membrane-Associated CDPK in Grape Berry**

Grape is a berry fruit, comprising the fleshy portion and seeds. The fleshy portion covers three parts, i.e. pericarp and endocarp that both are several cell layers thick, and mesocarp that makes the large fleshy portion. Using in-gel assays, we detected in the microsomes of the grape mesocarp a calcium-dependent autophosphorylated protein with a molecular mass of 58 kD (Fig. 1A). This 58-kd protein phosphorylates histone III-S, one of the best exogenous substrates for assaying CDPKs (Roberts and Harmon, 1992), and this kinase activity depends also on the presence of Ca\(^{2+}\).
Figure 1. Biochemical characterization of a 58-kD membrane-associated Ca\(^{2+}\)-dependent protein kinase in grape berry. A, A 58-kD membrane-associated Ca\(^{2+}\)-dependent protein kinase is present in grape berry. Soluble fraction (lanes labeled S, 40 \(\mu\)g of protein) and microsomes (lanes labeled M, 20 \(\mu\)g of protein) were separated on a 12% SDS-polyacrylamide gel. The separating gel for assaying kinase catalytic activity was polymerized in the presence of histone III-S. Autophosphorylation and kinase activities were assayed in the presence (\(+\)Ca\(^{2+}\)) or absence (\(-\)Ca\(^{2+}\)) of free Ca\(^{2+}\) as described in “Materials and Methods.” The molecular masses of protein standards are shown at the left of the sections in kD. Autophosphorylating (gels above) and histone-phosphorylating activity (gels below) of 58-kD kinase are shown in the left and middle sections, and immunoblotting in the right section. Immunoblotting was done with the antiserum directed against the CaM-like domain of soybean CDPKα as described in “Materials and Methods.” B, Ca\(^{2+}\)-dependent electrophoretic mobility shift of the 58-kD kinase in both the in-gel autophosphorylation (a) and histone-phosphorylating activity (b) assays. Ca\(^{2+}\) or EGTA to a final concentration of 2 mM was added to the microsomal proteins dissolved in SDS-PAGE sample buffer. After SDS-PAGE, the in-gel phosphorylation assays were done in the presence of Ca\(^{2+}\). \(-\)Ca\(^{2+}\) and \(+\)Ca\(^{2+}\) indicate the absence and presence of Ca\(^{2+}\) in the SDS-PAGE sample buffer, respectively. C, Ca\(^{2+}\) dependence of the in-gel autophosphorylation (indicated by 58-kD with arrow) and in vitro activity (columnar figures) of the kinase in a medium pH at 7.5 and in the presence of Mg\(^{2+}\) at 10 mM and EGTA at 0.45 mM. The in vitro and in-gel histone III-S-phosphorylating activities and the in-gel autophosphorylation of the kinase were assayed as described in...
(Fig. 1A). The 58-kD kinase was immunorecognized by an antiserum raised against the CaM-like domain of soybean (Glycine max) CDPKα (Bachmann et al., 1996; Fig. 1A). In the soluble fractions or in the absence of free Ca\(^{2+}\), neither the autophosphorylation nor histone-phosphorylating kinase activity was detected (Fig. 1A). These results suggest that the 58-kD membrane-associated kinase is a member of CDPK family.

Ca\(^{2+}\)-binding proteins such as CDPKs migrate in gels at different rates in the Ca\(^{2+}\)-bound versus Ca\(^{2+}\)-free state (Roberts and Harmon, 1992). To investigate the phenomenon, we added Ca\(^{2+}\) or EGTA to the protein sample just before electrophoresis and then the in-gel phosphorylation was analyzed in the presence of Ca\(^{2+}\). The assays of both in-gel autophosphorylation and histone-phosphorylating activity of the 58-kD kinase showed a clear mobility shift when the kinase migrates in the presence of Ca\(^{2+}\) (Fig. 1B). The in-gel autophosphorylation and in vitro histone-phosphorylating activities of the 58-kD kinase depended strongly on Ca\(^{2+}\) (Fig. 1C) and Mg\(^{2+}\) (Fig. 1D) concentrations and in a weaker extent on medium pHs (Fig. 1E). The assays of in-gel kinase activity gave the same results as the in vitro assays (data not shown).

We also analyzed the effects of the CaM antagonists trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W7) and inhibitor of Ser/Thr protein kinases K252a on the 58-kD kinase. The calcium-dependent in-gel autophosphorylation and histone-phosphorylating activity of the 58-kD kinase could be inhibited by TFP, W7, and K252a, and the dose dependence of W7 for the inhibition was observed (Fig. 1F). By contrast, CaM and N-(6-aminohexyl)-1-naphthalene sulfonamide (W5, an inactive analog of W7), had no apparent effect on autophosphorylation or phosphorylating activity of the 58-kD kinase (Fig. 1F). Taken together, all of the results consistently indicate that the 58-kD membrane-associated kinase is a CDPK.

### ABA Stimulates the 58-kD CDPK

To investigate the effects of ABA on grape CDPK, we used throughout this study a system to incubate in vivo the berry tissues in the ABA-containing medium essentially according to the technique of Peng et al. (2003), as detailed in “Materials and Methods.” The assays of in vitro phosphorylation of the total microsomal proteins extracted from ABA-free incubated tissues allowed the detection of two bands of calcium-independent phosphoproteins (CIPs; Fig. 2A, a, lane 1) and two other bands of calcium-dependent phosphoproteins (CDPs; Fig. 2A, a, lane 2). The molecular masses of the CIPs are 72 (CIP72) and 40 kD (CIP40), and those of CDPs 64 (CDP64) and 58 kD (CDP58), respectively (Fig. 2A, a). ABA appeared to stimulate three of them, CIP72, CDP64, and CDP58, but not CIP40 (Fig. 2Aa). However, only one band of phosphorylation at the point of 58 kD was detected by in-gel phosphorylation (Fig. 2A, a and b), probably because the other in vitro-detected phosphoproteins may be only substrates of protein kinase, or that phosphorylation of diverse proteins may require the complex in vitro system where total membrane proteins are present and protein-protein interactions related to the phosphorylation may be realizable. The CDP58 phosphoprotein detected in the in vitro system should be the identified 58-kD kinase (Fig. 1). ABA strongly stimulated the 58-kD kinase in both its autophosphorylation and histone-phosphorylating kinase activities (Fig. 2A, b and c). In accordance with this, the amount of the 58-kD kinase, estimated by immunodetection with the antiserum to CaM domain of soybean CDPKα, was also enhanced by ABA treatment (Fig. 2A, d). The ABA-induced stimulation of the 58-kD kinase was dependent on the dose of ABA application, and the ABA concentration of 10 μM maximized the effects (Fig. 2A, b–d). When ABA at concentrations above 10 μM was applied, the ABA-induced effects declined but still remained at steady, higher levels (Fig. 2A, b–d). It is noteworthy that the concentrations of ABA within the treated tissues corresponding to the concentrations of applied ABA below 10 μM did not overpass the highest level of the physiological concentrations in grape berries (Fig. 2A; also see Fig. 8).

We further analyzed time course of the ABA-induced effects. ABA rapidly stimulated the 58-kD kinase in its autophosphorylation and kinase activity, and the ABA-induced effects appeared to be transient (Fig. 2B, a and b). An incubation of 15 min could induce the effects, and the effects were maximized with a 60-min incubation (Fig. 2B). A longer incubation over 60 min allowed still a steady, higher level of the 58-kD kinase stimulation, though the effects declined (Fig. 2B, a and b). It is noteworthy, however, that the increase of the apparent amount of the 58-kD kinase began at the point of 45-min incubation, significantly later than did its autophosphorylation and kinase activity, and this enzyme amount did not decrease after 60 min of incubation, which, contrasting with the enzyme activities, showed a typical saturation

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**Figure 1.** (Continued.)

