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Gatekeeper tyrosine phosphorylation is autoinhibitory for Symbiosis Receptor Kinase

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ABSTRACT

Plant receptor-like kinases (RLKs) are distinguished by having a tyrosine in the 'gatekeeper' position. Previously we reported Symbiosis Receptor Kinase from *Arachis hypogaea* (*Ah*SYMRK) to autophosphorylate on the gatekeeper tyrosine (Y670), though this phosphorylation was not necessary for the kinase activity. Here we report that recombinant catalytic domain of *Ah*SYMRK with a phosphomimic substitution in the gatekeeper position (Y670E) is catalytically almost inactive and is conformationally quite distinct from the corresponding native enzyme. Additionally, we show that gatekeeper-phosphorylated *Ah*SYMRK polypeptides are inactive and depletion of this inactive form leads to activation of intramolecular autophosphorylation of *Ah*SYMRK. Together, our results suggest gatekeeper tyrosine autophosphorylation to be autoinhibitory for *Ah*SYMRK.

Structured summary of protein interactions:
AhSYMRK and **AhSYMRK** bind by molecular sieving (1, 2)
AhSYMRK and **AhSYMRK** phosphorylate by protein kinase assay (View interaction)

1. Introduction

Gatekeeper position in protein kinases is located on a conserved β5 strand, distal to the active site and is adjacent to an intrinsically flexible hinge region that connects the N- and C-terminal lobes of the enzyme [1–3]. The position is referred to as a gatekeeper because it flanks a hydrophobic pocket at the rear of the catalytic cleft and confers selectivity for binding nucleotides and small-molecule inhibitors in the pocket [4,5]. Apart from being the architect of this deep cleft, gatekeeper position has gained more importance because several evidences indicate that protein kinases have adopted different mechanisms of regulation mediated by their respective gatekeeper residue and/or the hinge region [4,6]. Bulkier hydrophobic residue at gatekeeper position has been shown to activate tyrosine kinases whereas smaller residue, like glycine, causes inactivation [6]. Structural analysis suggested that the bulkier residue stabilises the active conformation by strengthening a 'hydrophobic spine', whereas the smaller residue inactivates the kinase by disruption of the same hydrophobic connectivity [4]. Emrick et al. had demonstrated the role of the gatekeeper residue in restraining auto-activation of ERK2 in the absence of upstream signalling [7]. Recent evidences have directly demonstrated that backbone flexibility within the hinge region adjacent to gatekeeper residue is an important determinant of ERK2 activation [8].

Plant receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) share a common feature with the animal interleukin-1 receptor-associated kinase (IRAK)/Pelle family of soluble kinases that distinguishes them from all other kinases: the presence of a Tyr residue in their gatekeeper position [9]. Several evidences have indicated the importance of gatekeeper tyrosine in determining the active conformation of these receptor or non-receptor kinases. In IRAK4, the invariant glutamate from α-helix C forms hydrogen bonds with gatekeeper Tyr as well as the Phe residue in the DFG motif, suggesting the importance of gatekeeper Tyr in regulation of this kinase [10,11]. Structural analysis of gatekeeper residue arrangement in BRASSINOSTEROID-INSENSITIVE 1 (BRI1) and BRI1-associated kinase 1 (BAK1) of *Arabidopsis thaliana*

Abbreviations: RLK, receptor-like kinase; RTK, receptor tyrosine kinase; IRAK, interleukin-1 receptor-associated kinase; SYMRK, Symbiosis Receptor Kinase; WT, wild-type; Trx, thioredoxin; CD, circular dichroism

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suggests that hydrogen-bond interactions of the hydroxyl group of the invariant gatekeeper tyrosine with the conserved Lys/Glu salt-bridge (residue 911/927 and 317/334 in BRI1 and BAK1, respectively) are hall-marks of activated plant receptor-like kinases [12,13]. The importance of gatekeeper tyrosine in the functional outputs of RLKs/RLCKs is also evidenced. For example, structure–mimic substitutions of gatekeeper Tyr–Phe in BRI1 (Y956F) [14], or in an RLCK like Botrytis-induced kinase 1 (BIK1, Y150F) [15], blocks their *in vitro* kinase activity as well as biological function. In lysin motif domain-containing receptor like kinase-3 (LYK3), a gatekeeper mutant (Y390F) retained partial kinase activity, but mutant LYK3 was biologically active [9]. In contrast, in gatekeeper mutants Y363F of BAK1 [16] or Y670F of Symbiosis Receptor Kinase of *Arachis hypogaea* [17], the catalytic potential of the RLKs remain unaffected. This indicates that unlike BRI1 or BIK1, for RLKs like SYMRK or BAK1, the H-bonding network involving the free hydroxyl group of gatekeeper tyrosine was not essential for their *in vitro* catalytic activity. Considering the contrasting effects of gatekeeper substitution in RLKs, the involvement of free hydroxyl group of gatekeeper tyrosine in an H-bonding network cannot be considered as a quintessential signature of the activated state for all RLKs.

Symbiosis Receptor Kinase (SYMRK) is an orphan RLK involved in root nodule symbiosis [18,19]. Earlier, we have demonstrated that gatekeeper Tyr (Y670) is the predominant site of tyrosine autophosphorylation in *A. hypogaea* SYMRK (*Ah*SYMRK) both *in vitro* and *in planta* [17]. Catalytic activity of Y670F mutant of *Ah*SYMRK, indicated that autophosphorylation on gatekeeper Tyr was not a prerequisite for *Ah*SYMRK to be an active kinase [17]. Here we report that recombinant catalytic domain of *Ah*SYMRK with a phosphomimic substitution in the gatekeeper position (Y670E) is conformationally distinct with an almost loss of catalytic activity. We also show that the gatekeeper-phosphorylated *Ah*SYMRK polypeptides were almost inactive indicating that the negative charge of the phosphate group negatively affects the catalytic activity of *Ah*SYMRK. Depletion of this inactive form activated intramolecular autophosphorylation of *Ah*SYMRK, suggesting gatekeeper tyrosine autophosphorylation to be autoinhibitory for this RLK.

