Nicotiana tabacum Osmotic Stress-activated Kinase Is Regulated by Phosphorylation on Ser-154 and Ser-158 in the Kinase Activation Loop*

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Anna Maria Burza 1, Izabela Pękala 1, Jacek Sikora 1, Paweł Siedlecki 1, Paweł Małagocki 1, Maria Bucholc 1, Luiza Koper 1, Piotr Zielenkiewicz 1, Michał Dadlez 2, and Grażyna Dobrowska 1,2

From the 1Department of Plant Biochemistry, 4Department of Bioinformatics, and 5Mass Spectrometry Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawińskiego 5a, 02-106 Warsaw, Poland, the 2Faculty of Biology, Warsaw University, Miecznikowa 1, 02-096 Warszawa, Poland, and the 1Laboratory of Plant Molecular Biology, Warsaw University, ul. Pawińskiego 5a, 02-106 Warsaw, Poland

Nicotiana tabacum (Nicotiana tabacum osmotic stress-activated protein kinase), a member of the SnRK2 subfamily, is activated rapidly in response to hyperosmotic stress. Our previous results as well as data presented by others indicate that phosphorylation is involved in activation of SnRK2 kinases. Here, we have mapped the regulatory phosphorylation sites of NtOSAK by mass spectrometry with collision-induced peptide fragmentation. We show that active NtOSAK, isolated from NaCl-treated tobacco BY-2 cells, is phosphorylated on Ser-154 and Ser-158 in the kinase activation loop. Prediction of the NtOSAK three-dimensional structure indicates that phosphorylation of Ser-154 and Ser-158 triggers changes in enzyme conformation resulting in its activation. The involvement of Ser-154 and Ser-158 phosphorylation in regulation of NtOSAK activity was confirmed by site-directed mutagenesis of NtOSAK expressed in bacteria and in maize protoplasts. Our data reveal that phosphorylation of Ser-158 is essential for NtOSAK activation, whereas phosphorylation of Ser-154 most probably facilitates Ser-158 phosphorylation. The time course of NtOSAK phosphorylation on Ser-154 and Ser-158 in BY-2 cells subjected to osmotic stress correlates with NtOSAK activity, indicating that NtOSAK is regulated by reversible phosphorylation of these residues in vivo. Importantly, Ser-154 and Ser-158 are conserved in all SnRK2 subfamily members, suggesting that phosphorylation at these sites may be a general mechanism for SnRK2 activation.

Enzymes have to be regulated very strictly inside the cell. Several protein kinases involved in osmotic stress signal transduction in plants have been identified (for a review, see Ref. 1). Most of the available information concerns mitogen-activated protein kinase (MAPK)3 cascades, which in all eukaryotic organisms play a central role in stress signal transduction. All MAPKs, as well as MAPKKs, including plant enzymes, are activated by phosphorylation in their activation loops by specific upstream kinases, MAPKKs and MAPKK kinases, respectively (for a review, see Ref. 2), and become inactivated by phosphoprotein phosphatases (for a review, see Ref. 3).

Besides the enzymes belonging to the MAPK cascades and several other protein kinases conserved in all eukaryotic organisms ranging from yeast to human, plant genomes encode also plant-specific protein kinases, involved in the responses to harsh environmental conditions. Representative of such enzymes are some of the SNF1-related protein kinases (SnRKs). Plant SnRKs are classified into three subfamilies: SnRK1, SnRK2, and SnRK3 (for reviews, see Refs. 4–6). Enzymes belonging to the SnRK1 group structurally and functionally resemble the yeast and animal SNF1/AMPK kinases, which play a role in protecting cells against nutritional and environmental stresses (for reviews, see Refs. 4, 7, and 8). The SnRK2 and SnRK3 subfamilies are specific to plants (for reviews, see Refs. 4 and 5). Amassing data suggest that these enzymes participate in environmental stress signaling. Members of the SnRK3 subfamily, especially those that interact with calcineurin B-like proteins, are relatively well characterized. They take part in the protection of plant cells against different abiotic stresses (1, 9–11). These kinases are activated by interaction with calcineurin B-like proteins and most probably by phosphorylation, since in the case of several enzymes of this subfamily, substitution of a conserved threonine residue in the kinase activation loop with aspartic acid mimicking the phosphate group results in constitutively active enzymes (12, 13).

Increasing amounts of information concerning enzymes belonging to the SnRK2 subfamily also indicate their role in abiotic stress signaling in plants. It has been shown that in Ara-

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1 These two authors contributed equally to this work.

2 To whom correspondence should be addressed. Tel.: 48-22-5925717; Fax: 48-22-6584636; E-mail: dobrowol@ibb.waw.pl.

3 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; ABA, abscisic acid; SnRK, Snf1-related protein kinase; MBP, myelin basic protein; GST, glutathione S-transferase; GFP, green fluorescent protein; LC, liquid chromatography; MS, mass spectrometry.
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*bidopsis thaliana* and in *Oryza sativa*, there are 10 members of the SnRK2 family (14, 15). All of them, except SnRK2.9 from Arabidopsis, are activated by different osmolytes, such as sucrose, mannitol, sorbitol, and NaCl, and some of them also by abscisic acid, suggesting that these kinases are involved in a general response to osmotic stress (14, 15). The best known members of the SnRK2 group are wheat PKABA1 (protein kinase induced by abscisic acid), the expression of which is activated by ABA as well as by drought and salinity (16, 17); AAPK (ABA-activated protein kinase), activated by ABA in guard cells of *fava* bean (*Vicia faba*) in response to drought, involved in the regulation of stomatal closure and anion channels (18, 19); and *A. thaliana* SRK2E/OST1/SnRK2.6 protein kinase, an enzyme related to AAPK, mediating the regulation of stomatal aperture by ABA and acting upstream of reactive oxygen species production (20). The srk2e mutant cannot cope with a rapid decrease in humidity and has a wilt phenotype (21). Recently, it has been shown that *Arabidopsis* SRK2C/OSKL4/SnRK2.8 kinase is involved in drought stress signaling (22). The enzyme improves the drought tolerance of *Arabidopsis* plants by controlling stress-responsive gene expression. Also soybean SnRK2s involved in abiotic stress signal transduction have been described. Expression of genes encoding the SPK-3 and SPK-4 kinases from soybean, both members of the SnRK2 subfamily, is induced by dehydration and high salinity (23). Two other soybean protein kinases, SPK-1 and SPK-2 from the same group, are able to phosphorylate Ssh1p (soybean unusual phosphatidylinositol transfer-like protein), upon exposure of plant tissues to hyperosmotic stress (24).

