Role of Threonines in the Arabidopsis thaliana Somatic Embryogenesis Receptor Kinase 1 Activation Loop in Phosphorylation*

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The Arabidopsis thaliana somatic embryogenesis receptor kinase 1 (AtSERK1) gene encodes a receptor-like protein kinase that is transiently expressed during embryogenesis. To determine the intrinsic biochemical properties of the AtSERK1 protein, we have expressed the intracellular catalytic domain as a glutathione S-transferase fusion protein in Escherichia coli. The AtSERK1-glutathione S-transferase fusion protein mainly autophosphorylates on threonine residues (Km for ATP, 4 × 10⁻⁶ M), and the reaction is Mg²⁺ dependent and inhibited by Mn²⁺. A K330E substitution in the kinase domain of AtSERK1 abolishes all kinase activity. The active AtSERK1∗ can phosphorylate inactive AtSERK1K330E protein, suggesting an intermolecular mechanism of autophosphorylation. The AtSERK1 kinase protein was modeled using the insulin receptor kinase as a template. On the basis of this model, threonine residues in the AtSERK1 activation loop of catalytic subdomain VIII are potential targets for phosphorylation. AtSERK1 phosphorylation on myelin basic protein and casein showed tyrosine, serine, and threonine as targets, demonstrating that AtSERK1 is a dual specificity kinase. Replacing Thr-468 with either alanine or glutamic acid not only obliterated the ability of the AtSERK1 protein to be phosphorylated but also inhibited phosphorylation on myelin basic protein and casein, suggesting that Thr-468 is essential for AtSERK-mediated signaling.

Perception and transduction of external stimuli are essential for the growth and development of multicellular organisms. In many cases this involves membrane-associated receptor proteins of which the ligand binding domains perceive the extracellular signal molecule. Information is then transmitted through the kinase domains of the receptor that in turn lead to phosphorylation of target proteins (1, 2). Most animal receptor kinases have tyrosine kinase activity, only a few are serine/threonine kinases (2, 3) or can phosphorylate serine and threonine in addition to tyrosine (4). There is increasing evidence that protein kinases also play important roles in cellular signaling and metabolic regulation in plants. Rapid and transient changes in the phosphorylation pattern of plant membrane-associated proteins have indicated the involvement of protein kinases in signaling events (5, 6). Many plant genes encode receptor kinases sharing the same basic domain structures as shown in animal receptors and are called RLKs.1 Plant RLKs are involved in diverse processes such as hormone response (7), mitogenic induction (8), self-incompatibility (9), shoot meristem development (10), and disease resistance (11). Expression studies have implicated RLKs to be involved in embryogenesis, pollen function, and light responses (12). On the basis of amino acid sequences of their extracellular domains, the RLKs reported so far have been categorized into six different types (13): (a) the self-incompatibility domain type, with similarities to self-incompatibility locus glycoproteins in Brassica; (b) the epidermal growth factor receptor-like type; (c) the extracellular lectin domain type; (d) the tumor necrosis factor receptor domain type; (e) the pathogenesis-related protein domain type; and (f) the LRR type, with 3–26 leucine-rich repeats. The LRR motif occurs not only in receptor kinases but also in many other proteins as well and may be involved in protein-protein interactions (14). Analysis based on mutant phenotypes has revealed that in particular the LRR type is involved in plant development (13, 15). The ERECTA gene was the first putative receptor containing extracellular LRRs to be cloned from Arabidopsis and is involved in organ elongation (16). The best characterized of the plant LRR RLKs is the clavata 1 gene from Arabidopsis, which is similar in structure to ERECTA. Clavata 1 is involved in maintaining the balance between cell proliferation and organ formation in shoot and the inflorescence meristems (10). Another example is the brassinosteroid receptor 1 gene (17).

