Mutational Analysis of Stress-responsive Peanut Dual Specificity Protein Kinase

IDENTIFICATION OF TYROSINE RESIDUES INVOLVED IN REGULATION OF PROTEIN KINASE ACTIVITY

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We recently reported that Arachis hypogaea serine/threonine/tyrosine (STY) protein kinase is developmentally regulated and is induced by abiotic stresses (Rudrabhatla, P., and Rajasekharan, R. (2002) Plant Physiol. 130, 380–390). Other than MAPKs, the site of tyrosine phosphorylation has not been documented for any plant kinases. To study the role of tyrosines in the phosphorylation of STY protein kinase, four conserved tyrosine residues were sequentially substituted with phenylalanine and expressed as histidine fusion proteins. Mass spectrometry experiments showed that STY protein kinase autophosphorylated within the predicted kinase ATP-binding motif, activation loop, and an additional site in the C terminus. The protein kinase activity was abolished by substitution of Tyr297 with Phe in the activation loop between subdomains VII and VIII. In addition, replacing Tyr148 in the ATP-binding motif and Tyr317 in the C-terminal domain with Phe not only obliterated the ability of the STY protein kinase protein to be phosphorylated, but also inhibited histone phosphorylation, suggesting that STY protein kinase is phosphorylated at multiple sites. Replacing Tyr213 with the Thr-Glu-Tyr sequence motif with Phe resulted in a 4-fold increase in autophosphorylation and 2.8-fold increase in substrate phosphorylation activities. Mutants Y148F, Y297F, and Y317F displayed dramatically lower phosphorylation efficiency ($K_{cat}/K_m$) with ATP and histone, whereas mutant Y213F showed increased phosphorylation. Our results suggest that autophosphorylation of Tyr148, Tyr213, Tyr297, and Tyr317 is important for the regulation of STY protein kinase activity. Our study reveals the first example of Thr-Glu-Tyr domain-mediated autoinhibition of kinases.

Tyrosine phosphorylation plays an important role in the regulation of many cellular processes. The activity of the enzymes governing this modification, protein-tyrosine kinases and phosphatases, needs to be tightly controlled for tyrosine phosphorylation to proceed in an orderly manner. Improper phosphorylation due to malfunction of protein-tyrosine kinases and phosphatases can lead to diseases in humans and animals, including immunodeficiencies and cancer (1). In higher plants, several serine/threonine kinases have been identified, but the presence of protein-tyrosine kinases remains controversial. No tyrosine kinase has hitherto been cloned from plants. However, tyrosine phosphorylation in plants has been shown to be involved in various physiological processes (2–4). Dual specificity protein kinases have been reported in Arabidopsis (5, 6) and soybean (7), but the functions for these kinases remain unknown. We recently reported that serine/threonine/tyrosine (STY)$^3$ protein kinase from Arachis hypogaea is developmentally regulated and induced by abiotic stress (8). The site of tyrosine phosphorylation has not been identified for any dual specificity protein kinases in plants. However, MAPKs have been shown to be activated by dual phosphorylation of threonine and tyrosine in the Thr-Glu-Tyr (TEY) motif in the activation loop (9). Recently, the role of threonines in the phosphorylation of receptor-like kinase has been identified in plants (10, 11).

Most protein kinases retain their kinase domain conserved sequence motifs, which are separated into subdomains I–XI. The region between the conserved DFG sequence motif in subdomain VII and the APE sequence motif in subdomain VIII is referred to as the activation loop. Several protein kinases such as CAM-dependent protein kinase (12), a MAPK family (13), a MAPK kinase (MEK) family (14, 15), a cyclin-dependent protein kinase family (16), and c-Src tyrosine kinase (17) are activated by phosphorylation of residues within the activation loop (18). Phosphorylation negatively regulates a substantial number of protein kinases. For example, phosphorylation of cyclin-dependent protein kinase at threonine and tyrosine residues in the ATP-binding loop by wee1-related kinases leads to kinase inactivation (16), and phosphorylation of the Src tyrosine kinase at a tyrosine residue in the C terminus maintains the kinase in an inactive conformation (18).