“Materials and Methods.” Values in the columnar figures are means ± se (n = 5). D, The same assay as in C but in different concentrations of Mg\(^{2+}\) and in the presence of 0.55 mm Ca\(^{2+}\). E, The same assay as in C but in different medium pHs and in the presence of 0.55 mm Ca\(^{2+}\) and 10 mm Mg\(^{2+}\). F, Inhibition of both the in-gel autophosphorylation (indicated by 58-kD autophosph) and histone-phosphorylating activity (indicated by 58-kD histone phosphoph) of the 58-kD kinase by CaM antagonists or kinase inhibitors. CaM was used at 5 μM; TFP or W5 at 250 μM; W7 at 100 (W7*) or 250 μM (W7**), and K252a at 10 μM. These reagents were added, respectively, to the phosphorylation reaction medium (buffer B as described in “Materials and Methods”) for a preincubation and a subsequent reaction incubation for \(^{32}P\)-labeling to the kinase or its substrate histone as described in “Materials and Methods.” = and + indicate the absence and presence of Ca\(^{2+}\) in the reaction buffer, respectively.
ABA stimulates the 58-kD Ca\textsuperscript{2+}-dependent protein kinase. A, ABA stimulates the 58-kD Ca\textsuperscript{2+}-dependent protein kinase in a dose-dependent manner. A, ABA-stimulated in vitro phosphorylation in total microsomal proteins. The berry tissues were incubated in a medium (pH 5.5) containing 0 (control) or 10 \( \mu \text{M} \) (±)-ABA for 1 h. The microsomes prepared from the tissues were subjected to phosphorylation in vitro and separated by SDS-PAGE, and then phosphoproteins were detected by autoradiography. The detailed procedures of these assays were described in “Materials and Methods.” The phosphoproteins from the control tissues are shown after the in vitro phosphorylation in the absence (lane 1) or presence (lane 2) of Ca\textsuperscript{2+}. The phosphorylation level of some phosphoproteins increases in the microsomes from the ABA-treated tissues after the in vitro phosphorylation in the presence of Ca\textsuperscript{2+} (lane 3). The calculated molecular masses according to the mobility rate of protein standards are shown at the left of the section in kD; 2 and 1 indicate the absence and presence of Ca\textsuperscript{2+}, respectively. B, Time course of the ABA-induced stimulation of the 58-kD Ca\textsuperscript{2+}-dependent protein kinase. Berry tissues were incubated for different durations of time from 0 to 180 min in the medium containing 10 \( \mu \text{M} \) ABA (ABA) or the same amount of ethanol for solubilizing ABA (Ethanol) as described in “Materials and Methods.” The microsomes prepared from the treated tissues were used for analysis of the 58-kD Ca\textsuperscript{2+}-dependent protein kinase as described in A. a, In-gel autophosphorylation of the 58-kD kinase. b, In-gel histone-phosphorylating activity of the 58-kD kinase. c, 58-kD immunosignal detected by the rabbit antiserum directed against the calmodulin-like domain of soybean CDPKα.
ABA-Induced Stimulation of ACPK1 Is Specific to the Physiologically Active Form of ABA

To determine the stereo specificity of ABA in the ACPK1 stimulation, two ABA isomers (−)-2-cis, 4-trans-ABA [(−)-ABA], and 2-trans, 4-trans-(±)-ABA (trans-ABA), were used in the experiment. The used (±)-ABA in the experiments is a mixture of (+)-2-cis, 4-trans-ABA [(+)-ABA] and (−)-ABA with (+)-ABA being physiologically active.

The two ABA isomers (−)-ABA and trans-ABA are structurally similar to physiologically active (+)-ABA, but functionally inactive (Balsevich et al., 1994; Hill et al., 1995; Walker-Simmons et al., 1997). We previously showed also that the two ABA isomers were unable to be bound to ABA-specific binding proteins that possess potential receptor nature (Zhang et al., 1995; Walker-Simmons et al., 1997). The assays of both the in-gel autophosphorylation and histone-phosphorylating activities showed that the two ABA isomers were unable to stimulate the ACPK1 kinase (Fig. 3A), revealing that the ACPK1 kinase stimulation induced by ABA is stereo specific, only physiologically active (+)-ABA being effective.

We further analyzed the effects of some other phytohormones on activities of the ACPK1 kinase. In the assayed substances auxin IAA, GA₃, synthetic cytokinin N-benzyl-6-aminopurine, and brassinolide, none of them had any effect on ACPK1 in either its autophosphorylation or histone-phosphorylating kinase activity (Fig. 3B). This result indicates that the ACPK1 kinase appears to be stimulated specifically and exclusively by ABA.

ABA Indirectly Stimulates ACPK1, and Influx of Ca²⁺ May Be Involved in This Stimulation

To investigate whether ABA directly acts on the ACPK1 kinase, ABA was added directly to the reaction medium of the in-gel phosphorylation instead of incubating the tissues in vivo in the ABA-containing medium. The results showed that ABA had no direct effect on the ACPK1 kinase in either its autophosphorylation or kinase activity (Fig. 3C), indicating that ABA indirectly stimulates the ACPK1 kinase.

We further investigate possible roles of Ca²⁺ influx in mediating the ABA-induced ACPK1 stimulation. Two Ca²⁺ chelators, 1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid (BAPTA) and EGTA, were used to incubate the tissues in vivo together with ABA. This treatment of eliminating apoplastic Ca²⁺ significantly reduced the ACPK1 stimulation by ABA, though BAPTA was more effective compared to EGTA (Fig. 3D). A further experiment was done with A23187, a Ca²⁺ ionophore that allows diffusion of Ca²⁺ through plasma membranes in an electrically neutral manner, thus creating an artificial influx of Ca²⁺ from apoplasm into cytoplasm. However, the treatment with A23187 had no significant effect on the ABA-induced ACPK1 stimulation (Fig. 3D, lanes 5 and 6). The treatment with A23187 alone did not stimulate ACPK1 (Fig. 3D, lane 7). These data suggest that influx of Ca²⁺ from apoplasm into cytoplasm may be involved in the ABA-induced events, which requires a mechanism such as calcium channels to mediate influx of Ca²⁺, but ABA signaling in these events may be more complex than dependent on influx of Ca²⁺.

Purification and Molecular Cloning of ACPK1

We adopted a technique to purify ACPK1 kinase from SDS-polyacrylamide gels after the total membrane proteins were separated by SDS-PAGE, because the approaches based on columnar chromatography that we used for this purification were unsuccessful. The exact position of the 58-kD kinase on gels (Fig. 4A) was determined by both autophosphorylation and immunodetection with antisera against the CaM-like domain of soybean CDPKα, and thus the portions of the gels containing the 58-kD kinase were excised and eluted. The lyophilized eluate was analyzed for assessing the purification. A total amount of 400 µg purified proteins was obtained from 30 mg crude microsomal proteins, i.e. 50 gels × 10 lanes with 60 µg crude microsomal proteins on each lane.

The purified proteins migrated as a single protein band at the point of 58-kD on SDS-polyacrylamide gel (Fig. 4B). The purified 58-kD proteins could autophosphorylate (Fig. 4C) and phosphorylate histone III-s (Fig. 4D) in the presence of Ca²⁺, but both of their autophosphorylation and kinase activities were lost in the absence of Ca²⁺ (data not shown). It is particularly noteworthy that SDS-PAGE of the apparently purified protein in the presence of calcium displayed two bands (Fig. 4E), of which one possessing Ca²⁺-dependent electrophoretic mobility shift should be the 58-kD kinase, and another may be a protein other than this kinase. This nature of Ca²⁺-dependent electrophoretic mobility shift should be the 58-kD kinase, and another may be a protein other than this kinase. This nature of Ca²⁺-dependent electrophoretic mobility shift of the 58-kD kinase was further shown in the assays of autophosphorylation (Fig. 4F), histone-phosphorylating activity (Fig. 4G), and immunoblotting with antisera against the CaM-like domain of soybean CDPKα (Fig. 4H).

The 58-kD proteins were further identified and purified by isoelectric focusing electrophoresis (IEF)/SDS-PAGE two-dimensional electrophoresis. Two protein bands were observed on SDS-polyacrylamide gel with the same molecular mass of 58 kD but different pIs (Fig. 4I, lanes b and c), which is consistent with the above results of SDS-PAGE. One of them (Fig. 4I, lane
c) was identified as the 58-kD ACPK1 kinase by its features of Ca\(^{2+}\)-dependent autophosphorylation (Fig. 4J), histone-phosphorylating activity (Fig. 4K), and immunorecognition by anti-ACPK1-N40 serum (Fig. 4L), a specific antibody to ACPK1 (see below). The IEF/SDS-PAGE-purified 58-kD kinase was sequenced by tandem mass spectrometry (TSM). The data from the first sequencing indicated that the sequence of the purified 58-kD kinase matches that of some plant CDPKs registered in GenBank in their conserved kinase, junction, and CaM-like domains. The sequences of matched peptides were: REIQIMHH, GGELFDRI, HRDLKPENFL, DVWSAGVI, KQFSAMNK, DTDNSGTITFDE, and DQDNDQIDYGGEF (Fig. 5A, indicated by Peptide 1–Peptide 7), which formed the basis for the cloning of ACPK1 by PCR. Using the designed degenerate primers that correspond to two of the matched sequences of the 58-kD kinase and also to the conserved domain of most CDPKs (-EIQIMHHL- and -KQFSAMNK-), a cDNA clone putatively coding for ACPK1 (Fig. 5A) was obtained and registered in GenBank. A second sequencing of the purified 58-kD kinase showed that the sequence of 58-kD kinase matched the predicted amino acid sequence from the cDNA clone in 10 pieces particularly covering all the N-terminal variable, conserved kinase, junction and