Expression of plant RLKs in Escherichia coli or in insect cells yielded purified proteins suitable for biochemical studies. Plant RLKs were thus shown to autophosphorylate on serine or threonine residues or both. A possible exception is the Petunia pollen-expressed receptor-like kinase 1 gene, which autophosphorylates on serine and tyrosine residues (18). Autophosphorylation has been shown to occur predominantly by intermolecular mechanisms (trans), and it has been suggested that oligomerization may be involved in the regulation of the kinase activity (19). The sites where phosphorylation occurs are likely to have been identified in RLK1 from Catharanthus roseus. Site-directed mutagenesis has identified Thr-720 in the subdomain XI important both for auto- and transphosphorylation of C. roseus RLK1 (20).

1 The abbreviations used are: RLK, receptor-like kinase; LRR, leucine-rich repeat; AtSERK1, Arabidopsis thaliana somatic embryogenesis receptor kinase 1; GST, glutathione S-transferase; IRK, insulin receptor kinase; PAGE, polyacrylamide gel electrophoresis; MBP, myelin basic protein.

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This report describes the biochemical characterization of an LRR RLK from Arabidopsis thaliana, AtSERK1. The encoding gene is expressed in ovules, zygotic embryos, and embryogenic suspension cultures of Arabidopsis. During somatic embryogenesis, AtSERK1 expression continues up to early globular stage embryos and is absent in later stages of embryo development. This transient expression pattern is also observed during zygotic embryogenesis. DNA sequence analysis suggests that AtSERK1 consists of an extracellular domain that has a leucine zipper domain followed by five tandemly repeated leucine-rich motifs and a specific proline-rich region. The extracellular domain is linked via a transmembrane hydrophobic region to a protein kinase catalytic domain that is homologous to serine/threonine kinases. The catalytic kinase domain of the AtSERK1 protein was expressed as a recombinant fusion protein in E. coli.

After assaying the activity of the AtSERK1 recombinant fusion protein, we found that AtSERK1 has an intrinsic protein kinase activity and autophosphorylates mainly on threonine, slightly on serine, but not on tyrosine residues. We report the identification of a threonine residue, Thr-468, that is involved in receptor autophosphorylation as well as in phosphorylation of substrate proteins. The kinetics of the autophosphorylation reaction suggest that it proceeds via an intermolecular mechanism.

MATERIALS AND METHODS

Expression of AtSERK1 Kinase Catalytic Domain Fusion Protein in E. coli—The cDNA sequence encoding the AtSERK1 (GenBank™ accession number A67827) kinase catalytic domain, corresponding to nucleotides 1000–2068 (Fig. 1), was amplified by PCR using the 2.1-kilobase pair cDNA cloned in vector pBluescriptII SK+ as a template. Primer Sma11000 (5'-TCCCCCGGGTATTCTCTGATGTCCTG-3') and primer NotI2068 (5'-ATAGAAGATGCCGGCCGCTTGAGACCA-GATA-3') amplified a PCR fragment of 1 kilobase pairs that was cloned into a SmaI- and NotI-cleaved pGEX-4T1 (Amersham Pharmacia Biotech), resulting in the AtSERK1kin construct. Site-directed mutagenesis of highly conserved lysine, Lys-330, essential for phosphorylation, was performed according to the manufacturer's instructions (Stratagene), resulting in the AtSERK1K330E construct. Site-directed mutagenesis of all three threonines, Thr-462, Thr-463, and Thr-468, to glutamic acid resulted in an AtSERK1T463E construct, whereas the single amino acid mutations were made, resulting in AtSERK1T459E, AtSERK1T462E, AtSERK1T463E, and AtSERK1T468E constructs. The same amino acids were also mutated to alanine residues, resulting in AtSERK1T459A, AtSERK1T462A, AtSERK1T463A, and AtSERK1T468A. All plasmids were verified by restriction endonuclease digestion and DNA sequencing before being transformed into the E. coli B21 strain for protein expression.

