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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

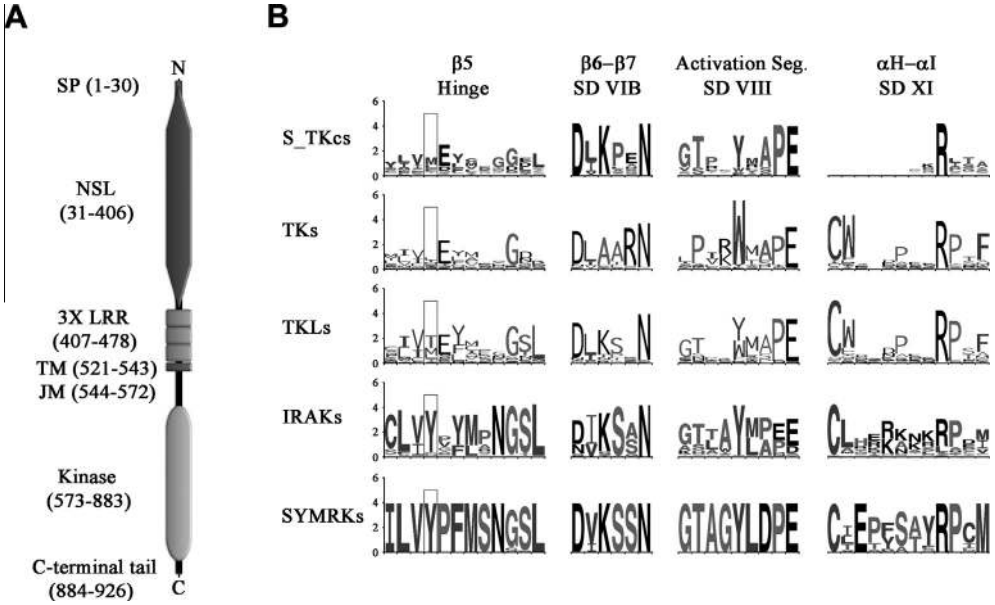

Fig. 1. Domain organization of *Ah*SYMRK. Schematic representation of *Ah*SYMRK (with detail in text) (A). Sequence web logo of indicated subdomains of SYMRKs, Ser/Thr kinases (S_T Kc), Tyr kinases (TKs) Tyrosine kinase-like (TKLs) and IRAKs. The boxes indicate the gatekeeper position (B).