Although there is evidence that SnRK2s function in abiotic stress signaling in plants and are activated very rapidly in response to osmotic stress, there is still very little information concerning the mechanism of their activation. Our previous results concerning NtOSAK of the SnRK2 subfamily (25), the very recent ones by Belin results concerning NtOSAK of the SnRK2 subfamily (25), the very recent ones by Belin et al. (26) on *Arabidopsis* OST1 kinase suggested that these enzymes were activated by phosphorylation. Contrary to this, Hoyos and Zhang (27) claimed that high osmolality stress-activated kinase from tobacco, whose properties resemble those of NtOSAK, was activated by dephosphorylation. In order to clarify this controversy and to establish the mechanism of SnRK2 activation under osmotic stress conditions, systematic studies on NtOSAK activation were undertaken. The results presented here show that phosphorylation of serines 154 and 158 in the NtOSAK activation loop is involved in the kinase activation in response to hyperosmotic stress. The identified sites are conserved in all members of the SnRK2 family, which suggests that this can be a general mechanism of regulation of the activity of these kinases.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—BY-2 tobacco cells, kindly provided by Dr. Witold Filipowicz (Friedrich-Miescher Institute, Basel, Switzerland), were cultured as described by Nagata et al. (28). The cells were grown in Murashige and Skoog’s medium supplemented with 100 mg/liter *myo*-inositol, 1 mg/liter thiamine HCl, 255 mg/liter KH$_2$PO$_4$, 0.2 mg/liter 2,4-dichloropenoxyacetic acid, and 3% sucrose. The cells were subcultured every 7 days.

The cells were treated with NaCl (100 or 250 mM) or sorbitol (500 or 900 mM) for the indicated times, harvested by centrifugation, quickly frozen in liquid nitrogen, and stored at −80 °C until analyzed.

**Preparation of Protein Extracts**—Protein extracts were prepared as previously described (25, 29).

**Reverse Transcription-PCR Analysis**—Total RNA was isolated from untreated and stressor-treated BY-2 using TRI REAGENT (MRC, Cincinnati, OH) according to the procedure recommended by the manufacturer. The cDNA was synthesized from 1 μg of total RNA using the HSRT 100 kit (Sigma). Briefly, RNA was reverse transcribed for 60 min at 47 °C in 20 μl of reaction mixture containing 1 unit of enhanced avian reverse transcriptase, 500 μM each dNTP, 3.5 μM anchored oligo(dT) primer, 1 unit RNAse inhibitor. One microliter of the reverse transcription reaction was used for PCR in 20 μl containing 0.4 units of TaqDNA polymerase (Fermentas UAB, Vilnius, Lithuania), 200 μM each dNTP, 1.5 mM MgCl$_2$, and a 625 nM concentration of the appropriate primers. Routine PCR conditions were as follows: 3 min, 94 °C (first cycle); 30 s, 94 °C; 30 s, 55 °C; 1 min, 72 °C (40 cycles); and 10 min, 72 °C (final cycle). The PCR products were separated on 0.8% agarose gels and visualized by EtBr staining. NtOSAK-specific primers were as follows: forward, 5′-CTTTCTTCTTCTTTCTTT-ATTTCATCTTCCCC-3′, based on NtOSAK untranslated region sequence; reverse, 5′-CTATGATCTCCTCAACACTCTG-3′, based on NtOSAK coding sequence. As a control of the amount and quality of RNA, the level of actin transcript was monitored using the following primers: 5′-ATGGCA-GACGTTGAGGATCTTCA-3′ and 5′-GGCTTTGCAATC-CACATCTTCTTTG-3′.

**Immunoblotting**—Western blot analysis was generally performed as described previously (29) using polyclonal anti-NtOSAK antibodies raised against the C-terminal peptide (KQVQQHESGEVRTL) of the kinase or phospho-specific polyclonal antibodies raised in rabbit against the phosphopeptide (KpSTVGT; where pS represents phosphoserine) and purified by affinity chromatography (BioGenes, Berlin, Germany). The phospho-specific antibodies were designated as anti-Ser(P)-158. In the case of blots probed with anti-Ser(P)-158 antibodies, in order to block nonspecific binding, the membranes were incubated with 5% bovine serum albumin and 5% milk in TBST buffer (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) overnight at room temperature and then for 2 h in the same solution with the antibodies at 1:500 dilution at room temperature. After incubation with alkaline phosphatase-conjugated secondary antibodies, the results were visualized by staining with p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt.

**Purification of NtOSAK**—NtOSAK was purified from BY-2 cells treated for 5 min with 250 mM NaCl according to the method described previously (25).

**Expression of GST-NtOSAK in Escherichia coli**—Preparation of the pGEX-NtOSAK construct, expression of GST-NtOSAK, and purification of the recombinant protein were described previously by Kelner et al. (29). Usually, expression was per-
formed for 3 h at 30 °C and only when indicated overnight at 18 °C.

**Site-directed Mutagenesis, Expression, and Purification of NtOSAK Mutated Forms**—The pGEX-NtOSAK construct was used as the template for site-directed mutagenesis with the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Eight individual constructs were generated with the following substitutions: GST-NtOSAK(K33A), GST-NtOSAK(S154A,S158A), GST-NtOSAK(S154A,S158E), GST-NtOSAK(S154E), GST-NtOSAK(S158E), GST-NtOSAK(S154A), GST-NtOSAK(S158A), and GST-NtOSAK(K33A,S154E). All constructs were sequenced to verify the introduced mutations and lack of unintended ones.

The obtained constructs were transformed into E. coli BL21 (DE3), and the GST fusion proteins were expressed and purified using glutathione-agarose beads according to the manufacturer's instructions (Amersham Biosciences), as described previously for wild type GST-NtOSAK (29).

**Immunoprecipitation**—Immunoprecipitation was performed as described previously (25, 29) with some minor changes. Proteins from crude extracts (4 mg) were incubated with anti-NtOSAK antibody (120 μg) in immunoprecipitation buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 100 μM Na3VO4, 2 mM dithiothreitol, 500 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin, 1 μM aprotenin, 1% Triton X-100, 150 mM NaCl) at 4 °C for 4 h on a rocker. An approximately 50-μl packed volume of protein A-agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added, and the incubation was continued for another 2 h. Agarose bead-protein complexes were pelleted by brief centrifugation and washed three times with immunoprecipitation buffer and two times with the following buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 2 mM EGTA, 50 mM β-glycerophosphate, 100 μM Na3VO4, 2 mM dithiothreitol, 500 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin, 1 μM aprotenin). After washing, the immunocomplexes were divided into two pools in a ratio of 1:10. The smaller portion was used directly for immunocomplex kinase activity assay, whereas the major part was used for determination of phosphorylation sites by mass spectrometry (MS). In parallel, cell extracts were analyzed by Western blotting with anti-Ser(P)-158 antibodies.