A 2-ml overnight culture was transferred to 200 ml of LB medium, and the cells were grown at 37 °C to an A600 of 0.6 and then induced with 0.1 mM isopropyl-β-thiogalactopyranoside for 3–4 h at 30 °C. The cells were collected by centrifugation, resuspended in 6 ml of phosphate-buffered saline lysis buffer containing a protease inhibitor mixture (Mini; Roche Molecular Biochemicals), sonicated, and cleared by centrifugation at 12,000 × g. The soluble GST fusion proteins were purified from the supernatant by glutathione-Sepharose 4B (Amersham Pharmacia Biotech) chromatography according to the manufacturer's instructions.

Computer Modeling Studies—The x-ray structures of IRK (inactive IRK, determined at 1.9 Å resolution; Ref. 22) and IRKP (activated human IRK, refined to 2.1 Å resolution; Ref. 23) were used as templates for modeling the AtSERK1 kinase domain. These two templates produced the best E value when using BLAST against the Protein Data Bank data base. The sequence of the kinase domain of AtSERK1 was aligned with the sequence of the insulin receptor kinase using the advanced BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Models of the kinase domain of AtSERK1 in its activated and inactive form were built using the Modeller-5 program of Sali and Blundell (24).

Modeller is an excellent program for comparative structural modeling, because it results in a very accurate, detailed, and explicit model of protein structure (25, 26).

The quality of the three-dimensional models was evaluated using PROCHECK and Prosa II version 3.0 (27). The two programs can evaluate the quality of three-dimensional homology models very well (26). Improvement of the models could be obtained by an iterative sequence-structure alignment procedure, yielding finally the sequence alignment between the AtSERK1 kinase domain and IRK. This improvement in the sequence-structure alignment procedure was especially important for the loop (amino acids 498–515), which is present in the AtSERK1 kinase domain but not in the insulin receptor kinase.

Phosphorylation Assays—The kinase activity of the AtSERK1kin and the AtSERK1kin mutant GST fusion proteins was demonstrated by incubating 500 ng (100 μg/ml) of protein in 20 μm Tris, pH 7.5, 50 μm NaCl, 0.01% Triton X-100, 10 mM MgCl2, 1 mM dithiothreitol, 50 μM unlabeled ATP, and 10 μCi of [γ-32P]ATP in a final volume of 30 μl. After incubation for 30 min at 30 °C, the reaction was quenched by adding Laemmli SDS-PAGE sample buffer, boiled at 95 °C for 5 min, and separated by 10% SDS-PAGE. The gel was stained with Coomassie brilliant blue to verify equal loading and then dried. The radioactivity was quantified with a Phospholmager using the ImageQuant program (Molecular Dynamics).

To remove the GST tag from AtSERK1kin and AtSERK1T459E, proteinase K treatment of each was treated with 10 units of thrombin (Amersham Pharmacia Biotech) and incubated at 16 °C for 4 h. Phosphorylation assays were performed in a similar way by incubating the cleared AtSERK1kin or AtSERK1T459E proteins with AtSERK1K330E in concentrations ranging from 0 to 500 ng. For phosphorylation assays of AtSERK1 with artificial substrates, 1 μg of casein (Sigma) or MBP (Sigma) was incubated with 500 ng of AtSERK1kin or with AtSERK1mutant proteins. The reaction conditions were the same as described above.