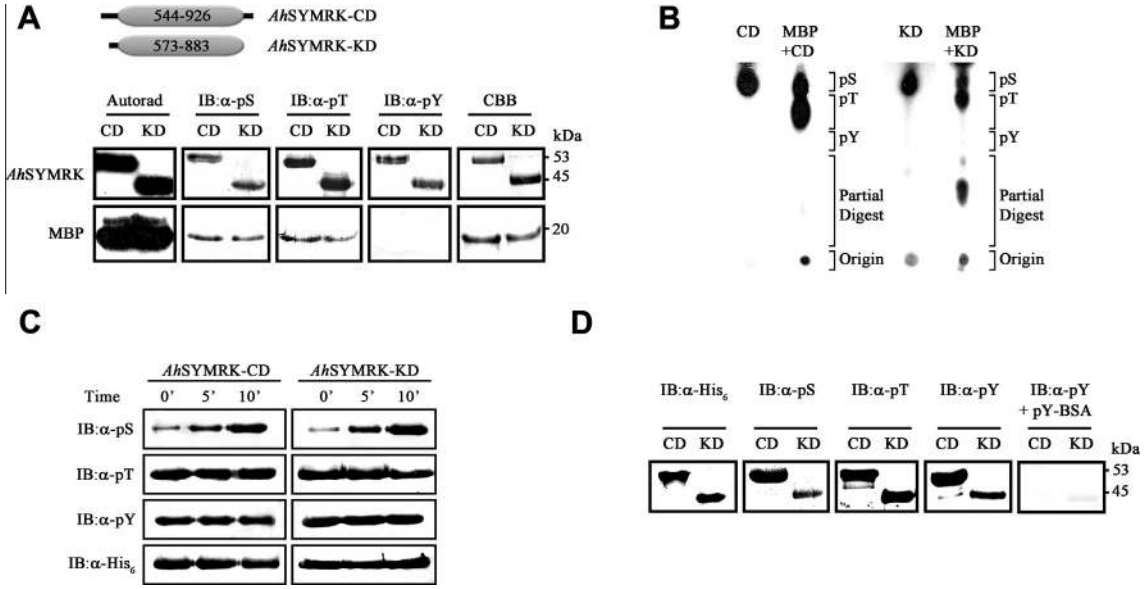

Fig. 2. Recombinant *Ah*SYMRK expressed in *E. coli* codes for a functional protein kinase. Post-purification catalytic activities of His_6-*Ah*SYMRK-CD and His_6-*Ah*SYMRK-KD (A–C); autoradiographs of autophosphorylation (SYMRK) and substrate phosphorylation (MBP) and corresponding immunoblots with indicated antibodies (A); TLE analysis of *Ah*SYMRK polypeptides and MBP(B); Immunoblot analysis of progress of autophosphorylation of *Ah*SYMRK polypeptides with indicated antibodies (C). Pre-purification phosphorylation status of *Ah*SYMRK in *E. coli* and specificity of α-pY;immunoblot analysis of *Ah*SYMRK-CD and *Ah*SYMRK-KD isolated under denaturing conditions with indicated antibodies (D); inhibition of *Ah*SYMRK detection with α-pY by prior incubation of the antibody with pY-BSA (right most panel, D). Autorad, autoradiograph; CBB, coomassie brilliant blue; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; α-pS, anti-phosphoserine; α-pT, anti-phosphothreonine; α-pY, anti-phosphotyrosine; α-His_6, anti-His_6 pY-BSA, phosphotyrosine-conjugated BSA.

aa encoded by *Ah*symrk is a type I transmembrane protein featuring a signal peptide (1–30), a NORK sequence-like domain (NSL, 31–406), three LRR motifs (407–478) in the extracellular region, a transmembrane domain (521–543) and the cytoplasmic domain (544–926) containing the core kinase domain (573–883) (Fig. 1A). The core kinase domain of *Ah*SYMRK shows significant homology with other dual-specificity RLKs like *At*BAK1 (43% identity: 63% similarity), *At*SERK1 (41% identity: 63% similarity), *At*BRI1 (37% identity: 57% similarity) and *Mt*LYK3 (37% identity: 54% similarity) (Supplemental Fig. S1). *Ah*SYMRK also shows significant homology with *Hs*IRAK4 (38% identity: 55% similarity) and *Hs*IRAK1 (38% identity: 54% similarity), which is noteworthy because within the kinase complement of the human genome, the IRAK4 kinase domain is relatively distinct with 38% sequence identity to the kinase domain of IRAK1 and 32% or less sequence identity to all other human kinases. SYMRKs and IRAKs are classified as tyrosine kinase-like or TKLs, where domain VIB and domain VIII has signature features of Ser/Thr kinases but domain XI has those of Tyr kinases (Fig. 1B).

3.1. AhSYMRK codes for a functional Ser/Thr protein kinase

Previous studies on *At*BRI1 showed that without the juxtamembrane (JM) domain, the kinase could autophosphorylate on Ser residues but not on Thr or Tyr residues [29]. Our efforts toward characterizing *Ah*SYMRK were therefore initiated with the His_6-tagged whole cytoplasmic domain of *Ah*SYMRK-CD (544–926: CD) and the core kinase domain *Ah*SYMRK-KD (573–883: KD) (Fig. 2A). A structured extension of ~30 residues at the N-terminus was included in the core kinase domain as it is important for enzymatic activity in RLKs as well as IRAKs [3,10]. The purified *Ah*SYMRK-CD and *Ah*SYMRK-KD polypeptides were subjected to kinase reactions in vitro in presence of [γ-^{32}P]ATP. Both the polypeptides autophosphorylated as well as actively phosphorylated myelin basic protein (MBP) as an exogenous substrate (Fig. 2A). Immunoblot analysis indicated *Ah*SYMRK-CD and *Ah*SYMRK-KD to be phosphorylated on Ser, Thr and Tyr and MBP to be phosphorylated on Ser and Thr. However, thin layer electrophoresis (TLE) could only confirm autophosphorylation on Ser residues in vitro (Fig. 2B) though for MBP both Ser and Thr phosphorylation could be confirmed through TLE. We could successfully detect pTyr in another dual-specificity protein kinase (GenBank: AY027437) from *A. hypogaea* that predominantly phosphorylated Tyr (>80%) (33) indicating that the failure to detect phosphotyrosine (pTyr) in TLE analysis was not due to inefficiency of the technique (Supplemental Fig. S2). Therefore, detection of pSer through TLE analysis in Fig. 2B suggests that in vitro autophosphorylation of *Ah*SYMRK is primarily targeted to Ser residues.

Immunoblot analysis of the progress of autophosphorylation in vitro, indicated that both *Ah*SYMRK-CD and KD were detectable with α-pTyr/Thr/Ser before the kinase reaction and only the detection with α-pSer increased with the progress of the reaction (Fig. 2C).This is in consistence with our TLE results and confirms autophosphorylation on Ser residues to predominate as post-purification activity of the kinase. To clarify whether phosphorylation on bacterially-expressed *Ah*SYMRK was occurring before or during the procedure of extraction and purification, we harvested over-expressed *E. coli* cells under denaturing conditions in presence of 8 M urea, followed by clarification of the polypeptides through Ni-NTA agarose. *Ah*SYMRK-CD and KD polypeptides isolated under these conditions, reacted with α-pTyr/Thr/Ser suggesting that the kinase polypeptides get phosphorylated on all these sites in *E. coli* before they are denatured for extraction (Fig. 2D). To ensure specificity of α-pTyr, the antibody was pre-incubated with phosphotyrosine-conjugated BSA (pY-BSA) before using it for immuno-detection. Disappearance of bands in immunoblot ensured the specificity of α-pTyr (Fig. 2D).

