Molecular identification of phenylalanine ammonia-lyase as a substrate of a specific constitutively active *Arabidopsis* CDPK expressed in maize protoplasts

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Abstract Phenylalanine ammonia-lyase (PAL) is a key enzyme in pathogen defence, stress response and secondary metabolism and is subject to post-translational phosphorylation. In order to address the significance of this phenomenon it is necessary to identify the protein kinase (PK) responsible and place it in its regulatory circuit. Using protoplast transient expression of *Arabidopsis* kinase genes coupled to immunocomplex kinase assay, it has been possible to screen for specific PAL kinase. We show here that AtCPK1 (calcium dependent PK), but not other closely related PKs could phosphorylate both a recombinant PAL protein and a peptide (SRVAKTRTLTTA) that is a site phosphorylated in vivo. Identification of the specific CDPK as a PAL kinase now opens up the possibility of exploring the calcium link in biotic stress signalling, salicylate and phytoalexin production as well as the significance of PAL phosphorylation. The protoplast transient expression system is a potentially powerful method to determine and screen for plant gene functions utilising genomic and proteomic data.

Key words: Phenylalanine ammonia-lyase; Protein kinase; Calmodulin-like domain protein kinase; *Arabidopsis*

1. Introduction

Phenylalanine ammonia-lyase (PAL) is the first enzyme of phenylpropanoid biosynthesis and is therefore a key step in the biosynthesis of flavonoids, lignins, stilbenes and many other compounds. It is also probably involved in the biosynthesis of signalling molecules, notably salicylate. Regulation of the enzyme is complex and includes differential transcriptional activation of members of the gene family, stability of mRNA and post-translational control. These processes include modulation by phenylpropanoids of transcription and also of proteolytic turnover. A further post-translational regulation has been discovered recently that involves phosphorylation [1,3,4,7]. This phosphorylation seems to have a limited effect on the kinetics of PAL in vitro [1] and may be related to the increased rate of turnover in vivo in cells subject to biotic stress through application of fungal elicitors [3]. However, this latter effect may be coincidental. The possibility exists that phosphorylation targets PAL to membranes, as has been shown for enzymes such as sucrose synthase [22], and membrane proteins have been shown to turn over faster in microsomal fractions following elicitation [17]. Association of PAL with membranes has long been postulated as several of the subsequent enzymes of the phenylpropanoid pathway are endoplasmic reticulum localised and evidence for this and consequent metabolic channelling of intermediates has been presented [16].

Biochemical evidence has accumulated that confirms the PAL kinase from bean is a serine/threonine type [1,4,7]. Purification and further biochemical characterisation have shown that the PAL kinase from French bean is probably a calmodulin-like domain protein kinase (CDPK). However, insufficient protein was purified to acquire protein sequence to allow molecular cloning and absolute identification. As an alternative approach a number of heterologously expressed protein kinases [21] were tested for their ability to phosphorylate the model peptide having the known phosphorylated sequence in PAL [1] and recombinant PAL itself. Transient expression of kinases in protoplasts has been innovative in placing individual kinases in the signal transduction pathway for specific stimuli for both CDPKs [21] and components of the MAP kinase cascade [14,15]. The versatility of the system and the significance of its development have been recognised [9]. Transient expression of constitutively active kinase can also be used to link kinases with targets. In this way, a specific CDPK has been identified as capable of phosphorylating PAL in a specific manner in the present work.

2. Materials and methods

2.1. Materials

Purified recombinant poplar PAL was a kind gift of Prof. B.E. Ellis, University of British Columbia, Vancouver, Canada as described previously [1]. Syntide-2 and histone III-S were from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) was from NEN.

2.2. Expression of cloned protein kinases

Eight individual protein kinases as described previously [21] were expressed in etiolated maize protoplasts. All kinases were PCR products polymerised using the primers described previously [21]. Briefly, each kinase tested was inserted into a vector [21] after a 35SC4PPDK promoter and before the DHA tag and nopaline synthase terminator

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Abbreviations: PAL, phenylalanine ammonia-lyase; CDPK, calmodulin-like domain protein kinase

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sequence. Constitutive kinase sequences were numbered as described previously [21], but the CDPKs are also identified using newly recommended nomenclature [9,11]. Kinases expressed were ATCDPK (AK1), now AtCPK1, amino acids 1–413; ATCDPK1, now AtCPK10, amino acids 1–274; AtCDPK1a, amino acids 1–274; ATCDPK2, now AtCPK11, amino acids 1–289; ATPKa, amino acids 1–284; ATPKb, amino acids 1–283; ASK1, amino acids 1–265; ASK2, amino acids 1–265. A control vector containing a 35S promoter-green fluorescent protein (GFP) construct was used to measure the efficiency of transformation of protoplasts [21]. About 0.5×10^5 protoplasts were routinely transformed with 30 μg DNA for each sample processed for immunoprecipitation at an average efficiency of about 30%. Details of the preparation of protoplasts and their transformation have been described previously [6,20].