Phosphoamino Acid Analysis—The Coomassie brilliant blue-stained phosphorylated bands of AtSERK1kin, casein, and MBP were excised from the gel, rehydrated in 50 mM NaH2PO4, 0.1% SDS, and 0.25% β-mercaptoethanol, ground with a small pestle, boiled for 5 min, and then agitated for 10 min. The proteins in the supernatant were precipitated by adding 20 μg of bovine serum albumin and 20% (v/v) solid trichloroacetic acid and incubated overnight at −20 °C. The precipitate was collected by centrifugation, and the trichloroacetic acid was removed by lyophilization for 30 min. The sample was hydrolyzed in 50 μl of 6 M HCl for 1 h at 120 °C. The HCl was evaporated, and the pellet was resuspended in the ascending solvent of ethanol, glacial acetic acid, and water (1:1:1 v/v/v) containing 20 μM of each phosphoamino acid standard. The sample was applied to a 10 × 10 cellulose TLC plate (Merck) as described (28) using the ascending solvent for 90 min. The first dimension fractionation was followed by second dimension chromatography in a phosphopeptide buffer containing isobutyl alcohol, formic acid, and water (5:3:4 v/v/v) for 1 h. Phosphoamino acid standards were visualized by spraying the plate with 0.25% ninhydrin in acetone, and heating it at 85 °C for 30 min. The radiographic film was exposed to the TLC plate for 12 h, and the radioactivity was quantified with the ImageQuant program (Molecular Dynamics).

RESULTS

The deduced protein sequence of AtSERK1 (Fig. 1A) consists of 605 amino acids with a calculated Mr of 68,000. The predicted domain structure has the general structure of the RLK-type kinases (29): it consists of a hydrophobic N-terminal putative signal peptide, an extracellular domain consisting of a leucine zipper domain, five leucine-rich repeats, and a proline-rich region unique for the AtSERK1 family (30) followed by a single hydrophobic membrane-spanning domain of 24 amino acids and an intracellular C-terminal kinase catalytic domain. AtSERK1 has seven putative N-glycosylation sites, five of which are located in the extracellular LRR domain (Fig. 1). This distribution is characteristic for RLKs, and there is evidence that RLKs are indeed glycoproteins (31). The AtSERK1 protein kinase catalytic domain has all of the 11 subdomains as described for protein kinases (32) and is predicted to have a serine/threonine protein kinase activity based on conserved amino acids found in subdomains VIIb and VIII. Fusion proteins were constructed in which GST was fused in frame to the N-terminal end of the AtSERK1 kinase domain. After expres-
tion in E. coli Bl21, GST fusion proteins were affinity-purified on glutathione-Sepharose. In a standard assay with 10 mM divalent cations, the autophosphorylation was complete after 25–35 min (Fig. 2A); therefore, 30-min incubations were used routinely. There was no autophosphorylation when MgCl₂ was left out of the reaction mixture. The addition of MnCl₂ considerably reduced the activity, whereas addition of CaCl₂ had no influence on the autophosphorylation activity (Fig. 2B). Optimal autophosphorylation activity was achieved with 10 mM MgCl₂ (data not shown). The AtSERK1 kinase protein exhibited standard Michaelis-Menten kinetics with respect to ATP, with a \( K_m \) of 4.10⁻⁶ M and a \( V_{max} \) of 4.6 × 10⁻⁹ mg/min (data not shown).

To further confirm the specific kinase activity of the AtSERK1 protein, the AtSERK1 K330E mutant protein in which the essential lysine required for the phosphotransfer was replaced by glutamic acid and was analyzed for kinase activity. Fig. 2C shows that the AtSERK1K330E mutant protein no longer autophosphorylates, confirming that AtSERK1 is a functional receptor kinase. To determine which amino acids are autophosphorylated, the \( ^{32} \text{P} \)-labeled proteins were subjected to total hydrolysis. The subsequent analysis of the radioactive amino acids (Fig. 2D) showed that the autophosphorylation was mainly on threonines (≈80%), partly on serines (≈18%), and low on tyrosines (≈2%).

To identify the precise residues on which AtSERK1 autophosphorylates, we compared the predicted three-dimensional structure with the known domain structures of a eukaryotic protein kinase (Fig. 1). The predicted kinase domain of AtSERK1 is found in the kinase domain (amino acids 301–562). The lysine residues that were mutated in this study are shown in bold.

