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Autophosphorylation of gatekeeper tyrosine by symbiosis receptor kinase

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ABSTRACT

Plant receptor-like kinases (RLKs) share their evolutionary origin with animal interleukin-1 receptor-associated kinase (IRAK)/Pelle family of soluble kinases and are distinguished by having tyrosine as 'gatekeeper'. This position is adjacent to the hinge region and is hidden in a hydrophobic pocket of the catalytic cleft of protein kinases and is therefore least probable to be a target for any modification. This communication illustrates the accessibility of the gatekeeper site (Y670) towards both autophosphorylation and dephosphorylation in the recombinant cytoplasmic domain of symbiosis receptor kinase from *Arachis hypogaea* (*Ah*SYMRK). Autophosphorylation on gatekeeper tyrosine was detected prior to extraction but never under in vitro conditions. We hypothesize gatekeeper phosphorylation to be associated with synthesis/maturation of *Ah*SYMRK and this phenomenon may be prevalent among RLKs.

Structured summary of protein interactions:
AhSYMRK and **AhSYMRK** phosphorylate by protein kinase assay (1, 2, 3)

1. Introduction

Evolutionarily RLKs belong to a kinase family that predates the split of plants and animals and are closely related to some animal kinases like IRAKs and the Drosophila Pelle group of soluble Ser/Thr kinases [1]. Together RLKs and IRAKs belong to the tyrosine kinase-like (TKL) family of kinases that are closely related to the animal receptor Tyr kinase [2]. The feature that distinguishes RLKs and IRAKs from all other kinases is having a tyrosine residue in their gatekeeper position [1,3]. This position is located on a conserved β5 strand, distal to the active site and adjacent to an intrinsically flexible hinge region that connects the N- and C-terminal lobes of protein kinases [4,5]. It flanks a highly variable hydrophobic pocket at the rear of the catalytic cleft and confers selectivity for binding nucleotides and small-molecule inhibitors and hence is referred to as a gatekeeper residue [6]. Importance of the gatekeeper residue is primarily because it is the architect of this deep cleft and recent evidence indicates that the conformation of this cleft can also have significant impact on catalytic-independent functions of kinases [7].

Protein kinases have adopted different mechanisms of regulation mediated by their gatekeeper residues. Structural analysis suggests this residue to stabilize a 'hydrophobic spine' in the active conformation of Tyr kinases [4,5]. In general, bulkier hydrophobic residue at gatekeeper position activates protein kinases by strengthening the spine whereas disruption of this hydrophobic connectivity by a smaller residue, like glycine, causes inactivation. In ERK2, the gatekeeper residue is part of a novel structural unit that plays an important role in restraining its auto-activation in the absence of upstream signaling [8]. Crystal structure of active IRAK4 revealed gatekeeper Tyr to form hydrogen bond with a conserved glutamate (Glu) from α helix C suggesting the importance of gatekeeper Tyr in determining the structure and regulation of RLK/Pelle/IRAK family of protein kinases [3,9]. In consonance, substitution of gatekeeper Tyr with phenylalanine (Phe) has been shown to inactivate several RLKs, like LYK3 of *Medicago truncatula* [10], BRASSINOSTEROID-INSENSITIVE 1 (BRI1), and BRI1-ASSOCIATED KINASE 1 (BAK1) [11] of *Arabidopsis thaliana* indicating the role of gatekeeper Tyr in the catalytic activity of RLKs. The best studied examples of RLKs also include SOMATIC EMBRYOGENESIS

Abbreviations: RLK, receptor-like kinase; TKL, tyrosine-kinase like; IRAK, interleukin-1 receptor-associated kinase; SYMRK, symbiosis receptor kinase; Trx, thioredoxin; KD, kinase domain; CD, cytoplasmic domain; CIAP, calf intestinal alkaline phosphatase

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RECEPTOR-LIKE KINASE 1 (SERK1) [12] and FLAGELLIN SENSITIVE 2 (FLS2) [13] of *A. thaliana*, Xa21 of *Oryza sativa* [14], Symbiosis Receptor Kinase (SYMRK) [15] and NOD FACTOR RECEPTOR 1 (NFR1) [16] of *Lotus japonicas*; but the significance of gatekeeper Tyr in plant RLKs is yet to be understood.

Symbiosis receptor kinase (SYMRK) is a LRR-type receptor-like kinase involved in plant–microbe symbiosis [17]. Earlier investigations on *Lj*SYMRK demonstrated SYMRK to be a Ser/Thr kinase and have indicated phosphorylation on an activation segment threonine (T760) to be critical [15]. Here we demonstrate *Ah*SYMRK to be a dual-specificity kinase that autophosphorylates on gatekeeper Tyr (Y670).

2. Experimental procedures

2.1. Cloning, mutagenesis and expression of recombinant AhSYMRK

*Ah*SymRK was amplified from cDNAs prepared from nodulated roots of *A. hypogaea* and cloned following standard procedures [18,19]. pET28a-*Ah*SymRK-CD/KD and its point mutations generated with the QuikChange Site-Directed mutagenesis kit (Stratagene) were used to express the His_6-polypeptides in *Escherichia coli* strain BL21 (DE3). *Ah*SymRK-K625E was cloned in pET32a to express Trx-K625E-KD. The expressed proteins were affinity-purified using Ni-NTA bead (Qiagen) under denaturing or non-denaturing conditions according to manufacturer's protocol. The native proteins were dialysed against 20 mM HEPES pH 7.4, 1 mM EDTA, 10% glycerol and stored in aliquots at −80 °C. Protein concentrations were estimated using Bradford method [20].