Here we report a novel site of autoinhibition of STY protein kinase. In contrast to the above-mentioned kinases, we demonstrate here that the phosphorylation of Tyr148 in the ATP-binding domain of and Tyr317 in the C-terminal domain activates STY protein kinase. The replacement of Tyr297 with Phe in the activation loop resulted in a drastic reduction in the phosphorylation of peanut STY protein kinase with ATP and histone. This study provides direct evidence for the tyrosine phosphorylation of peanut STY protein kinase.

EXPERIMENTAL PROCEDURES

Materials—Histone H1 (type III), monoclonal anti-phosphotyrosine antibody, phosphoamino acids, and calf intestinal alkaline phosphatase were purchased from Sigma. $[^{32}P]$ATP (3000 Ci/mmol) was obtained from New England Nuclear.

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1 The abbreviations used are: STY, serine/threonine/tyrosine; MAPK, mitogen-activated protein kinase; TEY, Thr-Glu-Tyr; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MALDI, matrix-assisted laser desorption ionization; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; GSK, glycogen synthase kinase; MLK, mixed lineage kinase; SH3, Src homology 3.
from PerkinElmer Life Sciences. Restriction endonucleases were from MBI Fermentas (St. Leon-Rot, Germany). Pfu polymerase was from New England Biolabs Inc. (Beverly, MA). Nickel-nitrotriacetic acid-agarose was obtained from Qiagen Inc. (Chatsworth, CA). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland).

Bacterial Strains, Growth Conditions, and DNA Manipulations—
E. coli strain DH5α (Invitrogen) was the recipient for all plasmids used in subcloning. The BL21(DE3) pLysS strain (19) was used for bacterial expression of PrSET-C-STY and its protein kinase tyrosine mutants. LB medium with 50 μg/ml ampicillin was used for growing E. coli cells containing the plasmids. Plasmids were prepared by the alkaline lysis method (20). The DNA fragments were eluted from the agarose gel by the low melting agarose gel method. The preparation of competent cells and transformation were carried out as described (20).

Matrix-assisted Laser Desorption Ionization (MALDI) Mass Spectrometry—Two micrograms of STY protein kinase or autophosphorylated STY protein kinase was electrophoresed on SDS-polyacrylamide gel and visualized by Coomassie Blue staining. STY protein kinase bands were then excised from the gel, transferred to an acid-washed tube, rehydrated with water, crushed, washed three times for 20 min with 50 mM Tris-HCl (pH 8.0) and 50% acetonitrile, and dried. The sample was incubated for 6 h at 32 °C with 0.80 ng/ml trypsin in 25 mM Tris-HCl (pH 8.5) to digest the protein. The tryptic fragments were then extracted with 50% acetonitrile and 0.1% trifluoroacetic acid; dried; suspended in 10 ng/ml 4-hydroxy-α-cyanocinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid containing angiotensin as an internal standard; and applied to a MALDI sample plate, which was dried and washed with water to remove excess buffer salts. MALDI mass spectrometry analysis was performed on a Kratos PCCompactSeq 1.2.2 mass spectrometer in the linear mode. The masses obtained in these experiments were searched against predicted tryptic fragments of STY protein kinase using the program PeptideMass (21).

Site-directed Mutagenesis—Wild-type STY protein kinase (pRSET-C-STY) template (80 ng) and sense and antisense primers (25 pmol) were added to PCR tubes containing 0.2 mM dNTPs, 1 mM MgSO4, 100 ng template (80 ng) and sense and antisense primers (25 pmol) were used for bacterial expression of PrSET-C-STY and its protein kinase tyrosine mutants. LB medium with 50 μg/ml ampicillin was used for growing E. coli cells containing the plasmids. Plasmids were prepared by the alkaline lysis method (20). The DNA fragments were eluted from the agarose gel by the low melting agarose gel method. The preparation of competent cells and transformation were carried out as described (20).

Expression and Purification of Wild-type STY Protein Kinase and Its Mutants—The cDNA spanning the coding region of STY protein kinase (lacking 11 amino acids from the N terminus) was subcloned into the histidine-tagged fusion protein expression vector pRSET-C at BglII and KpnI restriction sites. The resultant construct was expressed in E. coli BL21(DE3) pLysS. The recombinant fusion protein was induced with 0.4 mM isopropyl-1-thio-D-galactopyranoside for 4 h. The recombinant protein was induced in large-scale (500 ml) and purified by nickel-nitrotriacetic acid-agarose chromatography. Protein concentrations were determined by the method of Bradford (22). Purified fractions containing the eluted protein were analyzed by 12% SDS-PAGE, followed by Coomassie Blue staining (23). Mutant proteins were purified similarly to wild-type STY protein kinase.