**Fig. 1.** Nucleotide sequence and predicted amino acid sequence of AtSERK1. The dark gray amino acid sequences represent the N-terminal signal peptide and the membrane-spanning region. The light gray region (amino acids 301–74) represents the leucine zipper domain, followed by five LRRs (74–202) in the extracellular domain. The potential N-linked glycosylation sites are underlined, and the lysine and threonine residues in the kinase domain that were mutated in this study are shown in bold. The 15 invariant amino acids found in almost all eukaryotic protein kinases are boxed.

**Fig. 2.** Autophosphorylation characteristics of AtSERK1. Bacterially produced AtSERK1 protein was purified, and aliquots of 500 ng were incubated with \( ^{γ-\text{32}P} \text{ATP} \) as described under "Materials and Methods." After separation on 10% SDS-PAGE, the gels were autoradiographed using a PhosphorImager. A, Time course of autophosphorylation activity. AtSERK1 protein was incubated for 5 min (lane 1), 10 min (lane 2), 15 min (lane 3), 25 min (lane 4), 35 min (lane 5), 45 min (lane 6), 60 min (lane 7), 75 min (lane 8), and 90 min (lane 9). B, Divalent cation requirement of autophosphorylation activity. AtSERK1 protein was incubated with 10 mM divalent cations MnCl₂ (lane 1), CaCl₂ (lane 2), MgCl₂ (lane 3), MgCl₂ and CaCl₂ (lane 4), and MgCl₂ and MnCl₂ (lane 5). C, Autophosphorylation of the AtSERK1 and AtSERK1 K330E proteins. D, Phosphoamino acid analysis of the HCl-hydrolyzed, autophosphorylated AtSERK1 protein. The hydrolyzed protein was spotted onto a TLC plate and resolved by chromatography in two dimensions. The autoradiograph of the phosphorylated amino acids after hydrolysis of AtSERK1 and the positions of the standard amino acids (dotted circles), visualized by spraying with 0.25% ninhydrin, phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY), are shown.
models of the AtSERK1 kinase domain with the well-characterized structure of the kinase domain of human insulin receptor. The Z scores of the AtSERK1 models as determined by Prosa II were −7.1 and −7.0. These values imply that both AtSERK1 models according to the quality assessment criteria developed by Sanchez and Sali (25) are very reliable (p(GOOD/Q-SCORE) = 0.99). The PROCHECK evaluation of the models showed that 98.9% of residues are in the β/α-most favored/ additionally allowed regions of the Ramachandran plot. In addition the side chain parameters χ-1 and χ-2 determined by PROCHECK are better than a protein x-ray crystal structure model with 2.0-Å resolution. Comparing the x-ray structures of the active (IRK3P) and the inactive human IRK has revealed that major structural changes occur on phosphorylation (22, 23). In the inactive IRK structure, the activation A-loop (residues 1149–1170) traverses the cleft between the N- and C-terminal lobes so that the protein substrate-binding site is largely blocked. Autophosphorylation of Tyr-1158, Tyr-1162, and Tyr-1163 results in a dramatic change in the confirmation of the IRK A-loop. One of the tyrosines, Tyr-1158, is displaced ∼30 Å from its position in the unphosphorylated A-loop. The conformational change of the phosphorylated A-loop permits unrestricted access to the binding sites for ATP and protein substrates (23). Although the homology between the predicted AtSERK1 kinase sequence and the IRK is no more than 26%, a comparison of the model of the AtSERK1 kinase domain and the inactive 1IRK shows that they superimpose very well except for an extra loop region in the AtSERK1 kinase (amino acids 498–515). Because it is known that the activation of the insulin receptor kinase occurs by autophosphorylation on residues present within the activation loop of subdomains VII and VIII (33, 34), we designated the same region in the AtSERK1 kinase domain as the AtSERK1 A-loop (Fig. 3, A and B). The AtSERK1 A-loop begins with an invariant asparagine in subdomain VII (Asp-447 in AtSERK1) and terminates with an invariant glutamic acid in subdomain VIII (Glu-475 in AtSERK1; Ref. 35). The AtSERK1 A-loop contains 4 threonine residues, Thr-459, Thr-462, Thr-463, and Thr-468, and the tyrosine, Tyr-456, that are potential phosphorylation sites. In the AtSERK1 kinase model, the major region of the AtSERK1 A-loop is quite identical to that of the A-loop of the inactive insulin receptor kinase.