**Immunocomplex Kinase Activity Assay**—Fifty microliters of sample buffer was added to the pelleted agarose bead-protein complex after immunoprecipitation, and the sample was heated at 95 °C for 3 min. After brief centrifugation, supernatant was analyzed by an in-gel kinase activity assay.

**Protein Kinase Activity Assays**—In-gel, as well as in-solution, kinase activity assays were performed as described previously (25) using MBP (0.5 mg/ml) as a substrate.

**Staining of NtOSAK with Pro-Q Diamond**—NtOSAK immunoprecipitated from BY-2 cells (untreated or treated with 250 mM NaCl for 5 min) was delipidated, desalted, subjected to SDS-PAGE, and stained with Pro-Q Diamond stain (Molecular Probes, Inc., Eugene, OR) according to the manufacturer’s protocol. Briefly, the polyacrylamide gel was fixed in 50% methanol, 10% acetic acid overnight, washed with three changes of deionized water for 10–20 min/wash, and then incubated in Pro-Q Diamond stain for 90 min and destained with three washes in 20% acetonitrile in 50 mM sodium acetate, pH 4.0, for 30 min each. Before imaging, the gel was rehydrated in deionized water for 30 min. The stained phosphorylated proteins were visualized using a laser scanner with 532-nm excitation and a 580-nm long pass emission filter.

**Mass Spectrometry**—Protein samples were analyzed by liquid chromatography-electrospray mass spectrometry with collisional fragmentation (LC-electrospray ionization-MS-MS/MS). Prior to analysis, the gel bands containing NtOSAK were excised, and proteins were reduced, alkylated when necessary, and digested with trypsin (sequencing grade; Promega, Madison, WI) following a standard protocol. An eluted peptide mixture was applied to a RP-18 precol- umn (LC Packings, Amsterdam, The Netherlands) using water containing 0.1% trifluoroacetic acid as the mobile phase and then transferred to a nano-HPLC RP-18 column of 75-μm inner diameter (LC Packings) using an acetonitrile gradient (0–50% acetonitrile in 30 min) in the presence of 0.05% formic acid at a flow rate of 200 nl/min. The column outlet was directly coupled to the ion source of a Q-Tof (Micromass, Manchester, UK) or LTQ FTICR (Thermo, Waltham, MA) electrospray mass spectrometer working either in the regime of data-de- pending MS to MS/MS switch (for peptide identification) or in the Mass Survey Scan regime (for signal quantitation). A blank run ensuring lack of cross-contamination from previous sam- ples preceded each analysis. The output list of parent and daughter ions was used to search the data base using the MAS- COT (MatrixScience, London, UK) program. Peptide identifi- cation and the presence of covalent modifications were verified by inspection of parent mass fragmentation patterns using the programs MassLynx (Waters, Milford, MA), ProteinProspector, Excalibur (Thermo, Waltham, MA), and also in-house LC-MS data analysis software.

**Computer Modeling**—Analysis of the amino acid sequence of NtOSAK revealed a number of structures that could be used as templates to build a homology model. The template structures were chosen from the Protein Data Bank according to the overall sequence homology to NtOSAK but also according to sequence similarities in the DFG-APE fragment of the activation loop.

The model was based on five template structures taken from the Protein Data Bank, 1AQ6, 1CDK, 1IA8, 1JKS, and 1QL6, all of them having a serine/threonine kinase fold. The activation loop was modeled according to the 1QL6 and 1JKS templates, since these two structures have identical loop length and highest homology to the DFG-APE part of the NtOSAK loop.

The MODELER module of INSIGHT 2000 was used to con- vert the NtOSAK sequence into a three-dimensional structure. Five variants of the model were created and validated using the Profile3D module of INSIGHT 2000 and the PROCHECK pro- gram. The final model was subjected to a minimization proce- dure using the DISCOVER3 module of INSIGHT 2000 and a CVFF force field. The procedure consisted of the following two steps: 1) backbone atoms fixed, only side chains minimized and 2) no atoms fixed, the entire model minimized until the maxi- mum derivative was less than 0.001 kcal/mol Å.

**Agrobacterium-mediated Transient Transformation**—To- bacco plants (Nicotiana tabacum LA Burley 21) were grown at
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22 °C in a growth chamber programmed for a 14-h light cycle. Approximately 7-week-old plants were used for experiments. cDNA encoding NtOSAK or its mutated forms was inserted into the XhoI site of the steroid-inducible pTA7002 binary vector (kindly provided by N.-H. Chua, Rockefeller University, New York) (31). Agrobacterium LBA4404 carrying different constructs were grown overnight in YM medium (Invitrogen) containing 100 μg/ml rifampicin, 50 μg/ml kanamycin, and 100 μM acetylsyringone. Cells were collected by centrifugation (4000 × g), resuspended to \(A_{600} = 0.8\) in Murashige and Skoog’s medium, pH 5.9, with 100 μM acetylsyringone, and infiltrated into fully expanded leaves. Expression of the transgene was induced by infiltration of dexamethasone (30 μM) 24 h later. Samples for protein preparation were collected after 40 h and subjected to Western blotting and kinase activity analysis.

Protoplast Transient Expression Assay—The preparation and electroparation of maize mesophyll protoplasts was performed according to the protocol described by Sheen (30). The cDNA encoding NtOSAK was inserted into the NcoI and NotI sites of the pRT100 plant expression vector under the control of the 35S promoter. The vector was kindly provided by Prof. H. Hirt (Vienna Biocenter). NtOSAK was fused at the C-terminus with GFP. Constructs carrying mutated forms of NtOSAK were obtained by site-directed mutagenesis with the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the pRT100-NtOSAK-GFP construct as a template. In each electroporation, about 2 \(\times 10^5\) protoplasts were transfected with 50 μg of plasmid DNA and 50 μg of carrier DNA. The transfected protoplasts were incubated at 22 °C for 16 h, and then they were subjected to 500 mM NaCl treatment. In control experiments, the transfected protoplasts were treated with protoplast medium. The activity of expressed NtOSAK and its mutated forms was analyzed by an in-gel kinase activity assay.

RESULTS

To understand the mechanism of NtOSAK activation in response to hyperosmotic stress, we analyzed the effect of osmotic stress on NtOSAK expression at the mRNA and protein levels and on NtOSAK phosphorylation in BY-2 tobacco cells subjected to osmotic stress.