Assay of Tyrosine Phosphorylation—Samples of the His6-tagged STY protein kinase and mutant fusion proteins were separated on 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and probed with monoclonal anti-phosphotyrosine antibody. In Vitro Kinase Assay—ATP dependence assays were performed by incubating the STY protein kinase and mutant fusion proteins (1 μg, with 7000 cpm containing 1.5 μCi [γ-32P]ATP in a volume of 20 μl of kinase buffer and stopped at 20 min by the addition of 15 μl of 3× gel loading buffer. Histone phosphorylation assays were performed by incubating the STY protein kinase and mutant fusion proteins with 2.5–40 μl histone H1 containing 50 μl unlabeled ATP and 1.5 μCi of [γ-32P]ATP in a total volume of 30 μl of kinase buffer and stopped at 20 min by the addition of 15 μl of 3× gel loading buffer. The reaction products were separated by 12% SDS-PAGE. The Coomassie Blue-stained protein bands were recovered and then measured using a PhosphorImager system (Molecular Dynamics, Sunnyvale, CA). Phosphoamino Acid Analysis—Purified STY protein kinase and the mutant proteins were labeled in vitro with [γ-32P]ATP as described.
above and electroblotted onto a polyvinylidene difluoride membrane. After autoradiography, radioactive bands of interest were excised and hydrolyzed in 200 μl of 6 M HCl for 2 h at 110 °C. The hydrolysate was dried in a SpeedVac concentrator and resuspended in 20 μl of water containing 1 mg/ml each of the phosphoamino acid markers such as phosphoserine, phosphothreonine, and phosphotyrosine. Two microliters of the hydrolysate was analyzed by ascending silica thin-layer chromatography (Merck) using a solvent system containing a mixture of ethanol and ammonia (3.5:1.6, v/v) (24). The positions of phosphoamino acid markers were detected by ninhydrin staining of the silica thin-layer plate (0.25% ninhydrin in acetone). The plate was then exposed for autoradiography to locate the positions of 32P-labeled amino acids.

Calf Intestinal Alkaline Phosphatase Treatment of the Recombinant Kinase—The histidine fusion protein of STY protein kinase was incubated with 1 unit of calf intestinal alkaline phosphatase for 30 min at 37 °C, and the phosphorylated and dephosphorylated kinases were resolved by 12% SDS-PAGE.

Circular Dichroism—CD studies were performed using a Jasco J-720 spectropolarimeter with a thermostatically controlled 10-mm cylindrical CD cell at 4 μM protein. For near-UV and UV-visible wavelengths, protein concentrations ranged from 0.8 to 1 mg/ml using a 1-cm path length. The CD spectra are the averages of five independent experiments on different samples of the same enzyme preparation recorded at a scan speed of 50 nm/min with a bandwidth of 2 nm and averaged automatically. All spectra were corrected for the appropriate blank solutions, recorded in the absence of enzyme. The values are expressed in terms of molar ellipticity.

RESULTS

Sequence Comparison of the STY Protein Kinase Activation Loop with Tyrosine Kinases and Dual Specificity Protein Ki-
nases Regulated by Tyrosine Phosphorylation—A schematic representation of the STY protein kinase catalytic domain with all 11 subdomains representing the site-directed mutations generated in this study is shown in Fig. 1A. STY protein kinase has four conserved tyrosine residues that are present in the ATP-binding domain (subdomain I), the TEY sequence motif (subdomain V), the activation loop (subdomain VIII), and toward the C terminus (subdomain IX). Short sequences between the conserved DFG motif in subdomain VII and the APE sequence motif in subdomain VIII of the protein kinase domains are referred to as the activation loop. STY protein kinase contains a single tyrosine (Tyr297) in the activation loop (Fig. 1B). The site of tyrosine phosphorylation has not been identified in plant kinases. Therefore, we compared STY protein kinase with the dual specificity protein kinases from all the species (Fig. 1B). The protein sequence of STY protein kinase has homology to the DYRK family of kinases from mammals, and DYRK is an intracellular dual specificity protein kinase that is regulated by tyrosine phosphorylation of the activation loop (25). Based on the assumption that the kinase activity of STY protein kinase may be regulated by phosphorylation of the tyrosine residue in the activation loop, we constructed an STY protein kinase mutant with Tyr297 replaced with Phe by site-directed mutagenesis. Alignment of Src tyrosine kinases and STY protein kinase revealed the presence of a conserved tyrosine in the TEY motif subdomain V (Fig. 1C).