On the basis of the prediction of the presence of the threonines in the AtSERK1 A-loop and the predominantly serine/threonine autophosphorylation properties (Fig. 2D), we performed site-directed mutagenesis on the three threonines that might be involved in the autophosphorylation of AtSERK1. This led us to making a mutant construct, AtSERK1T462E, in which Thr-462, Thr-463, and Thr-468 in AtSERK1 A-loop were changed to glutamic acid. Assays with bacterially expressed affinity-purified AtSERK1T462E mutant protein showed an almost complete absence of phosphorylated residues (Fig. 4A, lane 2). Only a long exposure of the film revealed a radioactive band, but it was too weak to attempt to identify the phosphorylated amino acids. It may represent the weak phosphorylation of serine that was observed after total hydrolysis of AtSERK1kin (Fig. 2D).

To investigate the role of the individual threonine residues, Thr-462, Thr-463, and Thr-468 in AtSERK1 autophosphorylation, all 3 threonine residues were mutagenized separately to glutamic acid or to alanine. To ensure that the fourth threonine residue, Thr-459, in the AtSERK1 A-loop did not have any influence on the phosphorylation status of AtSERK1 kinase, we also mutagenized Thr-459 either to glutamic acid or to alanine. The threonine mutations either to glutamic acid or to alanine residues were performed to ensure that the substitution of the threonines did not influence the protein structure and thus the kinase activity. The assays with purified proteins showed that there was a reduction in phosphorylation activity from 1.0 (a relative unit obtained after densitometric scanning of the gel containing the radiolabeled AtSERK1kin protein; Fig. 4A, lane 1) to 0.3 for the AtSERK1T462E mutant protein (Fig. 4A, lane 4) and to 0.01 for the AtSERK1T468E mutant protein (Fig. 4A, lane 6). There was also a reduction in phosphorylation activity from 1.0 (AtSERK1kin protein; Fig. 4A, lane 1) to 0.35 for the AtSERK1T468A mutant protein (Fig. 4B, lane 4) and to 0.00 for the AtSERK1T468A mutant protein (Fig. 4B, lane 6). The effect on phosphorylation activity with both types of substitutions was similar, which reveals that the resulting kinase activity is due to the removal of threonine residues and not likely to be due to the identity of the newly introduced amino acid residues.

The ability of AtSERK1 protein to phosphorylate other proteins was tested with common substrates such as casein and MBP (Fig. 5). Both proteins were clearly phosphorylated by AtSERK1kin. The AtSERK1kin protein phosphorylates casein (Fig. 5A, lane 1) less when compared with MBP (Fig. 5C, lane 1). Both casein and MBP phosphorylation were reduced when they were incubated either with mutant AtSERK1T462E or AtSERK1K468E proteins. There was a reduction in casein phosphorylation activity from 1.0 (casein phosphorylated by AtSERK1kin; Fig. 5A, lane 1) to 0.015 for casein phosphorylated by the AtSERK1K468E mutant (Fig. 5A, lane 2) and to 0.012 for the AtSERK1T462E mutant (Fig. 5A, lane 3). There was also a
in casein and MBP, the 32P-labeled protein bands were excised. To determine the identity of the phosphorylated amino acids, we incubated an AtSERK1T459A, AtSERK1 T462A, AtSERK1 T463A, and AtSERK1T468A mutant proteins with casein (Fig. 5A) and MBP (Fig. 5B), both casein and MBP were not phosphorylated by AtSERK1T462A and AtSERK1T468A mutant proteins with casein (Fig. 5C) and MBP (Fig. 5D). These results identified two threonine residues, Thr-462 and Thr-468, in the AtSERK1 kinase domain that are important for both autophosphorylation and phosphorylation of artificial substrates such as casein and MBP. Thr-468 appears to be indispensable for AtSERK1 activity, whereas Thr-462 may be predominantly involved in phosphorylation of substrates. Furthermore, AtSERK1 autophosphorylation and phosphorylation on MBP and casein show tyrosine, serine, and threonine as targets, suggesting that AtSERK1 is a dual specificity kinase.