Collectively, Tyr phosphorylation on *Ah*SYMRK was not dependent on the JM domain as there were no apparent differences in the observed catalytic properties of *Ah*SYMRK-CD and *Ah*SYMRK-KD. Subsequent experiments were therefore focused on the core kinase domain.

3.2. Recombinant AhSYMRK autophosphorylated on Tyr in E. coli but failed to autophosphorylate on Tyr after extraction and purification in vitro

To clarify the origin of Tyr phosphorylation in *Ah*SYMRK, a catalytically inactive thioredoxin (Trx)-fused *Ah*SYMRK-KD was generated by mutating the conserved catalytic Lys (K625E) in the "VAIK" phosphotransfer motif. The Trx-K625E-KD mutant was totally inactive and the polypeptide was not detectable with any of the anti-phosphoamino acid antibodies indicating pTyr in *Ah*SYMRK to be generated by autophosphorylation and not due to phosphorylation by *E. coli*-derived kinases (Fig. 3A). Both immunoblot and TLE analysis indicated the wildtype *Ah*SYMRK to phosphorylate the dead kinase Trx-K625E-KD only on Ser residues (Fig. 3A and B) suggesting that phosphorylation on Ser residues was intermolecular in nature. On the other hand, autophosphorylation on Thr and Tyr residues of *Ah*SYMRK appeared to occur only on the active WT kinase. This could be either undertaken through intramolecular reactions or hierchical intermolecular reactions which the dead kinase is unable to undertake.

We presumed that Tyr autophosphorylation in *Ah*SYMRK was not detectable during post-purification kinase reactions because the recombinant protein was already highly autophosphorylated on Tyr residues when isolated from *E. coli* as indicated in Fig. 2C. To eliminate the possibility of pre-occupancy of the target Tyr residues by phosphate moieties, *Ah*SYMRK-KD was dephosphorylated by calf intestinal alkaline phosphatase (CIAP) or *Mycobacterium* protein tyrosine phosphatase A (MptpA) before subjecting it to in vitro kinase reactions. CIAP rapidly dephosphorylated pTyr within 30 s. Dephosphorylation of pSer was almost complete by 60 min but pThr dephosphorylation was relatively resistant within the observed time frame (Fig. 3C). MptpA selectively dephosphorylated Tyr residues but the dephosphorylation was not complete even after 60 min though the phosphatase remained active (data not shown). The rapid dephosphorylation of pTyr in *Ah*SymRK by CIAP as opposed to incomplete dephosphorylation by MptpA could be due to hierarchical dephosphorylation which can only be undertaken by a non-specific phosphatase, like CIAP, but not a pTyr-specific phosphatase like MptpA. Autoradiographs shown in Fig. 3D indicate that MptpA treatment had no effect on the overall autophosphorylation as well as substrate phosphorylation activities. For CIAP-treated *Ah*SymRK-KD, the autophosphorylation activity was severely affected but the substrate phosphorylation potential was unaffected. Immunoblot analysis shows that neither MptpA nor CIAP-treated kinase could resume autophosphorylation on Tyr indicating that the failure of demonstration of in vitro Tyr phosphorylation in *Ah*SymRK was not due to pre-occupancy of Tyr residues by phosphate (Fig. 3C).

However, our inability to demonstrate autophosphorylation on Tyr in vitro is in tune to the paradox of Tyr phosphorylation in *At*-BRI1 where autophosphorylation on Tyr in vitro had not been observed to occur though the kinase was phosphorylated on Tyr [30–32].

3.3. Site-directed mutagenesis of conserved tyrosine residues in AhSYMRK-KD

With an overall sequence coverage of 95%, mass spectroscopic (MS) analysis identified 14 Ser and 4 Thr phosphorylation sites within the core kinase domain of *Ah*SYMRK but Tyr phosphorylation could not be detected (Table 1 and Supplemental Fig. S3) [24]. To identify the probable pTyr residue(s) in the core kinase domain of *Ah*SYMRK, we therefore generated site-directed mutants by individually replacing 11 conserved Tyr residues with Phe