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Homology Modeling—The x-ray structures of the mouse c-ABL kinase domain in complex with the inhibitor STI-571 (Protein Data Bank code 1IEPA), human protein-tyrosine kinase (code 2SRC), and chicken protein-tyrosine kinase (code 2PTK) were used as templates for modeling the STY protein kinase catalytic domain. These templates produced the best E value when using BLAST against protein kinase in the Protein Data Bank. A three-dimensional model of the catalytic domain of STY protein kinase was determined using SWISS-MODEL.

FIG. 3. Tyrosine phosphorylation of the STY protein kinase mutants in which the autophosphorylated tyrosines were replaced with phenylalanines. A, affinity-purified histidine fusion proteins of wild-type (WT) STY protein kinase and its mutants were run on 12% SDS-polyacrylamide gel, transferred onto a nitrocellulose membrane, and subjected to anti-phosphotyrosine (Anti-pY) immunoblot analysis. B, shown is an anti-STY protein kinase immunoblot illustrating equal loading of the fusion proteins. C, shown is the dephosphorylation of wild-type STY protein kinase with calf intestinal alkaline phosphatase (CIAP). Lane 1, 15% SDS-polyacrylamide gel representing molecular mass standards; lane 2, wild-type STY protein kinase; lane 3, calf intestinal alkaline phosphatase-treated STY protein kinase.

FIG. 4. Autophosphorylation and histone phosphorylation of STY protein kinase and its mutants. Bacterially produced STY protein kinase and its mutants were purified, and 500 ng of protein was incubated with \[\gamma^{32}\text{P}]ATP to determine kinase activity. After separation by 12% SDS-PAGE, the resulting gels were analyzed using a PhosphorImager. A, autoradiographs of autophosphorylation of wild-type (WT) STY protein kinase and mutants Y148F, Y213F, Y297F, and Y317F. As indicated, the proteins were preincubated with or without 0.5 mM unlabeled ATP in the same buffer for 30 min. B, histogram constructed with data from A. Black bars, autophosphorylated enzymes without preincubation with ATP; white bars, autophosphorylated enzymes preincubated with ATP. The error bars represent the S.D. of three independent experiments. C, histone phosphorylation of wild-type and site-directed mutants. Aliquots of 500 ng of purified STY protein kinase and its mutants were incubated with 1 \(\mu\)g of histone in the presence of \(\gamma^{32}\text{P}]ATP as described under “Experimental Procedures.” After separation by 12% SDS-PAGE, the resulting gels were analyzed using a PhosphorImager. Shown are autoradiographs of histone incubated with wild-type STY protein kinase, K160R, Y148F, Y213F, Y297F, and Y317F. D, histogram constructed with data from C. The error bars represent the S.D. of four experiments.

role of Tyr213 in the TEY motif, site-directed substitution of tyrosine with phenylalanine was performed.
of a phosphate (+80 Da) in the autophosphorylated samples were studied. One of the major peaks (2541.20 Da) fitting these criteria (peak b) has a mass that corresponds to a peptide from the activation loop of STY protein kinase. This tryptic peptide contains a single tyrosine (Tyr<sup>297</sup>). Two other peaks (peaks a and c) correspond to Tyr<sup>148</sup> (450 Da) in the ATP-binding domain and Tyr<sup>317</sup> (3850.02 Da) in subdomain IX, respectively. Corresponding unphosphorylated peptides were not detected in the MALDI time-of-flight mass spectra of the autophosphorylated kinase. We did not observe any evidence for phosphorylation of Tyr<sup>213</sup> in the TEY motif in STY protein kinase (Fig. 2). These results suggest the possibility of STY protein kinase being phosphorylated at multiple sites and that Tyr<sup>148</sup>, Tyr<sup>297</sup>, and Tyr<sup>317</sup> are the autophosphorylation sites.