2. Experimental procedures

2.1. Cloning, mutagenesis, expression and purification of recombinant AhSYMRK

Amplification, sub-cloning, mutagenesis, expression and purification of recombinant *Ah*SYMRK kinase domain polypeptides were performed as described previously [17]. The primers used for developing Y670E mutant were F-5′GATTCTCGTGGAGCCTTTTAT GTC3′ and B-5′GACATAAAAGGCTCCACGAGAATC3′. Purification of *Ah*SYMRK and its mutant polypeptides through FPLC was performed on Superose 6 10/300 GL column (GE Healthcare) at a flow rate of 0.5 ml/min using a buffer containing 25 mM Tris–HCl at pH 8.0, 150 mM NaCl and 2.5 mM EDTA and 250 μl fractions were collected. Standards for the gel-filtration column (Gel Filtration Calibration Kit, GE Healthcare) were Ferritin (440 kDa), Aldolase (158 kDa), Ovalbumin (44 kDa), and Carbonic Anhydrase (29 kDa). Eluted fractions corresponding to each peak were collected and distributed into 25 μl aliquots, quickly frozen in dry ice and stored at −80 °C for further analysis.

2.2. Kinase assay and phosphoamino acid analysis

Kinase autophosphorylation assays were carried out as described earlier [17,20,21]. Immunoblotting was conducted as per manufacturer's instructions with monoclonal α-pY (1:3000) and polyclonal α-pT (1:3000) from Cell Signaling Technology, polyclonal α-His_6 (1:2000) and monoclonal α-pS Q5 (1:100) from Qiagen, polyclonal α-SYMRK (raised in rabbit against the cytosolic domain of *Ah*SYMRK as described earlier [22]) and custom-made antibody against the synthetic peptide (665-QQILVpYPFMS-674) (Imgenex India) [17]. To monitor the phosphorylation status, Pro-Q Diamond Phosphoprotein Stain (Invitrogen) was used according to manufacturer's protocol and data were obtained using Typhoon Trio+ Scanner (GE Heathcare).

2.3. Circular dichroism (CD)

His_6-*Ah*SYMRK (wild-type (WT)) and its mutants (Y670F, Y670E and K625E) were dialyzed overnight against 5 mM Tris–HCl (pH 8.0) and 25 mM NaCl at 4 °C. Far-UV spectra with excitation wavelengths ranging from 200 to 260 nm were recorded for each protein (final concentration 0.1 mg/ml) in Jasco J-815 CD Spectrometer, in a 0.1 cm path length cuvette. The results were expressed in Molar Ellipticity [23].

2.4. Fluorescence measurements

*Ah*SYMRK and its mutants were dialyzed overnight against 5 mM Tris–HCl (pH 8.0) and 25 mM NaCl and were brought to A_{280} of 0.1 O.D. Measurements were conducted in a 1 cm cuvette at room temperature in a Cary Eclipse Fluorescence Spectrophotometer (Varian). Scans were performed at an excitation wavelength of 280 nm, a bandwidth of 5 nm with a scan speed of 240 nm/min [24].

2.5. Limited proteolysis

For limited proteolysis with trypsin, 40 μg of purified protein was diluted to 250 μl reaction volume containing 50 mM NH_4HCO_3, pH 7.8 and incubated at 37 °C with Trypsin (Promega) at a 1:1000 (w/w) enzyme: substrate ratio [24]. 25 μl aliquots were removed at fixed time points, and the reaction was stopped by the addition of 2X Laemmli buffer and boiling for 5 min. Digested protein samples (~4 μg/lane) were separated in a 7.5% SDS–PAGE and gels were stained with Commassie Brilliant blue R250.

2.6. Immunoprecipitation

*Ah*SYMRK expressed in *Escherichia coli* was immunoprecipitated with monoclonal α-pY antibody (Cell Signaling Technology) under binding conditions described previously with modifications [21]. In brief, 400 μg of protein was incubated in a total reaction volume of 300 μl in the presence of 20 mM HEPES pH 7.4, 150 mM NaCl and 1 μg of the corresponding antibody. Incubation was performed for 4 h at 4 °C. Protein A Sepharose Fast Flow beads (GE Healthcare) were added, gently mixed for 1 h and washed five times with 20 mM HEPES pH 7.4 and 150 mM NaCl. The bound *Ah*SYMRK from the immunoprecipitate was eluted with 2 M NaCl and immediately desalted. 0.2–0.5 μg of the bound and unbound fractions were used for immunoblotting and kinase activity assay.

2.7. Homology modeling analysis

*Ah*SYMRK kinase domain sequence (Uniprot ID: E6YC17) was subjected to HHPRED [25] server for the prediction of secondary and tertiary structure. Fold prediction results suggest strong structural similarity between *Ah*SYMRK kinase domain and human IRAK4 kinase domain complexed with AMPPNP (PDB ID: 2OID; chain B) [11]. Three-dimensional (3D) coordinates of *Ah*SYMRK was generated by MODELLER 9.8 [26] package using chain B of