NtOSAK Expression Is Transiently Up-regulated by Osmotic Stress—The level of NtOSAK transcript was analyzed by reverse transcription-PCR in BY-2 cells untreated and treated with 250 mM NaCl or 500 mM sorbitol for various times. The effect of both stressors was very similar. We observed an approximately 2-fold increase in the amount of the NtOSAK transcript in BY-2 cells exposed to either stress for 15 min (Fig. 1, A and B). Longer treatment caused a decline of NtOSAK transcription to the control level observed in untreated BY-2 cells. Very similar results were obtained when the cells were exposed to 100 mM NaCl or 900 mM sorbitol (data not shown). Expression of NtOSAK was detected in every tobacco tissue tested: roots, stem, and leaves. The highest expression was observed in roots and the lowest in leaves (Fig. 1C).

Effect of Hyperosmotic Stress on NtOSAK Protein Level—Protein extracts prepared from BY-2 cells (untreated and treated with 250 mM NaCl for the indicated times) were subjected to Western blotting analysis using specific anti-NtOSAK antibodies. The results showed that the level of NtOSAK did not change significantly upon NaCl treatment (Fig. 1D), indicating that the induction of NtOSAK expression observed at the mRNA level does not influence the amount of the kinase inside the cell, and the transient activation of NtOSAK upon osmotic shock, documented earlier (25), is not due to changes in NtOSAK synthesis or stability. Similar results were obtained with protein extracts from BY-2 cells exposed to sorbitol (data not shown).

Mapping of Phosphorylation Sites of NtOSAK—Our previous results (25) as well as the data presented by the Hattori group (14) and Belin et al. (26) suggested that the SnRK2 family members are activated by phosphorylation. In order to examine if the osmotic stress influences the phosphorylation level of NtOSAK, we immunoprecipitated the kinase from tobacco...
Osmotic stress triggers NtOSAK phosphorylation in BY-2 cells. A, changes in NtOSAK phosphorylation level in response to osmotic stress. NtOSAK immunoprecipitated from protein extracts (50 μg) prepared from BY-2 cells untreated and treated with 250 mM NaCl for 5 min was subjected to SDS-PAGE. Phosphorylation of NtOSAK was determined by ProQ Diamond stain. The data represent one of three independent experiments showing similar results. B, analysis of NtOSAK phosphorylation on Ser-158. Twenty micrograms of crude extracts from BY-2 cells untreated and treated with 250 mM NaCl for 5 min were subjected to Western blot analysis. The blot was probed with anti-Ser(P)-158 antibodies. The data represent one of four independent experiments showing similar results.

BY-2 cells (untreated and treated with 250 mM NaCl for 5 min) and stained the protein with the Pro-Q Diamond stain (Molecular Probes), which recognizes phosphorylated Ser, Thr, and Tyr residues. As is shown in Fig. 2A, NtOSAK undergoes dramatic phosphorylation in BY-2 cells exposed to NaCl. These results prompted us to map the amino acid residues that are phosphorylated in the active kinase. For this purpose, NtOSAK was purified from BY-2 cells treated with 250 mM NaCl for 5 min, according to the procedure described previously (25), and subjected to SDS-PAGE, and the protein band containing the kinase was analyzed by MS. Approximately 1 μg of purified NtOSAK was digested with trypsin, and the resultant mixture of peptides was analyzed by LC-MS-MS/MS to identify the site(s) of phosphorylation.

The mass spectrometry analyses revealed the presence of several phosphopeptides, based both on the molecular mass difference of 80 Da between the unphosphorylated and phosphorylated form and on the analysis of the collision-induced fragmentation patterns of corresponding peptides (Fig. 3A). All of the identified phosphopeptides were assigned to the region of the NtOSAK kinase domain corresponding to its activation loop (Fig. 3B). Analysis of the fragmentation pattern of peptide \( ^{15}e^{16}^{18}S^{19}L^{20}L^{21}S^{22}H^{23}R^{24}P^{25}K^{26} \) allowed us to localize the modification site to Ser-154. This conclusion is based on the inspection of the fragmentation spectrum of the + 2 charged signal at 552.78 m/z (Fig. 3C). In this spectrum, the most prominent ion series correspond to the \( y_{1}^{−}H_{2}P_{4}O_{7} \) to \( y_{9}^{−}H_{2}P_{4}O_{7} \) ions (denoted by green labels in Fig. 3C), although at lower abundances a clear \( y_{7}^{−} \) series of ions could be detected (labeled red in Fig. 3C). Distinct peaks could be attributed to the \( y_{4}^{−} \) to \( y_{8}^{−} \) series, indicating that the phosphate group must be attached to Ser-154. The \( b_{7}^{−} \) series (blue in Fig. 3C) could also be assigned, with the value of the \( b_{7}^{−} \) ion confirming the localization of the phosphopeptide to Ser-154. In the case of peptide \( ^{15}e^{16}^{18}S^{19}T^{20}V^{21}G^{22}T^{23}P^{24}A^{25}Y^{26}I^{27}A^{28}P^{29}E^{30}V^{31}L^{32}S^{33}R^{34} \), the fragmentation pattern did not allow distinguishing whether the modification was present at Ser-158 or Thr-159.

**Prediction of NtOSAK Structure by Computer Modeling**

**Suggests That Phosphorylation of Ser-154 and Ser-158 and Not Thr-159 Is Responsible for the Kinase Activation**

In order to follow the conformational changes of NtOSAK due to phosphorylation of Ser-154 and Ser-158 or Thr-159, the structure of NtOSAK, unphosphorylated and phosphorylated on Ser-154 and Ser-158 or Ser-154 and Thr-159, was predicted by computer modeling. The best alignment of the activation segment DFG-APE of NtOSAK with the template sequences places Ser-158 (not Thr-159) in front of the phosphorylated regulatory residue from other protein kinase structures. To align Thr-159 with Thr/Ser from other serine/threonine kinase sequences, one would need to introduce two very unfavorable gaps to keep the rest of the highly conserved activation segment in proper alignment. In the NtOSAK structure, the side chain conformation of Thr-159 is “outside” of the loop (Fig. 4). The phosphate group attached to Thr-159 would consequently be unable to interact with the Arg-122 residue, an interaction that facilitates proper orientation of the highly conserved Asp needed for ATP binding and catalytic reaction. The phosphate group of Ser-154 is too far from the Arg-122 residue to compensate for this effect. In contrast, the localization of Ser-158 in the structure is such that it easily allows for an interaction with Arg-122. These results suggest that the most likely phosphorylation site is Ser-158 and not Thr-159.