**Expression and Purification of Wild-type STY Protein Kinase and Substituted STY Protein Kinase Mutants**—Based on the comparative sequence analysis, homology modeling, and phosphopeptide mapping, the conserved Tyr<sup>148</sup>, Tyr<sup>213</sup>, Tyr<sup>297</sup>, and Tyr<sup>317</sup> residues of STY protein kinase were substituted with Phe by site-directed mutagenesis. All the mutant proteins were overproduced in *E. coli* BL21(DE3) pLysS using the T7 RNA polymerase expression system, induced by isopropyl-thio-β-D-galactoside, and purified by nickel-nitrotriacetic acid affinity chromatography. The expressed proteins were predominantly present in the soluble fraction, and yields of the proteins were in the range of 10–15 mg/liter. The expression levels of and the ability to purify the STY protein kinase mutants suggested that they were as stable as the wild-type protein.

**Tyrosine Phosphorylation of STY Protein Kinase Mutants**—To determine the degree of autophosphorylation, equal quantities of histidine fusion proteins of the wild-type and mutant STY protein kinases were subjected to anti-phosphotyrosine and anti-STY protein kinase immunoblot analyses. Notably, there was a significant increase in the reactivity of the anti-phosphotyrosine antibody with the Y213F mutant protein (Fig. 3A). The tyrosine mutations at positions 148, 297, and 317 diminished the reactivity of STY protein kinase with the anti-phosphotyrosine antibody. When the same blot was subjected to anti-STY protein kinase Western blot analysis, there was no difference in the reactivities of the wild-type and mutant proteins (Fig. 3B). There was a slight downshift in the mobility of the Y148F, Y297F, and Y317F mutant proteins with respect to the wild-type protein. When wild-type STY protein kinase was subjected to phosphatase treatment, we observed faster mobility of the protein upon SDS-PAGE compared with the untreated protein (Fig. 3C). The downshift in mobility of the protein kinase inactive mutants could be due to the phosphorylation status of the protein.

**Effect of Tyrosine Mutations on Kinase Activity**—The kinase activities of all the site-directed mutants were assessed by comparing their abilities to autophosphorylate and phosphorylate histone in *vitro* (Fig. 4). As a negative control, the conserved lysine in subdomain II involved in ATP binding was changed to arginine to form a kinase inactive mutant (K160R), and the mutant did not show autophosphorylation or substrate phosphorylation. Preincubation of STY protein kinase and the site-directed mutants with magnesium and unlabeled ATP resulted in a reduced level of labeled phosphate incorporation (Fig. 4, A and B). Replacement of Tyr<sup>213</sup> with Phe resulted in a 4-fold increase in autophosphorylation and a 2.8-fold increase in histone phosphorylation. Elevated tyrosine phosphorylation of the Y213F mutant protein was accompanied by reduced mobility upon SDS-PAGE. We analyzed the relative incorporation of <sup>32</sup>P label into the phosphoamino acids. Surprisingly, the Y213F mutant showed an increased band intensity of phosphoserine, phosphothreonine, and phosphotyrosine with respect to the

**Phosphorylation Site Mapping by MALDI Time-of-Flight Mass Spectrometry Analysis**—We carried out mass spectrometry experiments to identify autophosphorylation sites. Recombinant STY protein kinase was digested with trypsin before and after an extended autophosphorylation reaction, and the protein was analyzed by MALDI mass spectrometry (Fig. 2). Phosphorylation increased the peptide mass by 80 Da. Tryptic fragments of STY protein kinase that were found in the unphosphorylated state and those that were shifted by the mass
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**Table I**
Kinetic constants of wild-type STY protein kinase and its mutants