2.2. Kinase assays and phosphoamino acid analysis

Autophosphorylation and substrate phosphorylation were performed as described previously [21,22]. In general, for substrate phosphorylation 0.05 μg of His_6-*Ah*SYMRK was incubated with 1 μg myelin basic protein (MBP) for 15 min in 40 mM HEPES pH 7.4 supplemented with 10 mM MgOAc and [γ-^{32}P]ATP (200 μM 3000 cpm/pmol) in a reaction volume of 25 μl at 25 °C. For autophosphorylation reactions 2–5 μg of His_6-*Ah*SYMRK was used. Immunoblotting was done as per manufacturers' protocols with monoclonal α-pY (1:3000) and polyclonal α-pT (1:3000) from Cell Signaling Technology, monoclonal α-pS (1:200) and polyclonal α-His_6 (1:2000) from Qiagen. Custom-made antibody was generated in rabbit against the synthetic peptide (665-QQILV**pY**PFMS-674) that was sequentially affinity-purified by using the non-phosphorylated and phosphorylated antigen peptides (Imgenex India). For thin layer electrophoresis (TLE) labelled samples were transferred to PVDF membrane, bands excised and subjected to phosphoamino acid analysis [23]. For mass spectrometry analysis of phosphorylation sites data-dependent acquisition using LC/MS/MS and data-independent acquisition using LC/MS^E analysis were performed on a Waters nanoACQUITY ultra-performance liquid chromatograph coupled to a Q-Tof Premier mass spectrometer [24,25]. For details see Supplemental Materials and Methods.

2.3. Phosphatase treatment and analysis

Calf intestinal alkaline phosphatase (CIAP, Fermentas) and *Mycobacterium* protein tyrosine phosphatase A [26] (MptpA, kindly provided by Professor Amit Das, IIT, Kharagpur, India)digestions (0.01 u/μl) were done with 10–15 μg of *Ah*SYMRK-KD/CD at 25 °C in 25 μl reaction volume for indicated time periods. For rephosphorylation experiments the kinase polypeptides were affinity-purified using Ni-NTA agarose to remove the phosphatase after the digestion.

2.4. Immunoprecipitation

Transformation of *Arachis* root to express 35S::GFP-*Ah*SYMRK-KD was performed as previously described [27,28]. *Ah*SYMRK-KD expressed in transgenic *Arachis* roots was immunoprecipitated with monoclonal α-pY antibody (Cell Signaling Technology) and monoclonal α-GFP antibody (Abcam), respectively, under binding conditions described previously with modifications [19].

3. Results and discussion

A. hypogaea symrk (GenBank: FJ969396.2) was isolated by amplification of root cDNA using a degenerate priming approach based on legume symrk sequences. The predicted protein of 926

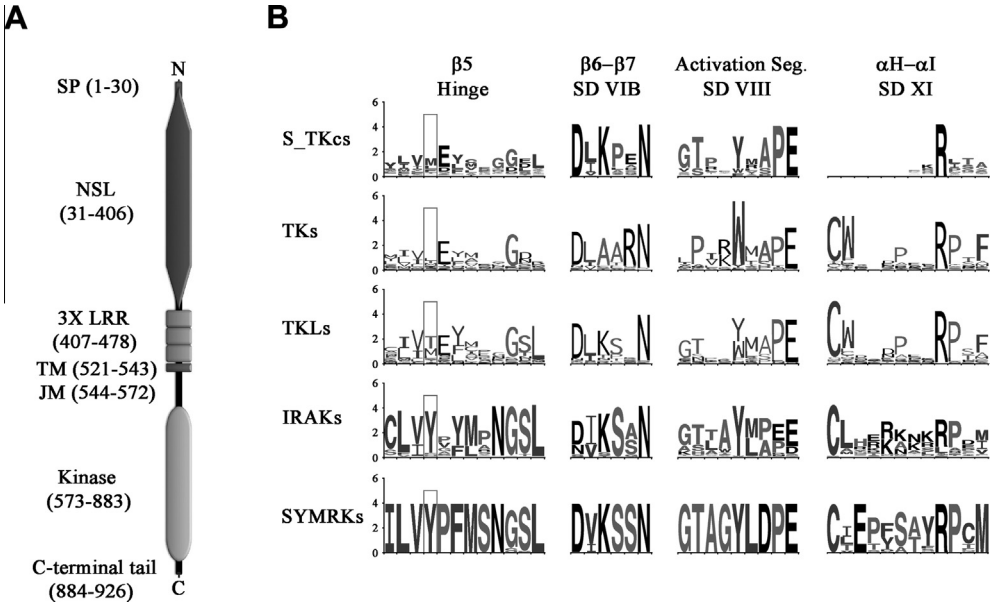

Fig. 1. Domain organization of *Ah*SYMRK. Schematic representation of *Ah*SYMRK (with detail in text) (A). Sequence web logo of indicated subdomains of SYMRKs, Ser/Thr kinases (S_T Kc), Tyr kinases (TKs) Tyrosine kinase-like (TKLs) and IRAKs. The boxes indicate the gatekeeper position (B).