The MS analysis showed that in peptide \( ^{15}e^{16}^{18}S^{19}T^{20}V^{21}G^{22}T^{23}P^{24}A^{25}Y^{26}I^{27}A^{28}P^{29}E^{30}V^{31}L^{32}S^{33}R^{34} \) from active NtOSAK, only one residue was phosphorylated, Ser-158 or Thr-159. To prove that in active NtOSAK, Ser-158 and not Thr-159 is phosphorylated, we generated phospho-specific antibodies, anti-Ser(P)-158, raised against the phosphorylated peptide KpSTVGT. The antibodies clearly recognized the phosphopeptide in a protein extract prepared from BY-2 cells subjected to hyperosmotic stress (250 mM NaCl) for 5 min (Fig. 2B), indicating that NtOSAK undergoes phosphorylation on Ser-158 in response to osmotic stress. Our results are in agreement with the data presented recently by Belin et al. (26), showing that in Arabidopsis OST1 kinase belonging to subclass I of the SnRK2 kinases according to the classification of Boudsocq and Lauriere (1), Ser-175 (corresponding to Ser-158 of NtOSAK) and not Thr-176 (corresponding to Thr-159 of NtOSAK) is required for the kinase activity and the enzyme function in planta.

**Residues Interacting with Ser(P)-154 and Ser(P)-158**

In the NtOSAK amino acid sequence, the highly conserved Arg-122 immediately precedes the catalytic Asp-123. The presence of this Arg-Asp motif (known as the RD motif) places NtOSAK in the group of RD kinases (32, 33). The RD kinases require some ionic interactions of the arginine with a phosphate or carboxylate group for proper orientation of the catalytic aspartic acid; therefore, several of those kinases are activated by phosphorylation on the activation segment (32). According to the predicted structure of NtOSAK, the phosphate group attached to Ser-158 interacts with Arg-122. Other residues forming hydrogen bonds with the phosphate group of Ser-158 include Arg-47 and Lys-148. The phosphate group of Ser-154 forms hydrogen bonds with Ser-150 and the backbone nitrogen from Leu-151 (Fig. 4); it may also interact with the Lys-148 side chain as some simulations indicate (data not shown). Additionally, the backbone nitrogen of Ser-154 forms a hydrogen bond with the backbone oxygen of Leu-151. All of these interactions create an energetically favorable turn of the activation loop, which could enforce a proper orientation of the positively charged cluster of...
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A

B

C

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residues that interact with the phosphate group attached to Ser-158 (Fig. 4).

**NtOSAK Phosphorylation Correlates with Its Kinase Activity in BY-2 Cells Subjected to Osmotic Stress**—It was important to examine whether phosphorylation of the identified serines correlates with NtOSAK activity in tobacco cells exposed to osmotic stress. For this purpose, we immunoprecipitated endogenous NtOSAK from BY-2 tobacco cells exposed to osmotic stress for varying times, and the resulting immunocomplexes were used for analysis of NtOSAK activity by an in-gel kinase assay using MBP as a substrate and its phosphorylation on Ser-154 and Ser-158. For estimation of NtOSAK activity, we made cell lysates from unstressed cells, NtOSAK was not phosphorylated on any of the studied residues (Fig. 5). The peptides containing phosphate groups on Ser-154 and Ser-158 appeared already after 1 min of treatment with NaCl and nearly vanished when cells were subjected to the osmotic stress for 120 min (Fig. 5). This conclusion is based on monitoring of the MS amplitude of unphosphorylated and phosphorylated peptide signal from peptide STVGTPAYIAPEVLSR containing Ser-158 (Fig. 5, A and B) and peptides 149SSLLHSRPK (Fig. 5, C and D) and 149SSLLHSRPK (data not shown) containing Ser-154. The data for the longer peptide containing Ser-154 (SSLLHSRPK) is not shown, because the signal of the phosphorylated form could be clearly identified only at 1 and 5 min after stress induction, and for longer times it was obscured by a nonmonoisotopic peak of a coeluting contamination; hence, its decay at later times could not be shown. For 1 and 5 min, the signals from this longer peptide are qualitatively the same as the ones shown in Fig. 5D. The amplitude of the phosphorylated peptide signal (marked by an asterisk in Fig. 5) is in general much lower than that of the unphosphorylated peptide signal. This is to be expected, since the addition of a phosphate group significantly decreases the MS sensitivity. Thus, the ratio of phosphorylated versus nonphosphorylated signal amplitude is not a quantitative measure of the ratio of the concentrations of the two forms of the peptide. However, for the Ser-154-containing peptides, the unphosphorylated signal disappears at 1 and 5 min of NaCl treatment and reappears later, which may indicate full phosphorylation of Ser-154 at these early times. In general, this experiment shows qualitatively the appearance and slow decay of phosphorylation of Ser-154 and Ser-158 during 120 min of osmotic stress.

The appearance of NtOSAK phosphorylation found by MS analysis correlates with the NtOSAK activity (Fig. 5E) and the Western blotting results with anti-Ser(P)-158 antibodies (Fig. 5F). NtOSAK becomes fully active in BY-2 cells within 1 min after NaCl application, and during the next 2 h its activity gradually declines to the basal level. Western blot analysis with anti-Ser(P)-158 antibodies shows the same pattern, with NtOSAK expressing the highest enzymatic activity, being also the most strongly decorated with the antibodies.

The mass spectrometry results indicated that in control unstressed cells, NtOSAK was not phosphorylated on any of the studied residues (Fig. 5). The peptides containing phosphate groups on Ser-154 and Ser-158 appeared already after 1 min of treatment with NaCl and nearly vanished when cells were subjected to the osmotic stress for 120 min (Fig. 5). This conclusion is based on monitoring of the MS amplitude of unphosphorylated and phosphorylated peptide signal from peptide STVGTPAYIAPEVLSR containing Ser-158 (Fig. 5, A and B) and peptides 149SSLLHSRPK (Fig. 5, C and D) and 149SSLLHSRPK (data not shown) containing Ser-154. The data for the longer peptide containing Ser-154 (SSLLHSRPK) is not shown, because the signal of the phosphorylated form could be clearly identified only at 1 and 5 min after stress induction, and for longer times it was obscured by a nonmonoisotopic peak of a coeluting contamination; hence, its decay at later times could not be shown. For 1 and 5 min, the signals from this longer peptide are qualitatively the same as the ones shown in Fig. 5D. The amplitude of the phosphorylated peptide signal (marked by an asterisk in Fig. 5) is in general much lower than that of the unphosphorylated peptide signal. This is to be expected, since the addition of a phosphate group significantly decreases the MS sensitivity. Thus, the ratio of phosphorylated versus nonphosphorylated signal amplitude is not a quantitative measure of the ratio of the concentrations of the two forms of the peptide. However, for the Ser-154-containing peptides, the unphosphorylated signal disappears at 1 and 5 min of NaCl treatment and reappears later, which may indicate full phosphorylation of Ser-154 at these early times.