2.3. Immunoprecipitation of protein kinases

Following transformation protoplasts were incubated for 16 h in the light (15 μE m^{-2} s^{-1}). Protoplasts were harvested and used fresh or following freezing at −20°C. Immunoprecipitation was carried out as described previously using an anti-double haemagglutinin (DHA) monoclonal antibody and protein A-Sepharose [14]. Beads were extensively washed to give the pure recombinant kinases [21].

2.4. Kinase activity

Kinase activity was assayed using a modification of the method of Roskoski [19]. Peptides or proteins were incubated at various concentrations in 50 mM MOPS pH 7.5, 10 mM $MgCl_2$, 1 μM Microcystin-LR, 100 μM [γ-^{32}P]ATP (0.05 μCi) with immunoprecipitated kinases in a total volume of 50 μl. Following incubation 40 μl aliquots were taken and spotted onto Whatman P81 phosphocellulose squares (2×2 cm) and immersed in 75 mM phosphoric acid to terminate the reaction. Squares were washed for 10 min, then washed with two further changes of phosphoric acid for 10 min each, air-dried and then subjected to liquid scintillation counting.

2.5. In-gel assay

Protoplasts were harvested and homogenised in extraction buffer (50 mM HEPES KOH (pH 7.6), 2 mM DTT, 2 mM EDTA, 20 mM β-glycerophosphate, 20% glycerol (v/v), 1 mM sodium vanadate, 1 mM NaF and 50 μg/ml protease inhibitors (Complete, Boehringer Mannheim)). The homogenate was centrifuged at 14 000×*g* for 10 min and the protein concentration determined for the supernatant. 20 μl samples were loaded directly on the gel or following incubation with 5 μl 2× standard SDS–PAGE sample buffer for 1 h at room temperature. Immunoprecipitates were also resuspended in 20 μl extraction buffer and incubated with 5 μl 2× standard SDS–PAGE sample buffer for 1 h at room temperature. Samples were separated on 10% acrylamide gels containing 100 μg/ml recombinant poplar PAL or 200 μg/ml histone III-S. Following electrophoresis, the gels were washed in 25 mM Tris–HCl pH 7.5, 0.5 mM DTT, 1 mM sodium vanadate, 5 mM NaF, 0.5 mg/ml BSA, 0.1% Triton X-100 while shaking at room temperature for 3×30 min. The gels were then equilibrated with 25 mM Tris–HCl pH 7.5, 0.5 mM DTT, 1 mM sodium vanadate, 5 mM NaF, at 4°C overnight and then incubated with 25 mM Tris–HCl pH 7.5, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM DTT and 1 mM sodium vanadate. The buffer was replaced with an identical solution containing 50 μCi [γ-^{32}P]ATP and incubated for 60 min at room temperature while shaking. The gels were fixed and extensively washed in 5% trichloroacetic acid containing 1% sodium pyrophosphate for at least 6 h at room temperature. The gels were dried and subjected to autoradiography.

3. Results

3.1. Activity of eight heterologously expressed kinases towards PAL peptide and syntide-2

Eight protein kinases from *Arabidopsis*, consisting of four CDPKs and four other protein kinases engineered for constitutive expression and previously shown to be active in vivo [21], were transformed in the present work into maize protoplasts and isolated by immunoprecipitation for testing in vitro. Following extensive washing, each kinase could be isolated in a pure form [21] and tested as an active immobilised