The AtSERK1 reaction showed second order kinetics (Fig. 6A), which suggests that AtSERK1 autophosphorylates by intermolecular phosphorylation (19). To provide additional evidence for intermolecular phosphorylation of AtSERK1, we tested whether a thrombin-cleaved AtSERK1kin-GST fusion protein was able to phosphorylate the mutant AtSERK1K330E GST fusion protein. The AtSERK1K330E protein was phosphorylated only in the presence of AtSERK1kin (Fig. 6B, lane 2). Although AtSERK1kin was able to phosphorylate AtSERK1K330E, the level of phosphorylation of the AtSERK1K330E-GST fusion protein was 3-fold less compared with the thrombin-cleaved AtSERK1kin protein. This was due to the presence of the GST protein rather than the K330E mutation, because a comparable reduction was observed when incubating an AtSERK1kin-GST fusion protein was a thrombin-cleaved AtSERK1 kin protein (results not shown). The phosphorylation of AtSERK1K330E in the presence of AtSERK1kin protein clearly demonstrates the intermolecular character of the AtSERK1 reaction. We also incubated the mix of AtSERK1KT3E and AtSERK1K330E proteins together with or without casein or MBP in a phosphorylation assay. The results show that AtSERK1KT3E protein is not able to phosphorylate the AtSERK1K330E protein (Fig. 6C, lane 1) regardless of the presence of a potentially active catalytic site in the AtSERK1KT3E protein. Furthermore, this mutant protein mix is not able to phosphorylate casein or MBP (Fig. 6C, lanes 2 and 3).

These results suggest that under in vitro conditions, phos-
Autophosphorylation of AtSERK1

**DISCUSSION**

The *Arabidopsis* AtSERK1 gene is a member of the diverse family of serine/threonine receptor kinases that have been identified in plants. In this study we have shown that the AtSERK1 catalytic domain indeed exhibits phosphorylation activity on serine and threonine. A single amino acid substitution of Lys-330 to Glu-330, an invariant lysine found in the catalytic site of all protein kinases, abolishes the AtSERK1 kinase activity completely. Using site-directed mutagenesis, it was shown that Thr-720 was required for phosphorylation of *C. roseus* RLK1 (20). Using matrix-assisted laser desorption/ionization mass spectrometry on the brassinosteroid receptor 1 kinase domain, the presence of five phosphorylation sites was shown, one each in subdomains I and VIa and three in subdomain VIII (40). Using computer-assisted molecular modeling in combination with the functional studies of various kinase domains of transmembrane receptors has given an insight into the conformational changes that take place once the receptors are activated (41). In our studies we used the x-ray crystal structure of the unphosphorylated (22) and phosphorylated (23) IRK as templates for the modeling experiments. The computer-modeled structure of the AtSERK1 kinase domain is observed. The major difference is the presence of threonines in the AtSERK1 A-loop instead of tyrosines in the IRK A-loop. On the basis of the prediction that the threonines in AtSERK1 might fulfill the same role as tyrosines in IRK, mutant proteins were constructed in which Thr-459, Thr-462, Thr-463, and Thr-468 were mutated individually to either alanine or to glutamic acid residues. After kinetic studies we used the x-ray crystal structure of the unphosphorylated kinase (39) plant RLKs, which all have a preference for threonine autophosphorylation. To our knowledge, only two previous studies have identified a specific target site for autophosphorylation in plant RLKs. Using formic acid digests and site-directed mutagenesis, it was shown that Thr-720 was required for phosphorylation of *C. roseus* RLK1 (20). Using computer-assisted molecular modeling in combination with the functional studies of various kinase domains of transmembrane receptors has given an insight into the conformational changes that take place once the receptors are activated (41). In our studies we used the x-ray crystal structure of the unphosphorylated (22) and phosphorylated (23) IRK as templates for the modeling experiments. The computer-modeled structure of the AtSERK1 kinase domain is observed. The major difference is the presence of threonines in the AtSERK1 A-loop instead of tyrosines in the IRK A-loop. On the basis of the prediction that the threonines in AtSERK1 might fulfill the same role as tyrosines in IRK, mutant proteins were constructed in which Thr-459, Thr-462, Thr-463, and Thr-468 were mutated individually to either alanine or to glutamic acid residues. After...
nase activity measurements, it appeared that Thr-468 was the most likely single target for intermolecular AtSERK1 kinase activity. However, definite confirmation of the role of Thr-468 in vivo will require additional analysis using mass spectrometry of phosphorylated peptide fragments of AtSERK1 and site-specific antibodies directed against the A-loop region. Both of these experiments are currently in progress.