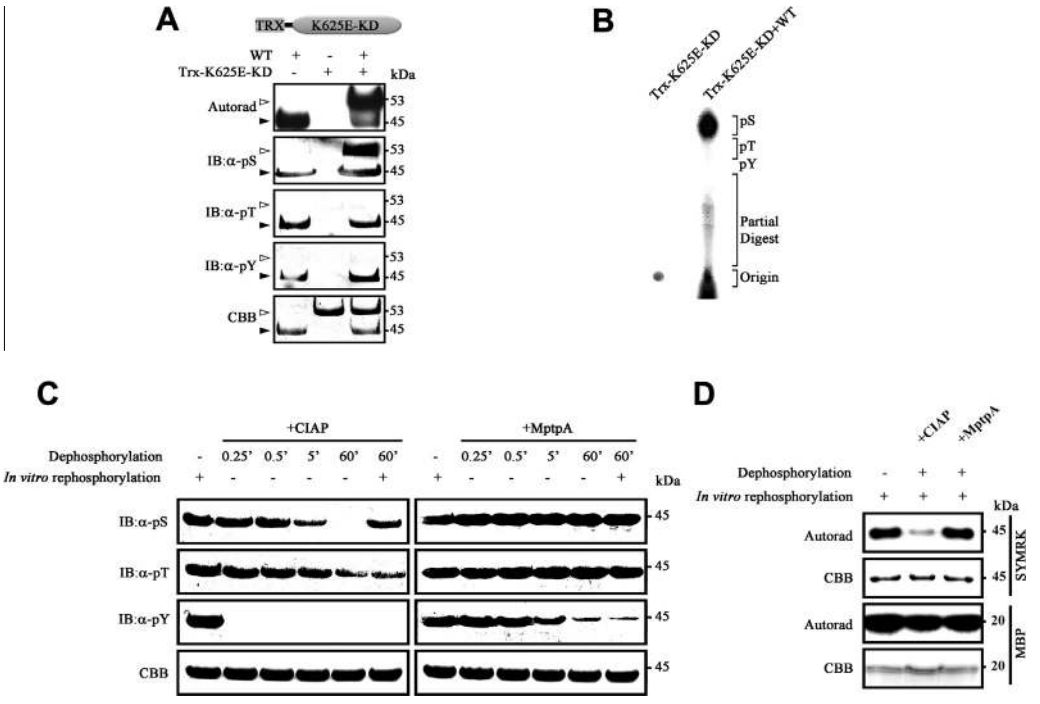

Fig. 3. Phosphorylation on tyrosine residues in *Ah*SYMRK is autocatalytic. Catalytically dead Trx-K625E-KD phosphorylation status before and after trans-phosphorylation by active His_6-*Ah*SYMRK-KD in vitro (A and B); autoradiographs and immunoblots with indicated antibodies; white and black arrowheads indicate the positions of Trx-K625E-KD and His_6-*Ah*SYMRK-KD, respectively (A) and TLE analysis of the indicated polypeptides (B). Dephosphorylation of His_6-*Ah*SYMRK-KDby CIAP or MptpA followed by rephosphorylation in vitro (C and D). Immunoblot analysis of the progress of dephosphorylation and rephosphorylation of His_6-*Ah*SYMRK-KD after removal of phosphatases (C); autoradiographs of autophosphorylation and substrate phosphorylation by dephosphorylated and phosphatase-free His_6-*Ah*SYMRK-KD (D). pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; Autorad, autoradiograph; CBB, coomassie brilliant blue; α-pS, anti-phosphoserine; α-pT, anti-phosphothreonine; α-pY, anti-phosphotyrosine; CIAP, calf intestinal alkaline phosphatase; MptpA *Mycobacterium* protein tyrosine phosphatase A.

(Fig. 4A and Supplemental Fig. S1) Out of these 11 residues, Y591/Y599 within the N-terminal extension of the kinase domain and Y771/Y772 in the P+1 loop were predicted by NetPhos2.0. Another 3 residues were chosen because they are phosphorylated in other RLKs: Y612 near the Gly-rich loop (*Lj*NFR1-Y338) [16], Y670, the gatekeeper Tyr (*At*BRI1-Y956) [29] and Y766 in the activation segment (*Lj*NFR1-Y484) [16]. In addition, Y683 in the α-helix D, Y747 in activation segment, Y836 before α-helix H and Y882 at the C-terminal end of the core kinase domain were included in our analysis because of their conservation in SYMRKs.

All the Y/F mutant proteins were subjected to in vitro kinase assay in presence of [γ-^{32}P] ATP (Fig. 4B) and immunoblot analysis with α-pTyr and α-pThr antibodies (Fig. 4C). The corresponding radioactivity incorporated in the in vitro assays and the densitometric analyses of the immunoblots are shown in Fig. 4D. While substitutions of Y599F, Y683F, Y747F, Y772F, and Y836F had little effect on *Ah*SYMRK activity, substitutions Y591F, Y612F, Y766F, Y771F, and Y882F affected both autophosphorylation and substrate phosphorylation activities indicating the Tyr residues in the later group to be important for *Ah*SYMRK activity. But with all these Y/F mutants detection with α-pTyr was either unaffected or increased, indicating that they may not be the predominant sites of Tyr phosphorylation (Fig. 4C). In contrast, Y670F that substitutes the gatekeeper tyrosine was the only mutant that was completely undetectable by α-pTyr, strongly suggesting this residue to be a potential site of phosphorylation. Alternatively it could also be a potential determinant of Tyr phosphorylation in *Ah*SYMRK. The Y670F mutant was barely detected by α-pThr which is also unlike all other Y/F mutants where detection by α-pThr were either unaffected or generally increased (Fig. 4D). Thus, Y670F substitution in the gatekeeper position strongly affected phosphorylation on Tyr and Thr without having any adverse effect on in vitro autophosphorylation of this kinase that is solely targeted to serine residues (Supplementary Fig. S4).