Figure 3. Some features of ABA-induced ACPK1 kinase stimulation. A, Specificity of the stimulation of the ACPK1 kinase to the physiologically active form of ABA. Berry tissues were incubated in the medium containing various concentrations (indicated by the numbers under lines as \(\mu\)M) of (\(\pm\))-ABA or ABA isomers for 1 h as described in “Materials and Methods.” In-gel phosphorylation (a) and histone-phosphorylating activity (b) of the 58-kD kinase in the microsomes prepared from the tissues treated by (\(\pm\))-ABA (as a control) or its isomers (\(-\))-ABA or trans-ABA were analyzed. B, ACPK1 appears to be stimulated uniquely by ABA. In-gel phosphorylation (a) and histone-phosphorylating activity (b) of the 58-kD kinase were assayed in the microsomes prepared from the tissues treated as described in A by (\(\pm\))-ABA (as a control) or other plant hormones auxin IAA, GA\(_3\), synthetic cytokinin N-benzyl-6-aminopurine (6-BA), and brassinolide (BL). The numbers under lines indicate the applied concentrations as \(\mu\)M. C, ABA stimulates ACPK1 kinase indirectly. Microsomes were prepared from nontreated tissues. In-gel phosphorylation (a) and histone-phosphorylating activity (b) of the 58-kD kinase were assayed in the microsomes in the presence or absence of 10 \(\mu\)M ABA or Ca\(^{2+}\) or both; – and + indicate the absence and presence, respectively. D, Effects of apoplastic Ca\(^{2+}\) and influx of Ca\(^{2+}\) on ABA-induced stimulation of ACPK1 kinase. Berry tissues were preincubated in a medium (the equilibration buffer as detailed in “Materials and Methods”) containing 5 mM BAPTA (a Ca\(^{2+}\) chelator, lane 3) or 5 \(\mu\)M A23187 (a Ca\(^{2+}\) ionophore, lanes 5–7) in the presence of Ca\(^{2+}\) (lanes 3, 5, and 7), or 1 mM EGTA in the absence of Ca\(^{2+}\) (lanes 4 and 6), and then ABA (10 \(\mu\)M except for lane 7 where ABA was absent) was added to the medium for a further incubation of 1 h. The incubation in the presence of Ca\(^{2+}\) but absence of ABA (lane 1) was taken as a control, and that in the presence of both Ca\(^{2+}\) and ABA (lane 2) as another control. The microsomes were prepared from the treated tissues. In-gel autophosphorylation (a) and histone-phosphorylating activity (b) of the 58-kD kinase in the microsomes were analyzed in the presence of Ca\(^{2+}\); – and + indicate the absence and presence, respectively.
CaM-like domains, and variable C-terminal tail domain (Fig. 5A; see also Supplemental Fig. 1; Supplemental Table I), demonstrating that the isolated cDNA clone encodes the 58-kD ACPK1 kinase. Based on the cDNA cloning, an ACPK1-specific antiserum against the N-terminal 40 amino acids covering the full variable domain and an adjacent portion of kinase domain (11 amino acids) of ACPK1 was prepared and recognized the 58-kD IEF/SDS-PAGE-purified kinase as mentioned above, confirming that the ACPK1 is encoded by the isolated cDNA clone.

Sequence Features of ACPK1

ACPK1 is a full-length clone with an open reading frame (ORF) having 1,491 nucleotides and encoding a protein of 496 amino acids with a predicted molecular mass of 56 kD and a pI at 5.3. The predicted amino acid sequence exhibits the modular structure typical for CDPKs (Harmon et al., 2001) comprising an N-terminal variable region, a kinase domain, an autoinhibitory junction domain, and a CaM-like domain with four conserved EF-hand motifs implicated in Ca\textsuperscript{2+} binding. Five potential myristoylation sites are present in the full-length amino acid sequence of ACPK1, and two of them are localized in the N-terminal region, suggesting possible membrane-associated nature of ACPK1 via N-terminal myristoylation (Harmon et al., 2001). Myristoylation may be partly responsible for the higher molecular mass of the biochemically identified 58-kD kinase than that of the isolated cDNA clone.

Figure 4. Purification of the ACPK1 kinase. The portions at the 58-kD point (arrow) of SDS-polyacrylamide gels (A, Coomassie Brilliant Blue R-252-stained gels) were excised, crushed, and eluted for purification of 58-kD proteins as described in “Materials and Methods.” The collected 58-kD proteins were subjected to SDS-PAGE (B, Coomassie Brilliant Blue R-252-stained gels), immunorecognized by anti-soybean CDPK\textsubscript{a} serum (C), and detected by in-gel autophosphorylation assay (D). The purified 58-kD proteins were further analyzed for their Ca\textsuperscript{2+}-dependent electrophoretic mobility shift with the assays of SDS-PAGE (E, Coomassie Brilliant Blue R-252-stained gels), in-gel phosphorylation (F), histone-phosphorylating activity (G), and immunoblotting by anti-soybean CDPK\textsubscript{a} serum (H). IEF/SDS-PAGE two-dimensional electrophoresis of the purified 58-kD proteins displays two polypeptides at the 58-kD point but with different pl (I, Coomassie Brilliant Blue R-252-stained gels). One of the two polypeptides (lane c in sections I–L) is shown to be the 58-kD ABA-stimulated Ca\textsuperscript{2+}-dependent protein kinase with the assays of in-gel autophosphorylation (J), histone-phosphorylating activity (K), and immunoblotting by anti-ACPK1-N\textsuperscript{40} serum (see “Materials and Methods”), an antibody specific to the 58-kD ACPK1 kinase (L). The molecular masses of protein standards are shown at the left of the sections in kD. Lane a in section I shows the protein standards on gel; −Ca\textsuperscript{2+} and +Ca\textsuperscript{2+} in sections E to H indicate the absence and presence of Ca\textsuperscript{2+}, respectively.
Figure 5. Alignment and phylogenetic relationships of ACPK1 and its related members of the CDPK family. We added the following prefixes to the names of some cited CDPKs in this paper for distinguishing them easily if their original names do not contain the prefixes: At for Arabidopsis, Gm and GmS (S indicates seed) for soybean, Os for rice, St for potato, Vf for broad bean, and Zm for maize. A, Alignment of deduced amino acid sequences of ACPK1 and one of its closest homologs, soybean seed CDPKa (GmS-CDPKα), shows the presence of conserved features in Ser/Thr (S/T) protein kinase domain, junction domain, and CaM-like domain.
of the predicted 56 kD. Comparison of the deduced ACPK1 amino acid sequence with a soybean CDPK from seeds (GmS-CDPKa) shows a strong homology that extends all over the conserved domains (Fig. 5A). The highest identity is shared with potato (Solanum tuberosum) StCDPK (83%), broad bean Vicia faba CDPK (81%), and two soybean CDPKs (81%), GmCDPKa (soybean CDPKα) and GmS-CDPKa (Fig. 5B). The two soybean CDPKs should be orthologs with amino acid sequence identity of 99% between them (Fig. 5B). The other closer homologs of ACPK1 are Arabidopsis AtCPK4, AtCPK11, and AtCPK12; soybean CDPK β (GmCDPKβ); soybean seed CDPKb (GmS-CDPKb); rice OsCDPK; and maize (Zea mays) ZmCDPK (Fig. 5B).

ACP1 has a lower amino acid sequence identity (53%) with the three previously identified ABA-responsive CDPKs (Fig. 5B; Sheen, 1996; Yoon et al., 1999). The analysis of phylogenetic relationships among the closet homologs of ACPK1 and the three ABA-responsive CDPKs based on amino acid sequence alignment shows that ACPK1 may be classified as a different group from the three previously identified ABA-responsive CDPKs (Fig. 5B).

Genomic Southern Analysis of ACPK1

Genomic Southern-blot analysis was done with a probe corresponding to full-length ACPK1 cDNA. Genomic DNA was digested with BamHI, KpnI, EcoRI, and HindIII restriction enzymes. The probe generated two hybridizing bands in BamHI or KpnI digestion, and four bands in EcoRI or HindIII digestion (Fig. 6). Since there is a catalytic site within the cDNA sequence of ACPK1 for both EcoRI and HindIII, these results suggest that the grape genome contains two copies or more than one copy of ACPK1-related genes.