| ATP | Histone |
|-----|---------|
| | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
| | $s^{-1}$ | $\mu M$ | $\mu M^{-1} s^{-1}$ | $s^{-1}$ | $\mu M$ | $\mu M^{-1} s^{-1}$ |
| Wild-type | 5.25 ± 0.46 | 34.4 ± 2.9 | 0.153 | 9.15 ± 0.89 | 6.7 ± 0.54 | 1.365 |
| Y148F | 1.02 ± 0.08 | 107.1 ± 9.3 | 0.009 | 3.09 ± 0.3 | 10.7 ± 0.19 | 0.288 |
| Y213F | 14.5 ± 0.12 | 18.5 ± 1.6 | 0.769 | 18.4 ± 1.6 | 6.4 ± 0.54 | 2.575 |
| Y297F | 0.54 ± 0.04 | 80.5 ± 7.3 | 0.006 | 3.71 ± 0.32 | 10.8 ± 0.9 | 0.342 |
| Y317F | 0.26 ± 0.02 | 58.8 ± 5.2 | 0.004 | 4.74 ± 0.39 | 11.5 ± 0.8 | 0.412 |

**Table II**
Fraction of secondary elements as calculated from respective CD spectra

| | $\alpha$-Helix | $\beta$-Sheet | Random coil |
| | % | |
| Wild-type | 37 | 16 | 48 |
| Y148F | 38 | 17 | 45 |
| Y213F | 37 | 15 | 48 |
| Y297F | 31 | 15 | 54 |
| Y317F | 37 | 15 | 48 |

wild-type protein, suggesting that serines, threonines, and tyrosines were phosphorylated (Fig. 5). Substitution of Tyr$^{148}$ in the ATP-binding domain with Phe decreased both autophosphorylation and substrate phosphorylation. The site-specific mutation of phenylalanine strongly decreased both autophosphorylation and substrate phosphorylation. There is a single tyrosine (Tyr$^{297}$) in the activation loop upstream of the APE sequence in STY protein kinase, and its replacement with phenylalanine strongly decreased both autophosphorylation and substrate phosphorylation. The site-specific mutation of Y317F in the C-terminal motif also resulted in a drastic reduction in protein kinase activity (Fig. 4, A–D). Phosphoamino acid analyses of the Y148F, Y297F, and Y317F mutant proteins revealed a drastic reduction in phosphorylation of tyrosine with respect to the wild-type protein (Fig. 5). These results suggest that the mutated sites could play a role in the regulation of STY protein kinase.

**Kinetics of Autophosphorylation of STY Protein Kinase Mutants**—STY protein kinase exhibited Michaelis-Menten kinetics with respect to ATP. The apparent $K_m$ value for ATP was calculated to be 34.4 $\mu M$ (Table I). The Y213F mutant showed an increase in phosphorylation activity with respect to the wild-type protein; hence, we investigated whether the kinetic properties of this mutant deviated significantly from that of the wild-type enzyme. The mutation decreased the apparent $K_m$ from 34.4 to 18.8 $\mu M$ and increased the $k_{cat}$ value (Table I). The phosphorylation efficiency of this mutant ($k_{cat}/K_m$) was 5-fold higher than that of the wild-type enzyme.

The same analyses were performed with mutants Y148F, Y297F, and Y317F (Table I). There was a significant reduction in the affinity of the Y148F mutant for ATP, and the $K_m$ of the mutant increased by 3-fold and the $k_{cat}$ was reduced by 5-fold compared with those of the wild-type enzyme. A drastic reduction (17-fold) in the phosphorylation efficiency of this mutant was observed compared with that of the wild-type protein. The Y297F mutant also showed a 26-fold reduction in phosphorylation efficiency compared with the wild-type enzyme. The $K_m$ of the Y317F mutant deviated slightly from that of the wild-type protein, but the $k_{cat}$ and phosphorylation efficiency of this mutant protein were drastically reduced by 20- and 38-fold, respectively, compared with those of the wild-type protein. These results suggest that the tyrosine mutations reported in this study significantly altered the phosphorylation activity.

**Kinetics of Histone Phosphorylation of STY Kinase Mutants**—The wild-type enzyme has an apparent $K_m$ of 6.71 $\mu M$ (Table I). The Y213F mutation showed no apparent effect on the $K_m$, but led to an increase (2-fold) in the $k_{cat}$. The Y148F mutant was substantially less active than the wild-type enzyme, with 5-fold lower phosphorylation efficiency and a 1.6-fold higher $K_m$ for histone. A decrease in $k_{cat}$ and histone phosphorylation efficiency was observed with the Y297F mutant. There was a 3.3-fold reduction in the histone phosphorylation efficiency of the Y317F mutant protein.