Two important conclusions are derived from the characteristics of Y670F mutant. Firstly, autophosphorylation on Tyr and/or Thr residues is not a prerequisite for *Ah*SYMRK to be an active kinase. Rather for several mutants like Y591F, Y771F, Y612F, Y766F, Y882F and Y670F autophosphorylations on Tyr and Thr residues appear to be inhibitory (Fig. 4B). Secondly, since substitution of Y670F did not have any adverse effect on the catalytic activity of *Ah*SYMRK (Fig. 4B), the free hydroxyl group of gatekeeper Tyr does not appear to be important for *Ah*SYMRK activity. This is in contrast to what has been claimed earlier for IRAK4 [3] or LYK3 [10] where activated state of these kinases were stabilized by an H-bonding network involving the gatekeeper tyrosine. This is in consistence with finding increased catalytic potential of Y670F in *Ah*SYMRK in contrast to significant reduction of activity by similar substitution in gatekeeper position of other RLKs like *At*BRI1 (Y956F) and *At*BAK1 (Y463F) and *Mt*LYK3 (Y392F) [10,29,33]. The H-bonding network therefore cannot be considered as a generalized signature of activated state for all RLKs having tyrosine as gatekeeper.

3.4. Gatekeeper (Y670) is the predominant site of tyrosine autophosphorylation in AhSYMRK cytoplasmic domain

To understand whether Y670 was actually a site of phosphorylation, we developed sequence and modification-specific antibody against *Ah*SYMRK: 665–674 with phosphorylation on Y670. α-pY670 (665–674) readily detected recombinant *Ah*SYMRK-KD and thus established Y670 as a phosphorylation site in *Ah*SYMRK expressed in *E. coli* (Fig. 5A). The specificity of α-pY670 (665–674) was demonstrated by complete inhibition of the immunoblot signal in presence of the synthetic antigen peptide pY670-(*Ah*SYMRK: 665–674), and its inability to detect the kinase-inactive (Trx-K625E-KD) mutant and the CIAP-treated *Ah*SYMRK-KD (Fig. 5A). The α-pY670 (665–674) detected all the mutant polypeptides of the Y/F series except Y670F, further confirming Y670 as the site of phosphorylation (Fig. 5B). The level of reaction of these mutants with α-pY670 (665–674) was overall similar to what was observed with α-pTyr, suggesting Y670 to be a predominant or sole site of Tyr phosphorylation in recombinant *Ah*SYMRK. Even in the entire cytoplasmic domain, Y670 appeared to be the predominant site,

Table 1
Phosphorylation sites of *Ah*SYMRK-KD identified by LC/MS/MS and LC/MS^E analysis.

Peptide	Calculated mass[b]	Measured mass[b]	Chg[c]	Localization probability[d]	AScore[d]	Mascot score	Site
573 G**pS***EF*SKDDFFIK 580	1498.6381	1498.6492	2	100	38.73	43.3	*Vec Seq*[a]
573 *GSEF***pS**KDDFFIK 580	1498.6381	1498.6522	2	100	53.70	55.76	573
581 SV**pS**IQAFTLEYIEEATEK 599	2136.9868	2136.9964	2	99	23.99	65.9	583
599 YK**pT**LIGEGGFGPVYR 613	1735.8335	1735.8328	2	100	46.21	51.68	601
664 DQQILVYPFM**pS**NGSLQNR 681	2188.9976	2189.0066	2	100	38.73	57.34	674
691 ILDWPTRL**pS**IALGAAR 706	1831.9709	1831.9942	2	50	0	NA*	699
707 GLAYLH**pT**FPGR 717	1310.6173	1310.6186	2	100	17.48	55.82	713
723 DIKS**pS**NILLDH**pS**MC[e]AK 738	1990.8294	1990.8142	2	100,100	23.98	77.89	727,734
723 DIK**pS**SNILLDHSMC[e]AK 738	1910.8631	1910.8642	3	33	0	61.15	726
723 DIKS**pS**NILLDHSMC[e]AK 738	1910.8631	1910.8948	2	100	23.98	75.62	727
739 VADFGF**pS**K 746	949.3947	949.3900	2	100	1000	47.33	745
747 YAPQEGD**pS**NVSLEVR 761	1742.7512	1742.7828	2	100	34.32	54.82	754
747YAPQEGD**pS**NV**pS**LEVR 761	1822.7176	1822.7290	2	100,100	81.55, 90.00	33.7	754,757
762 G**pT**AGYLDPEYYTTQQLSEK 780	2242.9671	2242.9788	2	3	0	73.63	763
762 G**pT**AGYLDPEYY**pT**TQQLSEK 780	2242.9671	2242.9818	2	94	19.22	65.65	763,773
807 NEW**pS**LVEWAKPYIR 820	1869.8814	1869.8874	2	100	169.45	76.79	810
821 A**pS**KIEEIVDPGIK 833	1477.7429	1477.7510	2	100	1000	47.05	822
844 VVEVALQC[e]IEPF**pS**AYRPC[e]MDDIVR 867	2946.3479	2964.3757	3	100	16.24	12.93	856
868 ELEDALIIEN[f]NA**pS**EYMQ[g] 883	2061.8489	2061.8960	2	100	33.19	31.87	880