Characterization of the Products of the ACPK1 Gene and Up-Regulation of Both Its Products and Transcripts by ABA

To confirm that ACPK1 encodes an active CDPK whose expression and activity are stimulated by ABA, the full-length ACPK1 (ACP1L) and the truncated form specific to ACPK1 (residues 1–40; ACPK1-N10) containing only the N-terminal variable region and its adjacent portion in the kinase domain (as mentioned above) were expressed in Escherichia coli as fusion proteins. The ACPK1L protein showed Ca2+-dependent autophosphorylation and histone-phosphorylating activities (Fig. 7A), and also Ca2+-dependent electrophoretic mobilities shift in SDS-PAGE, shown by its autophosphorylation (Fig. 7B). Both the autophosphorylation and histone-phosphorylating activities of the ACPK1L were inhibited by W7 but not by W5, and these activities were apparently not stimulated by CaM (Fig. 7C). Two-dimensional, thin-layer chromatography of the hydrolyzed autophosphorylated ACPK1L showed that 32P-labeled spots corresponded to the positions of phospho-Ser and phospho-Thr (Fig. 7D), suggesting that ACPK1 possesses Ser/Thr kinase

![Figure 5.](Image)

![Figure 5.](Image)

![Figure 6.](Image)

![Figure 6.](Image)
Figure 7. Characterization of the products of ACPK1 gene and up-regulation of both its products and transcripts by ABA. A, The purified recombinant protein (10 μg) of the full-length ACPK1 (ACPK1) was subjected to SDS-PAGE and then assayed for its in-gel autophosphorylation (a) and histone-phosphorylating activity (b) in the absence (−Ca²⁺) or presence (+Ca²⁺) of Ca²⁺. B, Ca²⁺-dependent electrophoretic mobility shift of the ACPK1, shown by its in-gel autophosphorylation; −Ca²⁺ and +Ca²⁺ indicate the absence and presence of Ca²⁺, respectively. C, Neither the in-gel autophosphorylation (a) nor histone-phosphorylating activity (b) is activated by CaM, whereas both activities are inhibited by CaM antagonist W7 but not by W5. CK, Control. D, Phosphoamino acid analysis of the autophosphorylated ACPK1. γ⁻³²P-labeled ACPK1 was hydrolyzed with HCl and subjected to two-dimensional thin-layer electrophoresis. The positions of phospho-Ser (p-Ser), phospho-Thr (p-Thr), and...
activity. Taken together, these data confirmed the nature of ACPK1 as an active CDPK.

The antiserum against either ACPK1L or ACPK1-N40 were prepared in rabbit. Both the anti-ACPK1L and -ACPK1-N40 sera recognized the ACPK1 signal from grape berry (Fig. 7E). The anti-ACPK1L serum can recognize other putative members of CDPK family as both this antiserum and the antiserum against the CaM-like domain of soybean CDPKα detected from apple fruit (Malus domestica) two immunosignals having molecular mass of about 60 and 67 kD, respectively, but the anti-ACPK1-N40 serum could not (Fig. 7E). This showed that the anti-ACP1-N40 serum is specific to ACPK1. The same in vivo incubation assays as used above when testing the ABA-induced 58-kD kinase stimulation were performed to confirm the action of ABA on CDPK1, but the specifically immunoprecipitated ACPK1 protein by anti-ACP1-N40 serum was used for the phosphorylation assays, and also ACPK1 mRNA was probed with 32P-labeled cDNA fragment corresponding to the variable domain of ACPK1 to assess the ACPK1 expression. The results showed that ABA stimulated the ACPK1 kinase in all levels of the gene transcription, mRNA translation and kinase autophosphorylation, and catalytic activities in a dose-dependent manner (Fig. 7F). It is noteworthy that in the immuno- and enzyme assays of ACPK1, the immunoprecipitated proteins with the anti-ACP1L serum gave substantially the same results as those with the ACPK1-specific anti-ACP1-N40 serum (data not shown), suggesting that ACPK1 may be a major CDPK having a predominant abundance in grape mesocarp as was observed above (Fig. 2A). All these assays based on molecular cloning of ACPK1 consistently confirmed the above-mentioned results from the biochemical approaches (Fig. 2A).

ACP1 Expression Is Fruit Specific and Altered Accordingly with Endogenous ABA Concentrations during Fruit Development

Northern-blot analysis showed that ACPK1 expressed only in the grape berry covering two essential portions of the fleshy mesocarp and seeds with much higher abundance of ACPK1 mRNA in the mesocarp than in seeds, but ACPK1 mRNA was undetectable in roots, young stems, or leaves (Fig. 8A). The amounts of mRNA and protein of ACPK1 as well as its autophosphorylation and histone-phosphorylating activities were all altered with changes in the endogenous ABA concentrations in the grape mesocarp during fruit development at different stages: I (early growth stage), II (first rapid growth phase), III (lag growth phase), and IV (second rapid growth phase or ripening stage; Fig. 8B).

ACP1 Is Localized in Both Plasma Membranes and Chloroplasts

Transient expression of the green fluorescent protein (GFP)-ACP1 fusion protein in chloroplast-free epidermis cells of onion (Allium cepa) showed that the fusion protein was targeted to the cell periphery, likely the plasma membrane (Fig. 9A, 1 and 2). A further assay of the transient expression of the fusion protein in Arabidopsis protoplasts showed that the fusion protein was localized to both plasma membranes and chloroplasts (Fig. 9B, 14). The localization of the enzyme in grape mesocarp by immunogold technique with the specific antiserum against ACP1-N40 showed clearly the double-localized ACPK1 in both plasma membranes and chloroplasts (Fig. 9, C–E). No substantial immunosignal was detected in the controls without antisera or using the preimmune serum instead of antisera (data not shown), indicating the specificity and reliability of the immunolocalization.

The ACP1-specific anti-ACP1-N40 serum recognized the ACPK1 in the total membrane proteins (microsomes), purified plasma membrane, and endomembrane fractions, but not in the soluble fraction, whereas the antiserum against the full-length ACP1 (ACP1L) recognized in these membrane fractions not only the 58-kD kinase ACPK1 but also a signal having higher molecular mass than ACPK1 (Fig. 9F). This immunosignal may be the 64-kD phosphoprotein.

Figure 7. (Continued.)

phospho-Tyr (p-Tyr) are indicated. E, Specificity of the anti-ACP1-N40 serum to ACPK1. The 58-kD ACPK1 immunosignal was detected in the microsomes of grape mesocarp (Grape) by both the anti-ACP1L (indicated by AL) and -ACP1-N40 (indicated by AN) sera. In the microsomes of apple flesh prepared with the same procedures as described in “Materials and Methods” for grape mesocarp, two immunosignals having molecular mass of about 60 and 67 kD, respectively, were detected by the antiserum against CaM-like domain of soybean CDPKα (indicated by AS) or anti-ACP1L serum (AL), but not by anti-ACP1-N40 serum (AN). F, ABA up-regulates gene expression, autophosphorylation, and kinase activity of ACPK1. Berry tissues were incubated in the medium containing different concentrations of (±)-ABA from 0 to 80 μM (indicated by ABA applied; unit: μM), and after the incubation, the concentrations of ABA present in the treated tissues (ABA in tissue; unit: μM) were determined by radioimmunoassay as described in “Materials and Methods.” Total RNA and microsomes were extracted from the treated tissues, respectively. Total RNA (20 μg), transferred on a nylon membrane after electrophoresed in an agarose gel, was hybridized with the 32P-labeled cDNA fragment corresponding to the N-terminal variable domain of ACPK1 to detect specifically mRNA level of ACPK1 (ACP1 mRNA). rRNA indicates loading control of the RNA samples stained with ethidium bromide. Total microsomal proteins (50 μg) were immunoprecipitated with 3 μg anti-ACP1-N40 serum protein. The immunoprecipitated proteins were subjected to SDS-PAGE and then to the assays for detecting the levels of ACPK1 protein (ACP1 protein, blotted with anti-ACP1-N40 serum), autophosphorylation (ACP1 Autophosphorylation), or histone-phosphorylating kinase activity (ACP1 Kinase activity) as described in “Materials and Methods.”
detected in the in vitro phosphorylation of the total membrane proteins (Fig. 2A, a), which may be a heavily modified form of 58-kD ACPK1 or a different protein kinase whose content is probably too low to be detectable in the crude extracts of membrane fractions (microsomes) but was sufficiently enriched in the purified membranes to be immunodetected by the anti-ACP1 kinase serum. These data from immunoblotting further demonstrated the specificity of the anti-ACP1-N40 serum to ACPK1 and confirmed the plasma membrane-associated nature of ACPK1. The signal of ACPK1 immunodetected in the endomembrane fraction (Fig. 9F) should be the chloroplast-associated ACPK1. It is finally noteworthy that neither the transmembrane domains nor signal to target ACPK1 to plasma membranes or plastids were predicted for this kinase with several programs (data not shown). So, ACPK1 may be myristoylated in its N-terminal region for targeting to the membranes (Dammann et al., 2003 and refs. therein).