**Circular Dichroism of STY Protein Kinase Mutants**—The secondary structures of these proteins were monitored by CD. The observed deficiency in enzyme activity for any of these mutant enzymes could have been caused by alterations in the secondary structures of the enzyme. The number of secondary structure elements was calculated from the CD spectra using Anthroprot software (29). The CD spectra of the wild-type and mutant STY protein kinases were similar and were characterized by a minimum at 204–205 nm and a shoulder in the 222–225 nm range, suggesting the presence of $\beta$-sheet and $\alpha$-helical structures, respectively. The results from the CD spectral analyses of STY protein kinase and its mutants are given in Table II.

**DISCUSSION**

Regulation of protein kinases is achieved through many different mechanisms, including protein phosphorylation by other kinases (30), autophosphorylation (31), and control by regulatory domains or subunits (32). A key feature of regulation in many protein kinases is thought to be the phosphorylation of one or more residues within the activation loop of the catalytic subunit. STY protein kinase activation by tyrosine phosphorylation within the activation loop is of interest because it suggests possible involvement of STY protein kinase in tyrosine kinase-mediated signaling pathways in plants. Most protein kinases that are activated by phosphorylation of residues in the activation loop belong to arginine/aspartate kinase family (18), in which the aspartate residue in the activation loop has an adjacent arginine. The arginine residue has been hypothesized to form an ionic bridge with the phosphorylated serine or threonine residue that stabilizes the catalytically active conformation (34). STY protein kinase also belongs to the arginine/aspartate kinase family. In this study, we have shown that a conserved tyrosine residue within the activation loop regulates STY protein kinase activity. There is a single tyrosine (Tyr$^{297}$) in the activation loop of peanut STY protein kinase, and the substitution of Tyr$^{297}$ with Phe led to a significant loss of catalytic activity in terms of both autophosphorylation and substrate phosphorylation toward histone. Activation of the mammalian dual specificity protein kinases is regulated by the tyrosine phosphorylation of the activation loop. Isoforms of the protein kinase C subfamily can be activated by tyrosine phosphorylation by Bruton’s tyrosine kinase or Src family tyrosine kinases (35, 36). Tyrosine phosphorylation of a member of the protein kinase C subfamily is dependent on the activity of a Bruton’s tyrosine kinase that may directly phosphorylate the protein kinase C (37). All other kinases that are regulated by tyrosine phosphorylation between subdomains VII and VIII,
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e.g., MAPK/ERK, JNK, and GSK3, are components of signaling pathways that transduce receptor-initiated signals to nuclear phosphorylation of transcription factors (38). Two requirements have been thought to be critical for the catalytic activity of protein kinases. One is the correct juxtaposition of catalytic groups contributing to the transfer of the γ-phosphate group from ATP to a serine, threonine, or tyrosine side chain of the substrate (34). The other is the accessibility and correct positioning of the substrate-binding site(s) (30). The mechanism of peanut STY protein kinase activation by phosphorylation could therefore be that phosphorylation promotes a conformation of the activation loop in which the catalytic and substrate-binding sites are correctly formed, resulting in a significant increase in kinase activity. STY protein kinase may also be activated in vivo through activation loop phosphorylation of the tyrosine residue by an upstream kinase(s).

In our studies, mutation of Tyr213 to Phe produced a recombinant enzyme with a 4-fold increase in autophosphorylation and a 2.8-fold increase in substrate phosphorylation activities. A similar observation was made earlier in several protein kinases (32, 39). The catalytic activity of many protein kinases is regulated by an autoinhibitory mechanism known as intraspecific regulation (32). Many protein kinases are maintained in low activity or inactive states through intramolecular or intramolecular association with inhibitory molecules or domains. These inhibitory molecules may be disrupted in response to appropriate stimuli, allowing the protein kinase to adopt an active conformation. Autoinhibitory sequences have been found in protein kinase C, calcium/calmodulin-dependent kinase, myosin light chain kinase, c-Src, the insulin receptor, and twitchin (32, 39, 40). The affinity of autoinhibitory sequences may also be regulated by autophosphorylation or other interactions, but the structural basis for these regulatory mechanisms remains to be elucidated. Casein kinases are also dual specificity kinases, and autophosphorylation has an inhibitory effect on protein kinase activity. Casein kinases are regulated by a C-terminal phosphorylation-dependent autoinhibitory domain (41). Autophosphorylation inactivates the kinase, and mutagenesis of the phosphorylation residues of the C-terminal domain reactivates the kinase. Serine and threonine to alanine substitutions of the phosphorylation residues in the kinase domain, Kuret and co-workers (42) described two forms of recombinant yeast casein kinase 1ε one form was autophosphorylated in the kinase domain and had a 4-fold decrease in activity compared with the unphosphorylated protein kinase.