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**FIGURE 3. Mass spectrometric localization of NtOSAK phosphorylation sites.** A, results of native NtOSAK analysis by LC-MS-MS/MS and protein sequence data base search carried out by MASCOT (MatrixScience), MassLynx (Waters), and in-house MudKit software. NtOSAK peptides identified in the sample are shown in red along the entire protein sequence (upper part) and as a list of peptides assigned to NtOSAK (lower part). The first column (Start-End) in the list indicates the positions of the peptide in the sequence. Observed, measured m/z value for the peptide; M,(calc), peptide mass calculated from observed m/z value; M, identified NtOSAK peptide mass resulting from in silico trypsin digestion of NtOSAK sequence; Delta, the mass difference between the measured and calculated peptide mass; Miss, number of missed tryptic cleavages in the identified peptide; Sequence, identified NtOSAK peptide sequence corresponding to the mass measured (red), its modification (black), and statistical score describing the fit of the peptide fragmentation pattern to one expected for the peptide of a given sequence (blue). Note the presence of peptide hits indicating phosphorylation of NtOSAK peptides (underlined), B, two fragments of two-dimensional maps resulting from LC-MS analysis showing peaks of phosphorylated and unphosphorylated versions of two NtOSAK peptides: SSSLHSSRPK (top panel) and STVGTPAYIAPEVLSR (bottom panel). In the panels, the horizontal axis denotes the measured m/z value, and the vertical axis shows the nano-HPLC retention time of the peak. Peaks are denoted as colored lines, with the number of counts (height of the peak) increasing from red to blue. The presence of a peptide results in a series of spots in the two-dimensional visualization, corresponding to the 13C isotope envelope. Monoisotopic peaks of the unphosphorylated (left arrow) and phosphorylated version (right arrow) are shown with the difference between their m/z values (indicated with a dotted line) equal to 40, as expected for doubly charged phosphorylated species. C, MS fragmentation results of the phosphorylated version of peptide SSSLHSSRPK. Black peaks indicate m/z values, and amplitudes of obtained peptide fragments are interpreted as y series ions (red), b series ions (blue), and the strongest y-H2PO4 ions (green). The mass difference between the y3 and y5 ions corresponds exactly to a phosphorylated serine residue, allowing the localization of the phosphorylation site to serine 6 in the peptide sequence, confirmed by the presence of the y4 peak.

**FIGURE 4. Modeled conformation of the activation loop DFG-APE (ribbon representation, magenta) with phosphorylated serines 154 and 158.** Residues forming hydrogen bonds (dashed green lines) with phosphate groups are shown. Yellow and blue ribbons represent different parts of the NtOSAK kinase. The conformation of threonine 159 is also shown; its side chain is pointing outside of the activation loop.

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A

B

C

D

Time [min]

0

1

5

15

30

120

E

F

NtOSAK activity

anti-Ser-158(P)
The substitution of both identified serines by glutamic acid residues caused the appearance of the kinase activity (Fig. 6). GST-NtOSAK(S158E), GST-NtOSAK(S154E), and GST-NtOSAK(S154E,S158E), in which Ser-158, Ser-154, or both, respectively, were replaced by glutamic acid, exhibited comparable levels of activity (using MBP as substrate). However, it has to be stressed that glutamic acid can substitute for phosphorylated residues only to some extent. The specific activity of the active forms of NtOSAK obtained by site-directed mutagenesis and expressed in E. coli was only about 1–4% of the activity of native NtOSAK isolated from BY-2 cells subjected to osmotic stress.

Surprisingly, GST-NtOSAK(S154A) exhibited an activity similar to that of GST-NtOSAK(S154E), suggesting that phosphorylation of Ser-154 is not required for the kinase activity. To explain this result, we analyzed both purified proteins and wild type GST-NtOSAK by mass spectrometry. MS showed the presence of a peptide containing phosphorylated Ser-158 (Table 1) in GST-NtOSAK(S154A) and GST-NtOSAK(S154E), whereas in the wild type GST-NtOSAK only the unphosphorylated peptide was found. The presence of phosphorylated Ser-158 in GST-NtOSAK(S154A) and GST-NtOSAK(S154E) was confirmed by Western blotting using anti-Ser(P)-158 antibodies (Fig. 6D). The phosphorylation of Ser-158 can explain why GST-NtOSAK with Ser-154 substituted either by Ala or Glu was active. Moreover, the obtained data support the hypothesis that phosphorylation of Ser-158 and not Ser-154 is crucial for the kinase activity. Modification of Ser-154 could be involved in facilitation or acceleration of NtOSAK phosphorylation on Ser-158.

To find out whether phosphorylation in GST-NtOSAK(S154E) or GST-NtOSAK(S154A) is catalyzed by one of the bacterial kinases or is a result of autophosphorylation of the recombinant proteins, we produced in E. coli an inactive form of GST-NtOSAK(S154E), in which the conserved lysine involved in ATP binding was replaced by alanine (GST-NtOSAK(K33A,S154E)), and checked if such a protein was phosphorylated on Ser-158. MS and immunoblot analysis revealed that GST-NtOSAK(K33A,S154E), which had no kinase activity and therefore was not able to undergo autophosphorylation, was not phosphorylated (Table 1 and Fig. 6D). These results indicate that in GST-NtOSAK(S154E) and presumably in GST-NtOSAK(S154A), Ser-158 was autophosphorylated.

All of the recombinant proteins described above were produced in E. coli for 3 h at 30 °C. The wild type GST-NtOSAK
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TABLE 1
MASCOT data base search program scores (ion scores) indicating the presence or absence of a phosphorylated (left column) and unphosphorylated (right column) form of the peptide containing Ser-158 in different variants of NtOSAK

For each peptide signal its *m/z*, charge, sequence, and MS/MS fragmentation ion score is given. –, lack of the phosphorylated form of the peptide in a given variant.

| GST-NtOSAK variant | Phosphorylated *m/z*, charge, sequence, ion score | Unphosphorylated *m/z*, charge, sequence, ion score |
|--------------------|-----------------------------------------------|--------------------------------------------------|
| wt 30              | 749.36, 2+, 15STVGTPAVIAPEVLSR273, 13          | 554.30, 3+, 15STVGTPAVIAPEVLSR273, 71             |
| wt 18              | 870.91, 2+, 15STVGTPAVIAPEVLSR273, 60          | 830.95, 2+, 15STVGTPAVIAPEVLSR273, 104            |
| S154A              | 870.92, 2+, 15STVGTPAVIAPEVLSR273, 81          | 830.93, 2+, 15STVGTPAVIAPEVLSR273, 66             |
| S154E              |                                                | 830.93, 2+, 15STVGTPAVIAPEVLSR273, 116            |
| K33A              |                                                | 830.95, 2+, 15STVGTPAVIAPEVLSR273, 117            |
| K33A,S154E         |                                                | 554.23, 3+, 15STVGTPAVIAPEVLSR273, 61             |

obtained in such conditions had a low activity. However, when we expressed GST-NtOSAK overnight at 18°C, we were able to obtain a more active kinase, which was phosphorylated on Ser-158 and on several other serine and threonine residues (but not on Ser-154). This phosphorylation was performed by GST-NtOSAK itself (autophosphorylation), since GST-NtOSAK(K33A) expressed in the same conditions was not phosphorylated (Table 1 and Fig. 6D). These data are in agreement with the results of Belin et al. (26), who showed that recombinant OST1 kinase expressed in *E. coli* undergoes autophosphorylation on Ser-175 in the activation loop and on several other residues.