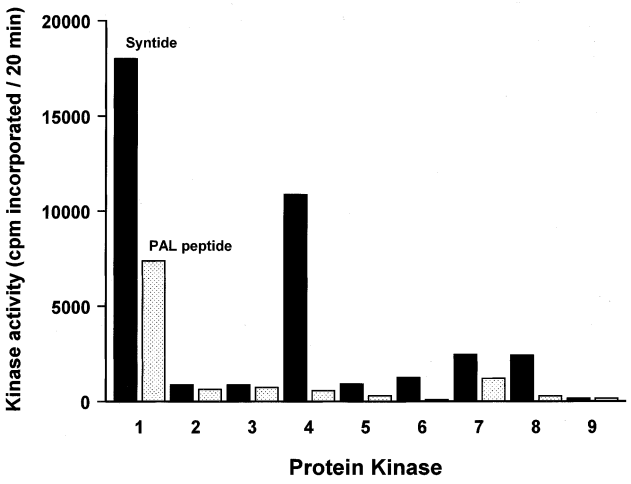

Fig. 1. Activity of eight expressed protein kinases towards syntide-2 and PAL peptide. Kinases were transiently expressed in maize protoplasts and subjected to immunoprecipitation. Kinases were as follows: 1, ATCDPK (AK1; AtCPK1); 2, ATCDPK1 (AtCPK10); 3, CDPK1a [21]; 4, CDPK 2 (AtCPK11); 5, ATPKa; 6, ATPKb; 7, ASK1; 8, ASK2. Lane 9 shows the activity with the immunoprecipitate from extracts of protoplasts transformed with the GFP control vector construct [21].

immunocomplex against PAL peptide (SRVAKTRTLTTA) and syntide-2 (PLARTLSVA). The expression of these epitope-tagged CDPKs and the purity of the immunoprecipitated kinases in the maize protoplast transient expression system have been well documented [21]. Phosphorylated peptide was bound to phosphocellulose paper and washed extensively. Incorporation for each substrate is shown for the kinases relative to the extract from protoplasts transformed with the GFP construct and treated in an identical manner (Fig. 1). This shows that only ATCDPK, using previous nomenclature [21], and which is also known as AK1 or AtCPK1 [9], phosphorylated both peptides with high efficiency. ATCDPK2 (AtCPK11 [9]) showed high activity towards syntide but not PAL peptide. Some low activity was shown towards syntide-2 by ASK1 and ASK2, but this was only 10% of that shown by ATCDPK. ASK1 showed slight activity towards PAL peptide but this too was only 10% of that shown by ATCDPK. This identifies ATCDPK (AK1; AtCPK1) as a likely PAL kinase. ATCDPK1 (AtCPK 10) and ATCDPK1a were previously shown to be activated in response to abscisic acid treatment of the protoplasts [21].

3.2. Time course and kinetics of ATCDPK activity using PAL peptide as substrate

A series of biochemical determinations were carried out on immobilised ATCDPK. Fig. 2 shows that incorporation into PAL peptide was linear up to 30 min and thus enabling determination of kinetic parameters. There was minimal background activity in control incubations without peptide using the phosphocellulose-binding assay. Phosphorylation of PAL peptide and syntide-2 by immunoprecipitated ATCDPK showed K_m values towards ATP of 26 and 40 μM respectively and typical of many protein kinases. However, either the engineering of ATCDPK to remove the regulatory site or the fact that it was assayed as an immunocomplex led to differences in substrate specificity compared to the native bean enzyme. The K_m values towards syntide-2 and PAL peptide

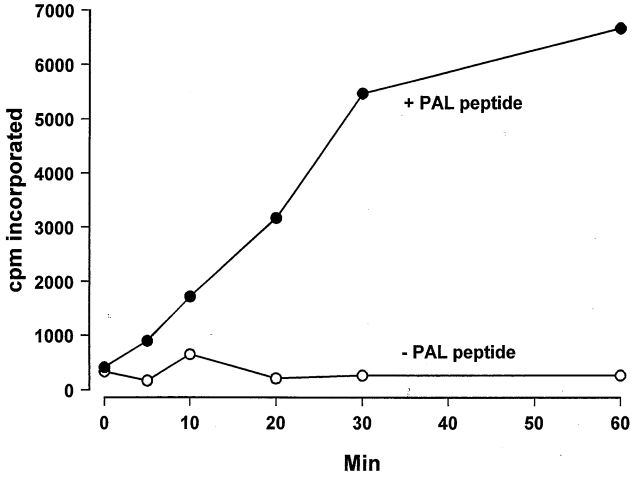

Fig. 2. Time course of activity of immunoprecipitated ATCDPK (AK1; AtCPK1) towards PAL peptide (2 mM).

were much higher than for the purified bean PAL-kinase enzyme [1]. The K_m for syntide was 311 μM compared with 7.7 μM, while that for PAL peptide was 191 μM compared with 7.5 μM.