Nevertheless, it is of interest to note that all three phosphorylation sites in subdomain VIII of brassinosteroid receptor 1 (40) lie within the predicted A-loop region, given that the activation of many protein kinases occurs by autophosphorylation of 1–3 residues within the activation loop of subdomain VIII (35). Like animal receptor kinases, the subdomains representing the AtSERK1 A-loop may also be important for the phosphorylation of plant RLKs in general. Not only do many RLKs have serine or threonine residues, some of which were shown to be phosphorylated in vitro, in the corresponding A-loop regions (Fig. 7), also genetic analysis points to the functional importance of this region. Numerous mutants affecting brassinosteroid signaling fall within the proposed A-loop region (12), including brassinosteroid receptor 1-104 and brassinosteroid receptor 1-115 (17). The importance of A-loop phosphorylation is supported by our finding that on mutation of Thr-468 (conserved in most of the plant RLKs; Fig. 7), all AtSERK1 phosphorylation activity is lost.

In the case of the A-loop shifts the equilibrium toward a conformation that accommodates protein substrate binding. Point mutations in the A-loop of various receptor kinases are presumed to alter this equilibrium (41) and thus influence protein substrate binding and subsequent phosphorylation of substrates. Indeed, in the AtSERK1 threonine mutant proteins, the AtSERK1 substrate phosphorylation activity was abolished, suggesting that the AtSERK1 A-loop may be involved in protein substrate binding.

Autophosphorylation activity and activation of the transmembrane receptor protein kinases is generally mediated by ligand-induced dimerization (43). The activation of autophosphorylation of the catalytic domain of the receptor is mediated by an intermolecular mechanism of phosphorylation. Dimerization and intermolecular autophosphorylation of a nuclear serine/threonine kinase from A. thaliana has been shown (21). In our studies we also examined the mechanism of the autophosphorylation reaction of AtSERK1. The observation that the AtSERK1 kinase can phosphorylate the AtSERK1 kinase protein suggests that the AtSERK1 protein catalyzes an intermolecular autophosphorylation in vitro.

On the basis of the experiments reported here, we propose that, whether by the presence of a minor population of catalytically active AtSERK1 molecules in vitro or by ligand-induced conformational changes in vivo, AtSERK1 activation requires intermolecular autophosphorylation. This results in movement of the AtSERK1 threonine-containing A-loop followed by improved accessibility of the catalytic domain to commence substrate phosphorylation activity and protein substrate binding. In this model, A-loop phosphorylation of one AtSERK1 monomer is also essential for intermolecular autophosphorylation activity of that monomer.

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