STY protein kinase has homology to the MLKs from mammals. A point mutation (Y52A) in the SH3 domain-binding site of MLK3 abolishes binding to the SH3 domain and increases the MLK3 activity (43). The Y52A mutant shows a 2-fold increase in autophosphorylation and a 2.5-fold increase in histone phosphorylation. SH3 domains are known to bind to proteins containing PXXP sequences that form polyproline type II helices (44). STY protein kinase also has a similar sequence motif in the C-terminal region. Apart from the other examples, substitution of Tyr148 with Phe in the intact insulin receptor results in an increase in basal protein kinase activity in the absence of insulin, consistent with an autoinhibitory role for Tyr148 (45). A point mutation in the TEY domain might disrupt the autoinhibitory domain, leading to a conformational change that activates the protein kinase, leading to the increased phosphorylation of all three amino acids. A crystal structure of the STY protein kinase molecule will be required to understand how autophosphorylation of Tyr213 might affect the conformation of STY protein kinase.

STY protein kinase possesses a tyrosine residue within the ATP-binding motif, whereas the vast majority of kinase family members have a phenylalanine at this site (1). The protein kinases with a tyrosine in the ATP-binding domain have been shown to be regulated by tyrosine phosphorylation. Tyr148 in the ATP-binding domain is conserved among STY protein kinase-related sequences. The replacement of Tyr148 with Phe drastically reduced the enzyme activity of peanut STY protein kinase. The tyrosine in the ATP-binding site of cyclin-dependent protein kinases and the phosphorylation of residues in this region are important for the inhibition of kinase activity (16).

Several protein kinases are regulated by tyrosine phosphorylation of the C-terminal region. STY protein kinase has structural similarity to Src tyrosine kinases from chicken and human (46, 47). Src family kinases have a regulatory tyrosine (Tyr527 in p60src) toward the C terminus and an autophosphorylation site (Tyr416). Data on the biological properties of p60src have shown that mutation of its C-terminal sites of tyrosine phosphorylation can serve to activate or suppress its transforming properties (48–50), indicating that this process is an important regulatory step. The C-terminal region of the epidermal growth factor receptor is important in regulating its biological activity (51). The point mutation of Tyr312 to Phe in the C-terminal region of STY protein kinase resulted in the reduction of autophosphorylation activity.

Our results show that STY protein kinase is a multisite-phosphorylated enzyme and suggest that its phosphorylation may be an intricate process that regulates its biological functions in very distinct ways. Multisite phosphorylation is a characteristic of MAPK-activated protein kinase-2, in which any two of three sites must be phosphorylated to achieve maximal activation (52). Protein kinase D is also phosphorylated at multiple sites such as the activation loop, C-terminal domain, and regulatory domain (33). STY protein kinase is developmentally regulated and is induced by cold and salt stresses (8). The distribution of STY protein kinase autophosphorylation sites in the kinase molecule suggests that different sites may be involved in distinct mechanisms mediated by STY protein kinase during the onset of stress response. Src family kinases and MLKs are dual specificity protein kinases regulated by both autophosphorylation and autoinhibition. Like Src family tyrosine kinases and mammalian MLks, STY protein kinase has a PXXP sequence motif in the C-terminal region. STY protein kinase might interact through these proline-rich sites with other kinases that are involved in signal transduction. Further studies are needed to identify the upstream kinase(s) that phosphorylate STY protein kinase in its catalytic segment and to fully elucidate the molecular mechanism of the regulation of STY protein kinase activity. These mutants provide an excellent opportunity to dissect in vivo function and regulation of STY protein kinase.

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