3.3. Activity of ATCDPK towards protein substrates and in-gel phosphorylation of PAL by ATCDPK

There is a choice of methodologies for demonstrating phosphorylation of specific protein targets. This can be carried out by incubating the kinase and target in the presence of [γ-^{32}P]-ATP, measuring incorporation and subsequent analysis by SDS–PAGE (in-tube assay). In the present case, phosphorylation of recombinant PAL protein and histone III-S (both 100 μg/ml) by ATCDPK gave much higher control values than for PAL peptide (2 mM) using the acid precipitation assay, presumably due to greater substrate entrapments by the precipitated protein. This was reflected in high backgrounds on analysis by SDS–PAGE of PAL protein phosphorylated by the in-tube assay as demonstrated previously [1], although ATCDPK showed much higher phosphorylation than the other kinases (data not shown). This background may also be due to a probable kinase contaminant in the recombinant poplar PAL preparation from insect cells [1]. Therefore in-gel assays were chosen to investigate specific phosphorylation of PAL protein as this methodology was not subject to such background interference [1].

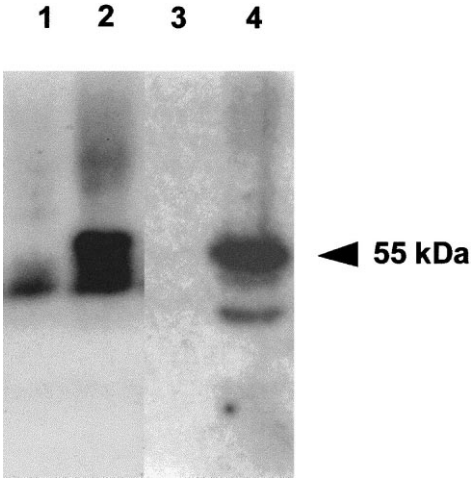

Fig. 3. Optimisation of the in-gel assay for expressed ATCDPK (AK1; AtCPK1) using histone III-S as substrate. Protoplasts were transformed with GFP or ATCDPK vectors and used directly without being frozen. Crude extracts or immunoprecipitated kinase were subjected to in-gel assay. Lanes: 1, crude extract from protoplasts transformed with GFP construct; 2, crude extract from protoplasts transformed with ATCDPK; 3, immunoprecipitate of the extract GFP; 4, immunoprecipitate of the extract from protoplasts transformed with ATCDPK. Gels were 10% with respect to acrylamide.

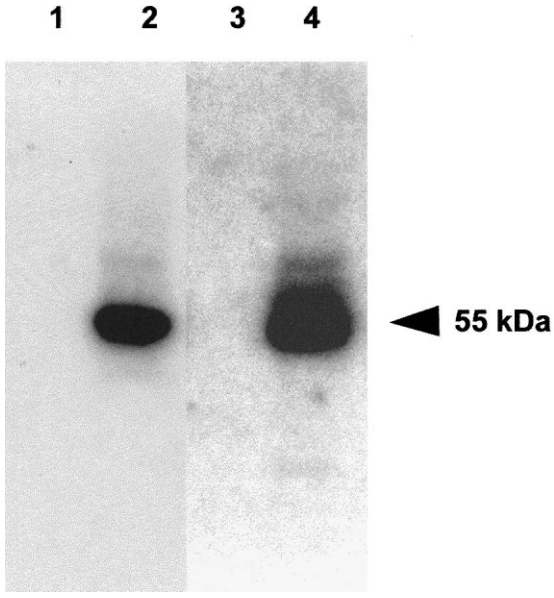

Fig. 4. In-gel assay of expressed ATCDPK (AK1; AtCPK1) in protoplasts following freeze thawing. Lanes: 1, crude extract from protoplasts transformed with GFP construct and histone III-S as substrate; 2, crude extract from protoplasts transformed with ATCDPK with histone III-S as substrate; 3, crude extract from protoplasts transformed with GFP construct with PAL as substrate; 4, crude extract from protoplasts transformed with ATCDPK with PAL as substrate. Gels were 10% with respect to acrylamide.

In-gel assays for the transiently expressed ATCDPK were first optimised using histone III-S as substrate due to limitations in the amount of recombinant PAL available. Fig. 3 shows in-gel assays of extracts from protoplasts that were transformed with either control vector carrying the GFP transcript or the vector containing in-frame ATCDPK engineered for constitutive activity. The GFP control was also used to confirm the level of transformation as at least 30% in each case. A number of CDPK bands that were common to both control and ATCDPK expression were observed when protoplasts were extracted directly (Fig. 3, lanes 1 and 2). However, the band corresponding to ATCDPK is clearly visible at M_r 55 000 in the latter case which corresponds to the migration of the expressed protein [21]. The identity of the ATCDPK was confirmed by immunoprecipitation followed by extraction into SDS–PAGE sample buffer under mild conditions followed by in-gel assay (Fig. 3, lanes 3 and 4). However, it was also found that when the protoplasts were extracted after freezing the endogenous background was reduced, leaving the main signal due to the M_r 55 000 ATCDPK (Fig. 4, lanes 1 and 2). It was not possible to carry out an extensive study using recombinant PAL due to limitations in its availability, so the chosen method was to use frozen protoplasts to maximise recoveries and avoid losses during immunoprecipitation

(Fig. 4, lanes 3 and 4). A band migrating at the same position as with histone III-S and specific to the expressed ATCDPK is clearly present with PAL as substrate, confirming the ability of ATCDPK to phosphorylate PAL as indicated using in-tube assays and the data relating to PAL peptide.