[a] Amino acids from the vector sequence (Vec Seq) are in italics, and the identified phosphorylated residues pS and pT are in bold.
[b] The deconvoluted neutral monoisotopic calculated/measured mass.
[c] The charge state of the peptide precursor ion subjected to CID fragmentation.
[d] Values determined by Scaffold PTM.
[e] Carbamidomethyl modification for the Cys residue.
[f] Deamidation for the Asn residue.
[g] The in-source fragment of a larger phosphopeptide (ELEDALIIENNApSEYMQ*ACGR*).
* Not applicable since this peptide was identified by LC/MS^E.

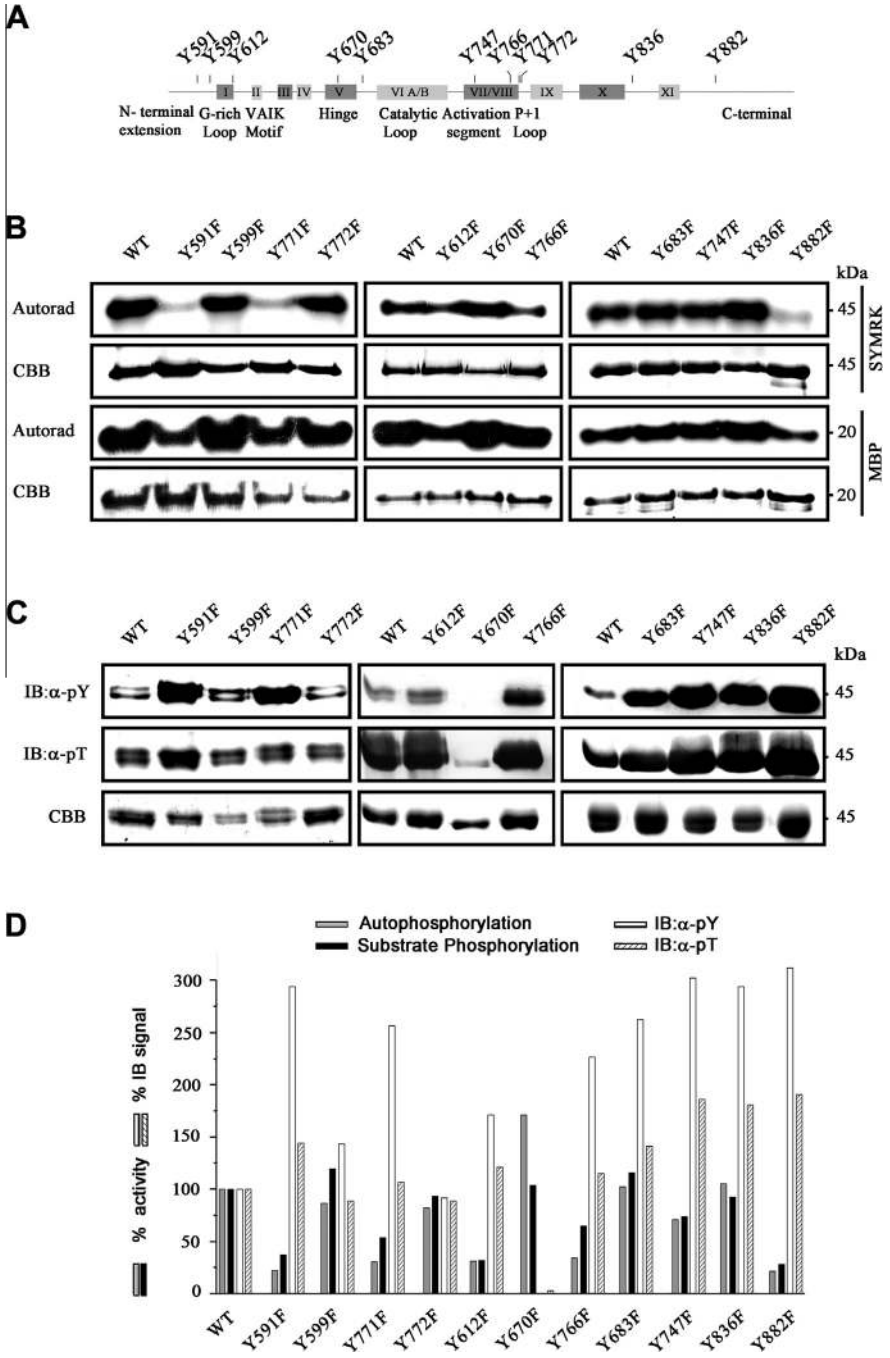

Fig. 4. Site-directed mutagenesis of conserved tyrosine residues in *Ah*SYMRK. Schematic representation of position of tyrosine residues targeted for mutagenesis in *Ah*SYMRK-KD (A). Autoradiographs of autophosphorylation (SYMRK) and substrate phosphorylation (MBP) of indicated Y/F mutants (B). Immunoblots of the autophosphorylated mutant polypeptides with α-pTyr and α-pThr(C). Radioactivity incorporation in bands presented in autoradiograph (panel B) and densitometric analysis of bands presented in immunoblots (panel C) (D). All data are normalized against WT.