The obtained results confirm the crucial role of Ser-158 phosphorylation for the kinase activation and suggest that modification of Ser-154 could have a role in facilitation of NtOSAK (auto) phosphorylation on Ser-158. However, considering the low activity of the recombinant proteins, the data cannot be directly extrapolated to the mechanism of regulation of NtOSAK activity in plant cells and should be treated with some skepticism.

Substitution of Ser-154 and Ser-158 by Glutamic Acid Is Not Sufficient to Obtain a Constitutively Active NtOSAK in Tobacco Plants—To obtain a constitutively active NtOSAK in tobacco plants, we used a steroid-inducible transient transformation system (31). cDNA encoding the mutated forms of NtOSAK, NtOSAK(S154E,S158E), NtOSAK(S154E), and NtOSAK(K33A), as well as wild type NtOSAK, in sense and antisense orientation, was introduced into the pTA7002 vector, which allows for inducible expression of a transgene, and *Agrobacterium tumefaciens*-mediated transient expression in tobacco plants was performed. Analysis of the protein expression level and protein kinase activity showed that although each of the introduced transgenes was expressed efficiently (Fig. 7A), none of the obtained proteins exhibited a detectable activity in unstressed plants (data not shown). In our hands, substitution of the serines whose phosphorylation is required for NtOSAK activation by acidic amino acid residues did not create an in-plant constitutively active kinase.

Studies on Ser-154 and Ser-158 Phosphorylation in Regulation of NtOSAK Activity in Maize Protoplasts—To verify the site-directed mutagenesis data obtained in the bacterial system and in tobacco plants using the *A. tumefaciens*-mediated transient expression system, we decided to express wild type NtOSAK and its mutated forms in protoplasts. The studied enzymes were produced as fusion proteins with GFP to differentiate the transiently expressed proteins from endogenous SnRK2s. Initially, we attempted to transform tobacco BY-2 cell protoplasts, but in our hands transformation of these protoplasts was inefficient; therefore, we performed our studies using maize mesophyll protoplasts. The maize protoplasts transient expression system was developed several years ago by Sheen and has been successfully used to study several signal transduction pathways (34, 35). Since NtOSAK is expressed in all plant tissues and its orthologs are present in every plant studied in this aspect, we decided that this system is appropriate for our studies. Maize protoplasts were transiently transformed with the pRT100 vector bearing cDNA encoding wild type NtOSAK-GFP or its point mutation variants with Ser-154 or Ser-158 substituted with Ala or Glu. In transformed protoplasts (untreated or treated with 500 mM NaCl) activity of the expressed variants of NtOSAK was analyzed by an in-gel kinase assay with MBP as substrate. None of the analyzed samples exhibited kinase activity (data not shown). The amount of RuBisco stained by Ponceau S was used as a protein loading indicator. The data represent one of three independent experiments. In the other two, the expression level of the wild type NtOSAK was similar to that of the mutated forms. B, transient expression in maize protoplasts. cDNA constructs were introduced into maize mesophyll protoplasts by electroporation. Protein expression was carried out for 16 h. After that time, protoplasts were subjected to osmotic stress (500 mM NaCl), and expressed proteins were analyzed by an in-gel kinase activity assay with MBP as substrate. The data represent one of five independent experiments.
with NaCl (Fig. 7B). In protoplast expressing NtOSAK(S158A)-GFP or NtOSAK(S158E)-GFP we were not able to detect any MBP kinase activity of the expressed proteins, either before or after NaCl treatment (Fig. 7B). These experiments confirmed our mutagenesis studies in the bacterial expression system indicating that phosphorylation of Ser-158 is crucial for the kinase activity. However, in contrast to the results obtained using the bacterial expression system, mutation of Ser-154 either to Ala or to Glu drastically reduced the NtOSAK activity, even when the expressing protoplasts were subjected to hyperosmotic stress (Fig. 7B). These results indicate an important role of both identified serines in NtOSAK activation in the plant response to osmotic stress.

**DISCUSSION**

NtOSAK, a member of subclass II of the SnRK2 family, according to the classification of Boudsocq and Lauriere (1), was identified as a kinase rapidly activated in tobacco cells subjected to osmotic stress (25). Our studies aimed to establish the mechanism of regulation of NtOSAK activity in response to osmotic stress. For this purpose, we analyzed changes in NtOSAK expression at the mRNA and protein levels and its phosphorylation in response to stress. NtOSAK expression is transiently up-regulated at the mRNA level in NaCl- or sorbitol-treated cells, although this does not cause any increase in the NtOSAK protein level. The amount of the NtOSAK protein is virtually the same in untreated and NaCl-treated cells, which means that NtOSAK activation is not due to its de novo synthesis or increased stability.

Our previous studies showed that NtOSAK activity was abolished by treatment with protein phosphatase PP2A (25), indicating that NtOSAK phosphorylation is required for its activity and suggesting that this might be the mechanism of its activation upon osmotic stress. This hypothesis was confirmed by Kobayashi et al. (14), who suggested that all members of the SnRK2 subfamily are activated by phosphorylation, and very recently by Belin et al. (26), who showed that phosphorylation is required for Arabidopsis OST1 protein kinase activity. In the present work, using mass spectrometry analysis, we mapped two phosphorylation sites in active NtOSAK purified to apparent homogeneity from hyperosmotically stressed tobacco cells. In the active kinase, Ser-154 and Ser-158 localized in the kinase activation loop were phosphorylated.

To prove that NtOSAK becomes phosphorylated on the above residues in BY-2 cells in response to osmotic stress, we analyzed the time course of NtOSAK phosphorylation on Ser-154 and Ser-158 and the enzyme activation in tobacco cells exposed to 250 mM NaCl. We observed a good correlation of NtOSAK phosphorylation on the identified serines and its activity. The kinase was not active and not phosphorylated in BY-2 cells not exposed to osmotic stress as well as after a 2-h exposure to the stress. This suggested that phosphorylation is responsible for NtOSAK activation and that dephosphorylation is responsible for its inactivation.