ACPK1 Positively Regulates PM H⁺-ATPase in Vitro

We previously reported that ABA activates plasma membrane (PM H⁺-ATPase) in apple fruit (Peng et al., 2003). The similar results were obtained in grape berry (data not shown). To assess whether ACPK1 is possibly involved in this ABA-induced event, we first showed the presence of a 97-kD plasma membrane-localized H⁺-ATPase in grape mesocarp by both immunogold subcellular localization (Fig. 10, A and B) and immunoblotting of the subcellular fractions (Fig. 10C). This plasma membrane colocalization of the ACPK1 kinase with PM H⁺-ATPase may facilitate possible interactions between the two proteins. A preincubation of the microsomal proteins with the recombinant full-length ACPK1 kinase ACPK1L significantly enhanced the activity of PM H⁺-ATPase in an ACPK1L dose-dependent manner with the activity being doubled in the peaks of the activation (Fig. 10D). The controls of the precipitation in the ACPK1L free- or denatured ACPK1L-containing medium showed no significant effect on PM H⁺-ATPase (Fig. 10D), indicating that the ACPK1-related effects were specific. Removal of free Ca²⁺ from the preincubation medium abolished the PM H⁺-ATPase activation by ACPK1 (Fig. 10D), showing that the PM H⁺-ATPase activation was Ca²⁺ dependent. This is consistent with the nature of the action of a CDPK.

It has been reported that the posttranslational modification of PM H⁺-ATPase involves the C-terminal auto-inhibitory domain of the enzyme, and cleavage of the C-terminal autoinhibitory domain by mild trypsin treatment or genetic deletion activates the enzyme with its pH optimum shifted to more alkaline (Palmgren...
In this experiment, the activation of PM H\textsuperscript{+}-ATPase by ACPK1 was enhanced when the medium pH was shifted from 6.5 to 7.0 (Fig. 10E), and a mild treatment of the microsomal proteins by trypsin also induced a significant PM H\textsuperscript{+}-ATPase activation, which was comparable to the ACPK1 treatment (Fig. 10F). The ACPK1 treatment did not induce any additional activating effect on PM H\textsuperscript{+}-ATPase in the trypsin-treated microsomes (Fig. 10F). These data suggest that ACPK1 regulates PM H\textsuperscript{+}-ATPase by acting on its C-terminal autoinhibitory domain.

To further assess the regulation of PM H\textsuperscript{+}-ATPase by ACPK1, we partially purified PM H\textsuperscript{+}-ATPase, which was tested by SDS-PAGE and immunoblotting (Fig. 10G, lanes 1 and 2). The assay of in vitro phosphorylation of the partially purified fraction in an ACPK1-free medium showed no phosphorylated protein band in the SDS-PAGE gel (Fig. 10G, lane 3), indicating that there was no phosphorylating activity in this partially purified fraction. Using this fraction, we showed that the PM H\textsuperscript{+}-ATPase was phosphorylated by ACPK1 (Fig. 10G, lane 5). Removal of free Ca\textsuperscript{2+} from the in vitro phosphorylation medium completely abolished the PM H\textsuperscript{+}-ATPase phosphorylation (Fig. 10G, lane 4), indicating the Ca\textsuperscript{2+} dependence of this phosphorylation event. These data suggest that ACPK1 activates PM H\textsuperscript{+}-ATPase by phosphorylating the enzyme.
Figure 10. Presence of plasma membrane-localized H^+-ATPase in grape berry and positive regulation of its activity by ACPK1. A, Immunogold labeling of PM H^+-ATPase in the grape mesocarp with antiserum against Arabidopsis PM H^+-ATPase AHA3.
DISCUSSION

Identification of an ABA-Stimulated CDPK, ACPK1, from Grape Berry

Numerous studies have demonstrated that calcium is involved in ABA signal transduction (for review, see Finkelstein et al., 2002; Himmelbach et al., 2003; Fan et al., 2004). However, many of the Ca\(^{2+}\)-binding sensory proteins as the components of the ABA-signaling pathway remain to be elucidated. In this study, we identified and purified a 58-kD ABA-stimulated CDPK from the mesocarp of grape berries (Figs. 1–4), designated ACPK1. Based on sequencing of the two-dimensional electrophoresis-purified ACPK1, we isolated a cDNA clone encoding a putative CDPK (Fig. 5). This cDNA clone was demonstrated to encode ACPK1 essentially by two mutually linked approaches. One is the second time of sequencing of the purified natural ACPK1 protein, showing that the predicted amino acid sequence from the cDNA clone matches the sequence of the natural ACPK1 protein (Fig. 5, see also Supplemental Fig. 1; Supplemental Table I), and another is the assay of immunoblotting where the purified 58-kD ACPK1 kinase was specifically immunorecognized with the anti-ACPK1-N\(^{10}\) serum specific to the full-length putative ACPK1 cDNA-encoded protein (Fig. 4). The analysis of the recombinant full-length protein expressed in E. coli further demonstrated that the isolated ACPK1 cDNA clone indeed encodes an active number of CDPK family (Fig. 7, A–D), and the further assays based on the molecular cloning of ACPK1 revealed that ABA stimulates the ACPK1 kinase in both protein (amounts and activities) and mRNA levels (Fig. 7F), confirming the ABA-induced effects of stimulating the ACPK1 kinase.

Biological Significance of ABA-Induced ACPK1 Kinase Stimulation

The features of the ACPK1 kinase stimulation by ABA are of importance to possible biological functions of ACPK1. The dose dependence of the ACPK1 stimulation induced by a physiological range of ABA concentrations (<3.7 \(\mu M\)) within the tissues (Figs. 3 and 7) is suggestive of action of endogenous ABA on ACPK1. For this action of endogenous ABA, further supporting evidence was provided by correlating the developmental changes of grape berries in their ABA concentrations with their ACPK1 expression and enzyme activities (Fig. 8B). The dose dependence of the ABA-induced ACPK1 stimulation essentially exhibited a saturation-like phenomenon with a slight decline of the ABA-induced effects with the over-physiological higher levels of ABA (>3.7 \(\mu M\)) resulting from excessively applied ABA (20–80 \(\mu M\); Figs. 2 and 7). This phenomenon of saturation, difficult to explain, may be due to a possible CDPK turnover mechanism involving a degradation pathway induced by excessive expression of the enzyme as observed in tobacco NtCDPK2 by Romeis et al. (2001) or probably to a decline of ABA-signaling pathway resulting from a typical saturation-binding nature of ABA to its putative receptor in this fruit (Zhang et al., 1999).

ABA functions in mediating both rapid responses such as stomatal apertures and slow events involving gene expression to regulate growth and development (Finkelstein et al., 2002; Himmelbach et al., 2003; Fan et al., 2004). The rapid stimulation of the ACPK1 kinase in 15 min by ABA without increase of the ACPK1 protein (Fig. 2B) suggests involvement of ACPK1 in mediating some rapid ABA-responsive events, while a slow and steady state of the ABA-stimulated ACPK1 activities paralleling de novo ACPK1 synthesis (Fig. 7).
plasmalemma $\text{H}^+$-ATPase-powered active uptake (Palmgren, 2001). The expression of ACPK1 exclusively in grape berry including the mesocarp and seeds (Fig. 8) appears to be associated with its possible specificity of biological function to this fruit. The expression pattern of ACPK1 during fruit development (Fig. 8) seems to be of particular significance. The lag growth phase stage III is a critical period when intracellular structural changes and active metabolism occur in spite of relatively lag appearance of berry growth, and this has been believed to prepare the onset of ripening (stage IV, see Fig. 8) during which nearly the totality of storage assimilates accumulates and fruit quality and yield are formed (Coombe, 1992; Zhang et al., 1997). The higher levels of ACPK1 in both its protein and mRNA amounts and enzyme activities in accordance with higher ABA concentrations (Fig. 8B) may be connected with its possible involvement in regulating postripening, triggering onset of ripening, and promoting rapid accumulation of carbon reserves during these two phases. It is difficult, however, to explain the high level of ACPK1 at stage I, an early stage after anthesis when berries begin growing, as the biological significance of the peak level of ABA at this stage remains currently unexplainable. Getting an insight into how ACPK1 works in fruit development will be of interest to understand ABA signal transduction in plants.