as α-pTyr as well as α-pY670 (665–674) failed to detect the Y670F mutant in *Ah*SYMRK-CD, the entire cytoplasmic domain of the receptor-like kinase (Fig. 5C). Accordingly, CIAP treatment of *Ah*SYMRK-CD abolished detection with these antibodies which is similar to what was observed with *Ah*SYMRK-KD, the core kinase domain (Fig. 5C). It may be noted at this point that *Ah*SYMRK-KD failed to phosphorylate the synthetic hinge region peptide *Ah*SYMRK: 665–674 suggesting that the sequence of the peptide was not enough for the recognition and phosphorylation of Y670 (data not shown).To check whether *Ah*SYMRK was phosphorylated on Y670 in vivo, we over-expressed *Ah*SYMRK-KD as a GFP fusion protein in *A. hypogaea* roots. Immunoprecipitated GFP-*Ah*SYMRK-KD from transgenic *Arachis* roots reacted with α-pTyr as well as with α-pY670 (665–674), thus establishing that phosphorylation on Y670 in *Ah*SYMRK occurs *in planta* (Fig. 5D); however, the same experiment performed with non-transgenic wildtype *Arachis* failed to show any reaction with these antibodies.

Although phosphorylation of Y670 is important, MS analysis was not able to confirm its identity. One plausible explanation is due to the presence of multiple sites of phosphorylation, such as S674, within the same peptide sequence 664-DQQILVYPFMpSNGSLQNR-681 which was readily detected as phosphorylated (Supplementary