Prediction of the NtOSAK three-dimensional structure indicated that phosphorylation of Ser-154 and Ser-158 has an essential impact on NtOSAK conformation and its enzymatic activity. Site-directed mutagenesis studies on NtOSAK expressed in bacteria as well as in plant cells confirmed this model.

Recombinant GST-NtOSAK expressed in *E. coli* has a low activity, which can be completely abolished by substitution of serine 158 with alanine, whereas substitution with glutamic acid, mimicking a phosphorylated residue, creates an active kinase, albeit of an activity much lower than the enzyme isolated from stressed plant cells. Detailed mutagenesis studies on the proteins expressed in *E. coli* showed that phosphorylation of Ser-158 is essential for the enzyme activity, whereas phosphorylation of Ser-154 most probably facilitates NtOSAK autophosphorylation on Ser-158.

The results of transient expression of NtOSAK and its mutated forms in maize protoplasts also indicated an important role of Ser-154 and Ser-158 in NtOSAK activation. Mutation of Ser-158, either to Ala or to Glu, eliminated the kinase activity, showing that in plants an acidic residue cannot substitute for phosphorylated Ser-158 of NtOSAK. The activity was undetectable in protoplasts expressing NtOSAK(S158A), as well as NtOSAK(S158E), untreated or treated with NaCl. Similarly, when Ser-154 was mutated either to Ala or to Glu, the expressed proteins exhibited no detectable activity in protoplasts not exposed to osmotic stress as well as after stress application. These results indicated an important role not only of Ser-158 but also of Ser-154 in NtOSAK activation in response to osmotic stress. The results obtained using bacterially expressed proteins seem to be partially inconsistent with the studies performed in the plant transient expression systems. These differences can be explained by the fact that, in the case of bacterially expressed proteins, we analyze purified proteins without any additional modifications, cellular components, and interacting proteins, which can influence the kinase regulation in plant cells. However, analysis of the bacterially expressed proteins confirmed the crucial role of Ser-158 phosphorylation for the kinase activity and suggested an involvement of Ser-154 phosphorylation in this process. Moreover, the results obtained in the bacterial system suggested different roles of Ser-158 and Ser-154 phosphorylation in NtOSAK activation.

Based on our results, we assume that NtOSAK activation in response to osmotic stress is a two-step process. At the first step, Ser-154 is phosphorylated by a kinase upstream of NtOSAK (this residue does not undergo autophosphorylation, at least in the recombinant kinase expressed in bacteria). Then Ser-154 phosphorylation triggers the next step, phosphorylation of Ser-158, which can be performed by an upstream kinase or by NtOSAK itself.

Comparison of the amino acid sequence of the NtOSAK activation loop with those of *Arabidopsis* and rice SnRK2 kinases shows that potential phosphorylation sites corresponding to Ser-154 and Ser-158 are conserved throughout the family (Fig. 8). Therefore, we suggest that all SnRK2 subfamily members are regulated by phosphorylation at sites equivalent to these serine residues. The very recent results of Belin et al. (26) partially confirm this hypothesis, showing that in OST1/SRK2.6/SRK2E kinase phosphorylation of Ser-175 corresponding to Ser-158 in NtOSAK is required for the kinase activity.

Considering the physiological role of Ser-154, we favor the idea that it takes active part in the kinase activation; however, it
is also possible that phosphorylation of Ser-154 influences the NtOSAK substrate specificity or its localization inside the cell. Several protein kinases contain secondary phosphorylation sites in their activation loops. Secondary phosphorylation sites are present in many CMGC kinases (e.g. MAP kinases, GSK3 kinases), a number of tyrosine kinases, and kinases from the OPK (other protein kinases) group (kinases), a number of tyrosine kinases, and kinases from the studied kinases. We estimate that the activity of GST-NtOSAK(S154E,S158E) or GST-NtOSAK(S158E) expressed in bacteria amounts to only about 1–4% of the activity of the enzyme purified from NaCl-treated BY-2 tobacco cells. However, it is also possible that phosphorylated serines and threonines in the kinase activation loop either to alanine or to aspartic acid abolished the kinase activity (45). The phosphomimetic mutation of Thr-295 in the kinase activation loop (crucial for Tpl2/Cot kinase activity) to aspartic acid (but not to glutamic acid) rescued a portion of the kinase activity in bacteria amounts to only about 1–4% of the activity of the same kinases phosphorylated by ERK activator (MAPKK) (43). In vitro autophosphorylation occurred primarily on tyrosine and, to a much lesser extent, on threonine in the kinase activation loop. Moreover, substitution of the regulatory threonine with alanine or glutamic acid enhanced autophosphorylation on the regulatory tyrosine; however, it did not create a constitutively active enzyme. Mutation of the threonine to alanine created the kinase that was not able to be activated by MAPKK, whereas mutation to glutamic acid created the kinase activated to a specific activity of about 10% of the specific activity of activated wild type ERK2 kinase (43). There are several other examples showing that substitution of the threonine or serine residue in the kinase activation loop by an acidic residue does not result in a constitutively active enzyme. In the case of the Xenopus mitotic kinase Aurora-A, mutation of the Thr-295 in the kinase activation loop either to alanine or to aspartic acid abolished the kinase activity (45). The phosphomimetic mutation of Thr-290 (crucial for Tpl2/Cot kinase activity) to aspartic acid (but not to glutamic acid) rescued a portion of the kinase activity in HEK-293T cells (46). According to the authors, their results indicate that phosphorylation of Thr-290 in the activation loop of Tpl2/Cot is necessary but not sufficient for kinase activity (46). It is also possible that phosphorylation of the identified residues of NtOSAK is required, but is not sufficient, for NtOSAK activation in planta. We cannot exclude the possibility that there are other phosphorylated residues that are needed for the kinase activation that were not detected in our MS experiments. We could miss some phosphorylated residues, since it is extremely difficult to cover the whole sequence of the studied protein by MS analysis; we achieved about 70% sequence coverage of NtOSAK isolated from tobacco cells. In addition to this, some phosphorylated serines and threonines are very difficult to detect by MS. When recombinant GST-
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NtOSAK was analyzed, several autophosphorylated serines and threonines were identified at the N-terminal and C-terminal parts of the molecule. Similarly, several autophosphorylated residues of recombinant OST1 were identified by Belin et al. (26). However, we did not see phosphorylation of those residues in NtOSAK isolated from NaCl-treated tobacco BY-2 cells in the MS analysis. This result does not prove that such phosphorylation does not exist; therefore, we will continue these studies.

Besides phosphorylation, other modifications or interactions with some specific cellular proteins can have a great impact on NtOSAK activity. Recently, it was demonstrated that NO with some specific cellular proteins can have a great impact on the MS analysis. This result does not prove that such phospho-

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