### MATERIALS AND METHODS

#### Plant Materials and Chemicals
Grape (Vitis vinifera × Vitis L. cv Kyoho) berries and other organs were sampled from a commercial vineyard in the western suburb of Beijing. We divided the sampling period of grape berries into four stages, i.e. early stage of berry growth (10 d after anthesis, stage I), first rapid growth phase (stage II, 10th–30th d after anthesis), lag growth phase (stage III, 30th–50th d after anthesis), and second rapid growth phase or ripening stage (stage IV, 50th–80th d after anthesis; see Fig. 8). Sampling for most of the assays was done during the stage III unless otherwise mentioned. Grape berries were picked for immediate use or frozen in liquid nitrogen and kept at $-80^\circ\text{C}$ until use. Rabbit polyclonal antibodies to the CaM-like domain of soybean Glycine max) CDPKs (Bachmann et al., 1996) were immunopurified on a column of immobilized soybean CDPKs and generously provided by Dr. Alice Harmon (University of Florida, Gainesville, FL). Rabbit polyclonal antibodies against Arabidopsis (Arabidopsis italiana) PM H$^+$-ATPase AHA3 (Pardo and Serrano, 1989) was also a generous gift from Dr. R. Serrano (Universidad Politècnica de Valencia, Valencia, Spain). [γ-32P]ATP was purchased from Amersham Pharmacia. All other chemicals were purchased from Sigma unless otherwise noted.

#### Preparation of Subcellular Fractions

The subcellular fractions were prepared as described by Shen et al. (2004). Grape pericarp was removed from berries to obtain mesocarp that was used to prepare microsomal and cytosolic fractions. Plasma membranes and endomembranes were isolated from the microsomes by an aqueous polymer two-phase system consisting of Dextran T500 and polyethylene glycol (PEG) 3350. Purity and biochemical activity of each subcellular fraction were evaluated by measuring the activity of the marker enzymes to ensure good quality of the subcellular fractions (Shen et al., 2004). Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.
Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out according to the method of Laemmli (1970). To detect Ca2+–induced electrophoretic mobility shifts of CDPK, CaCl2 or EGTA was added into protein samples in SDS-PAGE sample buffer to a final concentration of 2 mM. The protein samples were boiled for 2 min before being analyzed on a 12% SDS-polyacrylamide gel. IEF/SDS-PAGE two-dimensional electrophoresis of the purified CDPK (see Fig. 7) was done essentially according to the method of Zhang et al. (2002). The purified CDPK protein (90 μg) was subjected to IEF and then was applied to a 12% SDS-polyacrylamide gel for the electrophoresis of second dimension. Proteins in the gel were detected by Coomassie Brilliant Blue R-250 (Amersham Pharmacia).

Immunoblotting was done essentially as described by Harper et al. (1994). After SDS-PAGE and IEF/SDS-PAGE, the proteins on gels were electrophoretically transferred to nitrocellulose membranes (0.45 μm, Amersham Pharmacia). The membranes were blocked for 2 h at room temperature with 3% (w/v) BSA and 0.05% (v/v) Tween 20 in a Tris-buffered saline containing 10 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and then were incubated with gentle shaking for 2 h at room temperature in the rabbit polyclonal antibodies (against the CaM-like domain of soybean CDPK, the N-terminal fragment of ACPK1, or Arabidopsis PM H+-ATPase AHAS; all diluted 1:1,000 in the blocking buffer). After being washed three times for 10 min each in the Tris-buffered saline containing 0.05% (v/v) Tween 20, the membranes were incubated with the alkaline phosphatase-conjugated antibody raised in goat against rabbit IgG (diluted 1:1,000 in the blocking buffer) at room temperature for 1 h, and then washed three times for 10 min each with 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 0.1% (v/v) Tween 20. Protein bands were visualized by incubation in the color development solution using a 5-bromo-4-chloro-3-indolyl-P/nitroblue tetrazolium substrate system according to the manufacturer’s protocol.

In-Gel Autophosphorylation and Kinase Assays

Autophosphorylation of proteins in polyacrylamide SDS gels was done essentially as described by Li and Assmann (1996) based on the method of Kameshita and Fujisawa (1989). After SDS-PAGE or IEF/SDS-PAGE as described above, the gels were washed twice with 50 mM Tris-HCl, pH 8.0, containing 20% (v/v) 2-propanol for 1 h per wash, and then with buffer A composed of 50 mM Tris-HCl, pH 8.0, 5 mM mercaptoethanol, and 0.1 mM EDTA for 1 h at room temperature. Proteins in the gels were denatured by incubating the gels in buffer A containing 6 μg guanidine hydrochloride for two incubations of 1 h each at room temperature. Proteins were then renatured using buffer A containing 0.05% (v/v) Tween 20 for six incubations of 3 h each at 4°C. After preincubation at room temperature for 30 min with buffer B composed of 40 mM HEPES-NaOH, pH 7.5, 10 mM MgCl2, 0.45 mM EGTA (1 mM in the Ca2+-free medium), and 2 mM dithiothreitol (DTT) in the absence or presence of 30 μM CaCl2, the gels were incubated with buffer B containing 50 μM ATP and 10 μCi/mL [γ-32P]-ATP (3,000 Ci/mm; Amersham Pharma- cia) for 1 h at room temperature. The gels were then washed extensively with 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate until radioactivity in the used wash solution was barely detectable. The gels were then stained with Cooamassie Brilliant Blue R-250. After destaining, the gels were air dried between two sheets of cellophane, and autophosphorylated CDPK in gels was detected by autoradiography after exposition of the dried gels to Kodak X-Omat AR film for 2 to 3 d at room temperature.

In Vitro Kinase Activity Assay

In the assays to detect Ca2+, Mg2+, and pH dependence of the 58-kD kinase activity (see Fig. 2), kinase activity was analyzed in vitro (in solution) essentially as described previously (Shen et al., 2004) based on the method of Yao et al. (1995). Briefly, microsomes (5 μg) were mixed with histone III-S (0.5 mg mL−1) essentially in the buffer in the absence or presence of 0.55 mM CaCl2, with corresponding modifications in medium pHS and Ca2+ and Mg2+ concentrations when assaying their effects on kinase activity (see Fig. 2). The medium pH was modulated with 50 mM MES-NaOH at 5.5 to 6.5 and with 50 mM Tris-HCl at 7.5 to 9. The phosphorylation reaction (50 μL) was initiated by addition of 25 μM ATP containing 50 μCi/mL [γ-32P]-ATP. After 15 min at room temperature, the reaction was stopped by transferring 20 μL reaction mixture to P81 filter treated by TCA. After an extensive wash in 5% TCA solution at 4°C, P81 filter was dehydrated in ethanol and air dried. The bound radioactivity in the P81 filter was measured with a liquid scintillation counter.

In Vitro Phosphorylation of Total Microsomal Proteins

The microsomes (100 μg proteins) were added to the buffer B (50 mL) described above in the absence or presence of 0.55 mM CaCl2. After turbination, the mixtures were incubated in a water bath at 30°C for 10 min. The reactions were initiated by addition of 25 μM ATP containing 50 μCi mL−1 [γ-32P]-ATP. After further incubation at 30°C for 20 min, the reaction was stopped by addition of 10% (v/v) TCA. After the sample was centrifuged, the pellets were rinsed twice with ice-cold acetone. Precipitated proteins were dissolved in SDS-PAGE sample and boiled for 2 min. The samples were then electophoresed on a 12% SDS-polyacrylamide gel. The gels were air dried between two sheets of cellophane, and phosphorylated proteins in gels were detected by autoradiography after exposition of the dried gels to Kodak X-Omat AR film for 3 d at room temperature.

In Vivo Incubation of Berry Tissues in the ABA-Containing Medium

The treatment of the tissues of grape berry with ABA by an in vivo incubation was done essentially as described by Peng et al. (2003). After washing with tap and distilled water, the freshly harvested berries were precooled to 4°C. Discs of berry mesocarp, 10 mm in diameter and 1 mm in thickness, were prepared with a cork borer. The discs were immediately immersed in the equilibration buffer for 30 min. The equilibration buffer consists of 5 mM MES-NaCl, pH 5.5, 250 mM mannitol, 10 mM MgCl2, 5 mM ascorbic acid, 1 mM EGTA, and 1 mM CaCl2 (±) ABA (or ABA analogs or other plant hormones in the assay to test the specificity of ABA-induced effects, see Fig. 4) was added into the equilibration buffer to give the medium-containing ABA, or distilled water containing the same amount of ethanol for solubilizing ABA was added in this equilibration buffer to give the control medium. The equilibrated discs were incubated in vivo in this ABA-containing medium or in the control medium at a ratio of tissue fresh weight to buffer volume being 1 to 3 (v/v) by gentle shaking at 20°C for 1 h (except when analyzing time course of ABA-induced effects, see Fig. 3). Following washes with double-distilled water, the treated tissues were used immediately for assays or frozen in liquid nitrogen and kept at −80°C until use.