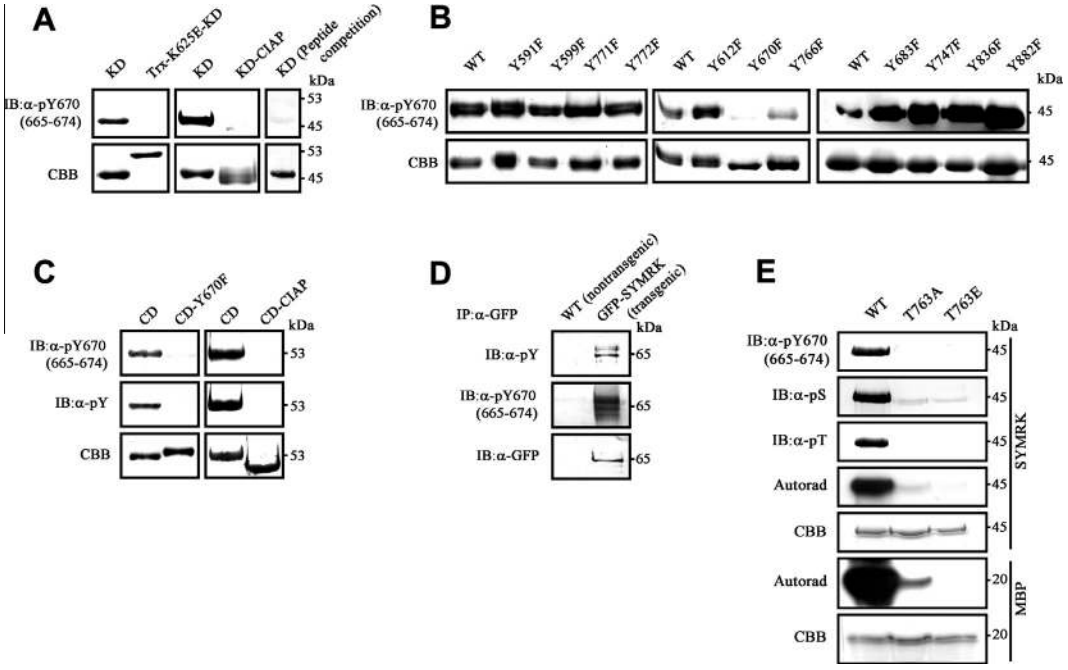

Fig. 5. Gatekeeper (Y670) is the predominant site of tyrosine autophosphorylation in *Ah*SYMRK cytoplasmic domain. Immunoblot analysis of *Ah*SYMRK-CD and KD using sequence and modification-specific antibody α-pY670 (665–674) (A–C); immunoblot of *Ah*SYMRK-KD, Trx-K625E-KD and CIAP-treated *Ah*SYMRK-KD with α-pY670 (665–674); inhibition of *Ah*SYMRK-KD detection with α-pY670 (665–674) by prior incubation of the antibody with antigen phosphopeptide pY670 (*Ah*SYMRK: 665–674) (rightmost panel) (A); immunoblot of autophosphorylated Y/F mutants with α-pY670 (665–674) (B); immunoblot of *Ah*SYMRK-CD, *Ah*SYMRK-CD-Y670F and CIAP-treated *Ah*SYMRK-CD with α-pY670 (665–674) (C). Immunoblot of GFP-*Ah*SYMRK-KD (65 kDa) expressed in transgenic *Arachis* plants: protein was immunoprecipitated (IP) from non-transgenic WT plants and transgenic plants using α-GFP antibody and was analyzed by immunoblotting with α-GFP, α-pY670 (665–674) and α-pY antibodies (D).Activation segment threonine (T763) is important for autophosphorylation on gatekeeper tyrosine (Y670) (E) WT *Ah*SYMRK-KD, *Ah*SYMRK-KD-T763A and *Ah*SYMRK-KD-T763Ewere autophosphorylated and subjected to immunoblot analysis against α-pY670 (665–674), α-pS, and α-pT antibodies (upper three panels). Autoradiograph and CBB staining of an in vitro kinase assay showing autophosphorylation (SYMRK) and substrate phosphorylation (MBP) of the corresponding proteins (lower four panels).

Fig. S5). This phosphorylated form of the peptide could preclude the detection of a low stoichiometry event at Y670 since both phosphopeptides would have a similar retention time, identical precursor mass, and many product ions in common.

3.5. Activation segment threonine (T763) is important for autophosphorylation on gatekeeper tyrosine (Y670)

Earlier investigations on *Lj*SYMRK indicated an invariant Thr (T760) (*Ah*SymRK: T763) to be phosphorylated and substitution of T760A strongly affected the *Lj*SYMRK kinase activity [15]. To investigate the importance of T763 in the enzymatic transfer of phosphate to gatekeeper Tyr (Y670) in *Ah*SymRK, we substituted T763 with Ala (T763A) or a phosphomimetic Glu (T763E). Both T763A and T763E, strongly reduced *Ah*SymRK activity and its autophosphorylation on Y670 were abolished (Fig. 5E). Absence of activity in the phosphomimetic substitution in T763E mutant however indicated that negative charge cannot replace the functional importance of a phospho-group that was shown to be present on this Thr (Table 1). These observations reaffirmed the importance of the activation segment Thr in determining the catalytic ability of SymRKs and also reconfirmed gatekeeper Tyr (Y670) autophosphorylation to be autocatalytic in *Ah*SymRK.

4. Conclusion

The main finding of this study is autophosphorylation of gatekeeper tyrosine by the recombinant core kinase domain of a plant RLK (*Ah*SYMRK). The phosphorylation could be physiologically significant because the gatekeeper tyrosine residue in *Ah*SYMRK was also phosphorylated *in planta* (Fig. 4D).It was intriguing to observe autophosphorylation on gatekeeper tyrosine because this position is buried in a hydrophobic pocket at the rear end of the catalytic cleft in a mature kinase [4], and therefore should be inaccessible. This rules out intermolecular catalysis on gatekeeper tyrosine but intramolecular transfer of phosphate was also difficult to justify because the gatekeeper position Y670 was distal from the conserved central structural region that is responsible for phosphotransfer [4,5]. The inability to autophosphorylate on tyrosine in vitro by *Ah*SYMRK can be explained by the inaccessibility of the gatekeeper position in a mature kinase [4]. On the other hand, it is highly probable that phosphorylation on gatekeeper Tyr was associated with the synthesis or maturation of the polypeptide. Cotranslational tyrosine phosphorylation of protein kinases has been reported earlier with GSK3 beta and DYRK kinases where a one-off auto-activation of these kinases takes place in a translational folding intermediate during or immediately after translation by an intramolecular reaction [34,35]. Whether similar mechanisms are adapted by *Ah*SYMRK for gatekeeper phosphorylation is yet to be understood. The only other plant receptor-like kinase that is phosphorylated in gatekeeper tyrosine is BRI1, the best studied RLK so far [29,36]. Gatekeeper autophosphorylation in BRI1 (Y956) has been claimed to be a post-translational event based on the delay of its onset but with the present state of understanding of protein kinase structure it is not clear how this could be achieved in a mature kinase [36]. In addition to phosphorylation, inaccessibility of the gatekeeper position also makes the observed rapid dephosphorylation from this site incomprehensible. These paradoxes of gatekeeper inaccessibility [4] and its observed modifications described in this report can only be explained by the dynamicity of the hinge region where a local structural readjustment forces the gatekeeper to be relatively exposed.

Further investigations are required to understand the significance of gatekeeper phosphorylation event and since gatekeeper tyrosine is a conserved feature in IRAK/Pelle kinases it remains to

be seen whether this modification is a cross kingdom conserved phenomenon.

Data deposition

*Ah*SymRK complete mRNA sequence has been deposited to GenBank under the accession No. FJ969396.2.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.050.

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