4. Discussion

Calcium-dependent protein kinases are a large gene family in plants and they differ from their animal counterparts in that the regulatory domains are part of the same polypeptide responsible for substrate recognition and phosphorylation [9]. We have recently shown that a PAL kinase can be purified from cells of French bean that was preliminarily characterised as a CDPK [1] and subsequently confirmed as a member of a family of CDPKs that undergo changes in activity following elicitor activation (Allwood, E.G., Davies, D.R., Bolwell, G.P., unpublished data). As with many protein kinases, the challenge is to identify the kinase responsible for each target protein at the molecular level and place it in an appropriate regulatory circuit. For various reasons, it has not yet been possible to accomplish the molecular cloning of the French bean kinase, so an alternative approach was used to identify a CDPK that could phosphorylate PAL utilising transient expression of constitutively active kinases in a heterologous mode [21]. It is possible to engineer constitutively active CDPKs from *Arabidopsis* so that they are expressed in a robust maize protoplast system [21]. In the present paper, it has been possible to demonstrate that one *Arabidopsis* CDPK, ATCDPK, which is now designated AtCPK1 [9,11], can phosphorylate a peptide corresponding to the target site on French bean PAL as well as recombinant poplar PAL protein. The highly heterologous nature of this system is consistent with PAL phosphorylation being a ubiquitous phenomenon. Although no crystal structure has been determined yet for PAL, it is available for *Pseudomonas putida* histidine ammonia-lyase (HAL). Following alignment of the sequences, the equivalent motif to the phosphorylation site on poplar PAL present in HAL can be modelled onto the polypeptide surface showing its availability for kinase action (Ellis, B.E., personal communication) and may be a feature of ammonia-lyases in general. The methodology presented in this paper also shows that immunoprecipitated kinases retain activity so they can be screened for targets. There are many more CDPKs and PKs arising from the genomic sequencing projects that could be similarly screened and their functions confirmed in plants using knockout mutations and transgenic plants.

Fortuitously, AtCPK1 and its homologue from soybean, CDPKα, are extremely well-described kinases [9] especially with respect to regulation by Ca^{2+} [8,10,12,23], the calmodulin domain [2,23] and phospholipids [2,10]. However, its physiological function is to our knowledge as yet unknown [9], although AtCPK1 has been shown recently to inhibit a calmodulin-stimulated Ca^{2+} pump that is endoplasmic reticulum located in *Arabidopsis* when they are expressed in yeast [13]. Indeed, using the maize transient expression system, of the four CDPKs expressed, only AtCPK10 (kinase 2 in Fig. 1) has been placed in a regulatory circuit so far [21], being involved in activating the expression of a stress, Ca^{2+}- and ABA-responsive reporter gene. However, other CDPKs have been shown to be involved in responses to biotic stress. M_r 68 000 and 70 000 CDPKs were shown to undergo reciprocal changes in activity following elicitation in the Avr9/Cf9 transformed tobacco system [18], suggesting a role for individual CDPKs in responses to biotic stress. Furthermore, the French bean CDPK that phosphorylates PAL is regulated by elicitation [1]. Elicitation opens Ca^{2+} channels leading to an influx of the cation, a process which is coupled to the recognition process possibly through cAMP as a signal [3,5]. The CDPK is then activated, phosphorylating PAL. Without molecular sequence data for the French bean kinase, similarities to AtCPK1 cannot be determined. By analogy therefore it is possible that AtCPK1 functions in stress responses including biotic stress. This would also be compatible with its activation by lipids [2,10] since there is growing evidence that lipid signals are also important in biotic stress besides ion fluxes [5]. The ability to phosphorylate PAL, which is a key enzyme in defence responses against plant pathogens such as the production of isoflavonoid phytoalexins in leguminous species, cell wall phenolic material and probably the formation of secondary intercellular signals is further evidence for a role in biotic stress. However, the precise function of PAL phosphorylation is still under investigation.

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