ABA Analysis

Grape tissues were immediately frozen with liquid N2, and then homogenized in double-distilled water at a ratio of sample weight to double-distilled water volume being 1 to 3 at ice-cold temperature. Samples were extracted with oscillation at 4°C in dark for 24 h and then centrifuged for 25 min at 20,000g, and the supernatants were used for the ABA analysis. The ABA assay was carried out using the radioimmunoassay method as described by Quarrere et al. (1988) and used in our laboratory (Peng et al., 2003). The anti-ABA antibody was provided by Dr. Quarrere (Cambridge Laboratory, John Innes Centre, UK). The crude extract (50 mL) was mixed with 200 μL phosphate-buffered saline (PBS; pH 6.0), 100 μL diluted antibody solution, and 100 μL [3H]-ABA solution. The reaction mixture was incubated at 4°C for 25 min, and the bound radioactivity was measured in 50% saturated (NH4)2SO4 precipitated pellets with a liquid scintillation counter. ABA concentration was calculated according to the standard radioimmunoassay curve.

Purification and Sequencing of the 58-kD ACPK1

The microsomal proteins extracted from berry mesocarp were separated on SDS-polyacrylamide gels. The portions of the gels containing the ABA-stimulated 58-kD ACPK1, indicated by the markers of molecular mass and autophosphorylation and immunorecognition signals of this kinase, were as precisely as possible excised and crushed with pestles in microcentrifuge tubes. The elution buffer containing 4 mM HEPES-NaOH (pH 7.5), 0.45 mM EGTA, 0.55 mM CaCl2, 10 mM MgCl2, and 2 mMEDTT was added into the tubes to elute proteins from the gels. The collected eluate, considered as the primarily purified 58-kD kinase, was lyophilized for identification of 58-kD kinase and further purification of the kinase by IEF/SDS-PAGE. For sequencing of the purified kinase, 58-kD kinase spots were excised from five two-dimensional gels (first dimension IEF followed by nondenaturing
Cloning of ACPK1 Gene

The data from the first sequencing by TMS indicated that the sequence of the purified 58-kD kinase matches that of plant CDPKs in their conserved domains, which formed the basis for the cloning of ACPK1 by PCR. For the cloning, single-stranded cDNA was synthesized from total RNA of grape berry using PowerScript reverse transcriptase and SMART III Oligonucleotide/CDSIII as the primer (CLONTECH). Using the cDNA as the template, the main part of a CDPK cDNA was amplified in a PCR reaction with two degenerate primers (forward primer, 5'-GA/G/A/TTCACAGTTGGCAGC-3'; reverse primer, 5'-TTTTCATGCAGAAAT(C/T)TGTCCT-3') that correspond to two of the matched sequences of the 58-kD kinase and also to the conserved domain of most CDPKs (EQIMHMLL- and -KIQSAMNK). PCR products were cloned into PMD-18T plasmid (TAKARA, Dalian Division) and were confirmed by sequence analysis. 5'-RACE PCR was performed by using a nested PCR with gene-specific primers derived from the cloned CDPK 5' end (reverse primer 1, 5'-CCAACAATGCGTTCCATCGGTT-3'; reverse primer 2, 5'-CCCTCAACATCCACATGGAAT-3') and SMART III Oligonucleotide primer as forward primer. 3'-RACE PCR was done with gene-specific primers derived from the cloned CDPK 3' end (forward primer 1, 5'-CTATGCGGAAATTTGCTGATGCGG-3'; forward primer 2, 5'-CCCATGGTCGAGATGATGATG-3') and CDS III as reverse primer. 5'-RACE and 3'-RACE PCR products were cloned into PMD-18T plasmid (TAKARA, Dalian Division) and were sequenced. Splicing three fragments, and thus designing a pair of specific primers (forward primer, 5'-GGCATTCGCCGAGGAGAGAGACGTCTCC-3'; reverse primer, 5'-AGCATCAAATGCCCCAAGAGCTTACCAAG-3'), a full-length cDNA encoding a putative CDPK from grape berry was obtained by PCR amplification. The data of the second sequencing of the purified 58-kD kinase by TMS (see Fig. 6) demonstrated that the cloned putative CDPK cDNA indeed encodes ACPK1.

Northern- and Southern-Blot Analysis

Total RNA (20 μg) was subjected to electrophoresis on formaldehyde/agarose gels and transferred to nylon membranes (Hybond-N+, Amersham Pharmacia Biotech) according to standard protocols. For Southern-blot analysis, genomic DNA was extracted from young leaf tissue using the method of Doyle and Doyle (1990). Ten micrograms of DNA was digested to completion with BamHI, KpnI, EcoRI, and HindIII restriction enzymes, electrophoresed through 0.8% agarose, and blotted onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech). RNA and DNA blots were probed with 32P-labeled full-length cDNA or a cDNA fragment corresponding to the kinase domain. The corresponding regions of the ACPK1 gene-specific primers derived from the cloned CDPK 3' end (forward primer, 5'-CTATGCGGAAATTTGCTGATGCGG-3'; reverse primer, 5'-AGCATCAAATGCCCCAAGAGCTTACCAAG-3') of the cDNA encoding the putative CDPK from grape berry was obtained by PCR amplification. The data of the second sequencing of the purified 58-kD kinase by TMS (see Fig. 6) demonstrated that the cloned putative CDPK cDNA indeed encodes ACPK1.

Expression, Purification, and Antiserum Preparation of Recombinant ACPK1 and ACPK1-N40

Two constructs were generated for the expression of ACPK1 in Escherichia coli as glutathione S-transferase (GST) fusion protein. Full-length ACPK1 (designated ACPK1) corresponds to the protein encoded by the entire ORF and ACPK1-N40 represents the N-terminal fragment of 40 amino acids (pH 7.5) containing 10 μCi Mgl2, 0.45 EGTA, 2 mM DTT, 0.55 mM CaCl2, 50 μM ATP, and 15 μl/μl [γ-32P]-ATP (3,000 Ci/mmole), the protein was precipitated, and then hydrolyzed in 6 N HCl at 110°C for 1 h. After centrifugation, the hydrolysate was dried in vacuum and spiked with phosphoamino acid standards (12 μg each of i-phospho-Ser, phospho-Thr, and phospho-Tyr). The mixture of the samples and the standards was spotted onto two-dimensional thin-layer chromatography plates. The first-dimensional thin-layer electrophoresis was done at 1.5 kV for 20 min in the pH 1.9 electrophoresis buffer, and the second dimension at 1.3 kV for 16 min in pH 3.5 electrophoresis buffer. The plates were dried in an oven at 60°C. Phosphoamino acid standards were visualized by spraying with ninyhydrin (0.25% [w/v] in acetone) followed by heat treatment, and the labeled amino acids were detected by autoradiography.

Immunoprecipitation

The microsomal proteins (50 μg) were resuspended in 0.5 ml immunoprecipitation buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na2HPO4, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg ml⁻¹ antipain, 5 μg ml⁻¹ aprotinin, 5 μg ml⁻¹ leupeptin, and 0.5% Triton X-100. The mixture was incubated with either the purified anti-ACP-K-N40 serum (about 3 μg protein) or the same amount of preimmune serum protein (as a control) at 4°C for 2 h. Then 25 μl protein A-agarose suspension was added to the mixture, and the mixture was incubated further for 2 h. Following a brief centrifugation, the immunoprecipitated proteins, after three washes with the immunoprecipitation buffer, were used for the assays of autophosphorylation, kinase activity, or immunoblotting.

Phosphoamino Acid Analysis

Phosphoamino acids were analyzed essentially as described by Shi et al. (1999). In brief, after ACPK1 (10 μg) was autophosphorylated in 0.5 ml of 40 mM MES-NaOH buffer (pH 7.5) containing 10 mM MgCl2, 0.45 EGTA, 2 mM DTT, 0.55 mM CaCl2, 50 μM ATP, and 15 μl/μl [γ-32P]-ATP (3,000 Ci/mmole), the protein was precipitated, and then hydrolyzed in 6 N HCl at 110°C for 1 h. After centrifugation, the hydrolysate was dried in vacuum and spiked with phosphoamino acid standards (12 μg each of i-phospho-Ser, phospho-Thr, and phospho-Tyr). The mixture of the samples and the standards was spotted onto two-dimensional thin-layer chromatography plates. The first-dimensional thin-layer electrophoresis was done at 1.5 kV for 20 min in the pH 1.9 electrophoresis buffer, and the second dimension at 1.3 kV for 16 min in pH 3.5 electrophoresis buffer. The plates were dried in an oven at 60°C. Phosphoamino acid standards were visualized by spraying with ninyhydrin (0.25% [w/v] in acetone) followed by heat treatment, and the labeled amino acids were detected by autoradiography.

Transient Expression of GFP-ACP1 in Onion Epidermis and Arabidopsis Protoplasts

For observation of the subcellular localization of ACPK1, the full-length ORF of ACPK1 cDNA was PCR amplified by using primers 5'--GGTCAAGATGAGAAGAAATCTGGCAGGAGA-3' (forward primer) and 5'--GGATCCCTTTGTCGAGATGATGATG-3' (reverse primer). The PCR product was then fused to the upstream of the enhanced GFP (Cormack et al., 1996) at the Xhol (5' end)/BamHI (3' end) sites in the cauliflower mosaic virus 35S-EGFP-Ocs 3' vector (p-EZS-NL vector, Dr. Ehhardt, http://deepgreen.stanford.edu). This vector does not express GFP well without adding coding sequence to the 5' end of the ORF of GFP, thus the control cells do not show fluorescence of GFP. Protoplasts were isolated from the leaves of 3- to 4-week-old plants of Arabidopsis (ecotype Columbia) and transiently transformed by using PEG essentially according to Ueda et al. (2001). The strips of onion (Allium cepa) bulb epidermis were bombarded essentially according to Scott et al. (1999) with gold particles coated with plasmids using a Bio-Rad PDS-1000/He particle delivery system. Fluorescence of GFP was observed by a confocal laser scanning microscope (Bio-Rad MRC 1024) after incubation at 23°C for 16 h.

Subcellular Immunogold Labeling

Subcellular immunogold labeling of ACPK1 was done essentially as described previously (Zhang et al., 2004). Briefly, the ultra-thin sections were prepared from Lowicryl KM-embedded specimen. They were first blocked for 30 min at room temperature by floating the grids on droplets of a PBS containing 8 mM Na2HPO4, 1.5 mM KH2PO4, 3 mM KCl, and 500 mM NaCl (pH 7.4) supplemented with 50 mM Glycerol and continuously blocked with the PBS supplemented with 0.1% (w/v) gelatin, 0.5% (w/v) BSA, and 0.1% (v/v) methylsulfonyl fluoride. For the localization of ACPK1, the microsomal proteins were resuspended in 0.5 ml immunoprecipitation buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM Na2HPO4, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 5 μg ml⁻¹ antipain, 5 μg ml⁻¹ aprotinin, 5 μg ml⁻¹ leupeptin, and 0.5% Triton X-100. The mixture was incubated with either the purified anti-ACP-K-N40 serum (about 3 μg protein) or the same amount of preimmune serum protein (as a control) at 4°C for 2 h. Then 25 μl protein A-agarose suspension was added to the mixture, and the mixture was incubated further for 2 h. Following a brief centrifugation, the immunoprecipitated proteins, after three washes with the immunoprecipitation buffer, were used for the assays of autophosphorylation, kinase activity, or immunoblotting.
Twen 20 (pH 7.4: PBGT). Without rinsing, the sections were incubated with rabbit antisera directed against the ACPK1L or ACPK1-N40 or Arabidopsis PM H+-ATPase AHA3 (all diluted 1:100 in PBGT buffer) overnight at 4°C. Following extensive washes with PBGT buffer, the sections were incubated with secondary antibody (goat anti-rabbit IgG antibody conjugated with 10 nm gold) at 1:100 dilution in PBGT buffer for 2 h at room temperature. The sections were rinsed consecutively with PBGT and double-distilled water. The sections were then stained with 2% uranyl acetate in 50% ethanol for 25 min and 25°C with and alkaline lead citrate for 15 min. After extensively washing with double-distilled water, the ultra-thin sections were examined with an electron microscope.

The specificity and reliability of the immunogold labeling were tested by two negative controls. In the first one, the antisera was omitted to test possible unspecific labeling of the goat anti-rabbit IgG antibody-gold conjugate. In the second one, the immune serum was used instead of the rabbit antisera before immunogold labeling to test the specificity of the antisera. More than three repetitions of the control experiments were conducted for each sample.

**Assays of Effects of ACPK1 on PM H+-ATPase Activity**

PM H+-ATPase activity was assayed as described by Oliviari et al. (2000) with modifications. The reaction medium (50 μL) contains 5 mM MgSO4, 0.1 mM Na2MoO4, 50 mM KNO3, 1 μg mL−1 oligomycin, 5 μM carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone, 50 μg mL−1 polyoxyethylene-20-cetyl ether (Brij 58), 40 mM 1,3-bis[1-(hydroxymethyl) methylamino]propane (BTP)-MES (pH 6.5) or BTP-HEPES (pH 7.0), and 50 μg microsomal protein. A given amount of EGTA or CaCl2 was added to the reaction medium as indicated (see text below). The reaction was started by adding 3 mM ATP plus or minus 200 μM vanadate, and the assay was performed at 30°C over 1 h of incubation. The PM H+-ATPase activity was evaluated as the activity inhibited by vanadate. Released inorganic phosphate produced from ATP hydrolysis was assayed as described by De Michielis and Spanswick (1986).

To assess the effect of ACPK1 on PM H+-ATPase activity and Ca2+ dependence of this effect (see Fig. 10D), the microsomal proteins (only 50 μg control 1) or the microsomal proteins (50 μg) together with either 10 μg denatured ACPK1L protein (boiling for 10 min, control 2) or 10 μg ACPK1L protein were added to the above-described reaction medium supplemented with 0.1 mM ATP and 0.1 mM CaCl2 or in the presence of 0.1 mM CaCl2, but with 0.3 mM EGTA, and the mixtures were subjected to a 15-min preincubation at 20°C before adding ATP and 0.3 mM EGTA to start the reaction at pH 6.5. The pH dependence of PM H+-ATPase activation (see Fig. 10E) was assayed essentially in the same way where the mixture of ACPK1 and microsomal proteins was preincubated in the presence of 0.1 mM CaCl2.

For the trypsin treatment assay (see Fig. 10F), microsomal proteins (2 mg mL−1) were incubated at 20°C with 100 μg mL−1 trypsin for 10 min in a medium containing 20 mM BTP-HEPES (pH 7.0), 5% glycerol, 0.1 mM CaCl2, 100 μg mL−1 Brij 58, 0.5 mM DTT, and 1 mM ATP. The reaction was blockaded by addition of 0.5 mg mL−1 soybean trypsin inhibitor. The control was run in the absence of trypsin. The treated microsomal proteins were used for assaying PM H+-ATPase activity as described above in the medium at pH 7.0 with 0.3 mM EGTA after a preincubation of ACPK1 and microsomal proteins in the presence of 0.1 mM CaCl2.

To assay phosphorylation of PM H+-ATPase by ACPK1 (see Fig. 10G), we partially purified PM H+-ATPase essentially according to the technique described by Jahn et al. (1997). Briefly, plasma membrane proteins containing PM H+-ATPase solubilized by dodecyl β-D-maltoside were precipitated in PEG 3350, and the precipitated proteins were subjected to a Sephacryl S-300 HR gel filtration column to obtain a partially purified PM H+-ATPase fraction. In vitro phosphorylation of the PM H+-ATPase fraction by ACPK1 was assayed as described above for “In Vitro Phosphorylation of Total Microsomal Proteins” with addition of 50 μg partially purified PM H+-ATPase and 50 μg ACPK1L protein to the buffer B (30 μL). The control was run in the absence of ACPK1 protein.

**Accession Numbers**

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers or locus tags numbers (for Arabidopsis only) with some references related to the gene cloning or characterization being cited in parenthesis: ACPK1, AY394009; ACPK4, At4g09570 (Hrabek et al., 1996); ACPK10, At1g18890 (formerly At1CDPK1; Sheen, 1996); ACPK11, At1g35670 (formerly At1CDPK2); ACPK12, At5g25380

**LITERATURE CITED**


Abscisic Acid-Stimulated Calcium-Dependent Protein Kinase
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Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K (1994) Two genes that encode Ca2+-dependent protein kinases are induced by drought and high-salt stresses in Arabidopsis thaliana. Mol Gen Genet 244: 331–340


