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Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase

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ABSTRACT

The plant hormone abscisic acid (ABA) triggers production of reactive oxygen species (ROS) in guard cells via the AtrbohD and AtrbohF NADPH oxidases, leading to stomatal closure. The ABA-activated SnRK2 protein kinase open stomata 1 (OST1) (SRK2E/SnRK2.6) acts upstream of ROS in guard cell ABA signaling. Here, we report that OST1 phosphorylates Ser13 and Ser174 on AtrbohF. In addition, substitution of Ser174 to Ala results in a ~40% reduction in the phosphorylation of AtrbohF by OST1. We also show that OST1 physically interacts with AtrbohF. These results provide biochemical evidence suggesting that OST1 regulates AtrbohF activity.

Structured summary:
MINT-7260179, MINT-7260147, MINT-7260165: *OST1* (uniprotkb:Q940H6) *phosphorylates* (MI:0217) *ATRBOHF* (uniprotkb:O48538) by *protein kinase assay* (MI:0424)
MINT-7260208: *OST1* (uniprotkb:Q940H6) and *ATRBOHF* (uniprotkb:O48538) *physically interact* (MI:0915) by *bimolecular fluorescence complementation* (MI:0809)

1. Introduction

Reactive oxygen species (ROS) function as second messengers to positively regulate abscisic acid (ABA) signaling [1]. A molecular genetic study has shown that two of the 10 NADPH oxidases in the Arabidopsis genome, *AtrbohD* and *AtrbohF*, are responsible for ABA-induced ROS production and subsequent events leading to stomatal closure [2]. However, it remains unknown how ABA activates these two NADPH oxidases.

NADPH oxidase function has been shown to be regulated by phosphorylation in both animal and plant cells [3–5]. For example, AtrbohD was shown to be phosphorylated on several amino acid residues in response to pathogen elicitors, suggesting that phosphorylation regulates its activity [6,7]. However, protein kinases that phosphorylate NADPH oxidases have remained largely unknown. To date, only two potato calcium-dependent protein kinases (CDPKs), StCDPK4 and StCDPK5, have been shown so far to phosphorylate the NADPH oxidase StrbohB [4].

In Arabidopsis, members of the CDPK and the SNF1-Related Kinase 2 (SnRK2) families have been shown to function in guard cell ABA signaling [8–11]. A mutation in the SnRK2 kinase *OST1* gene (also called *SRK2E/SnRK2.6*) impairs ABA-triggered ROS production in guard cells, suggesting that open stomata 1 (OST1) acts upstream of NADPH oxidases in this signaling cascade [10]. Despite these findings, it has remained unknown whether AtrbohF and AtrbohD are regulated by OST1. Our biochemical study on AtrbohF, the major NADPH oxidase in ABA regulation of stomatal movements [2], shows that AtrbohF interacts with and is phosphorylated by OST1, indicating that AtrbohF is a substrate of OST1.

2. Materials and methods

2.1. Phosphorylation assays and LC-MS/MS analysis

Phosphorylation assays were performed as described [12] using a peptide MALDRTRS^{174}SAQRKKK from the N-terminal region of

Abbreviations: ABA, abscisic acid; CDPK, calcium-dependent protein kinase; OST1, open stomata 1; ROS, reactive oxygen species

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AtrbohF that was purified to 93.08% by Genecust (http://www.genecust.com/). The peptide (1–100 μM) was phosphorylated at 25 °C in a 50 μl reaction containing 20 mM $MgCl_2$, 1 mM DTT, 25 mM β-glycerophosphate, 0.1 mM ATPγ^{32}P (0.14 μCi nmol^{-1} ATP), 20 mM HEPES, pH 7.4, and 200 ng of OST1 expressed in *Escherichia coli*. GST-AtrbohF and GST-AtrbohF^{S174A} (1 μg) were phosphorylated in the same condition with 500 ng of OST1 and 0.1 mM ATPγ^{32}P (0.46 μCi nmol^{-1} ATP). Proteins were resolved by SDS–PAGE and stained with Coomassie Blue. Protein phosphorylation was quantified by phosphoimaging. Details about the cloning and protein expression of GST-AtrbohF and GST-AtrbohF^{S174A}, in vitro phosphorylation assays, and LC-MS/MS analysis are described in Supporting information.

2.2. Bimolecular fluorescence constitution and OST1 localization

BiFC assays were performed as described [13,14]. The coding region of OST1 was cloned into the pUC-SPYNE vector, resulting in P35S:YFP^N-OST1. The coding regions of AtrbohF and AtGLR3.1 were cloned into the pUC-SPYCE and pUC-SPYNE, resulting in P35S:YFP^C-AtrbohF and P35S:AtGLR3.1-YFP^C, respectively. Particle bombardments onto onion epidermal cells were performed using the Helios Gene Gun (Bio-Rad) according to the manufacturer's manual with the following modifications: each shot delivered 125 ng of tested pairs of plasmid DNA with 0.6 μm gold particle (0.25 mg per shot) using 200 psi helium pulse. Fluorescence was observed 40 h after incubation at RT in the dark using a Zeiss Observer Z1 inverted microscope with 38 HE GFP Zeiss filters. *GFP-OST1* was used to transfect Arabidopsis mesophyll protoplasts, and fluorescent signals were analyzed using a Zeiss LSM 510 Confocal laser scanning microscope. Details about OST1 localization are described in Supporting information.

3. Results

3.1. OST1 phosphorylates Ser174 on AtrbohF NADPH oxidase

The AtrbohF amino acid sequence was examined to find Ser/Thr residues that are likely to be phosphorylated by OST1, which displays similar phosphorylation preferences as the related SnRK2.10 kinase (S. Merlot, B. Turk, unpublished data). Based on the fact the SnRK2.10 kinase preferentially phosphorylates Ser/Thr in the motif [LIMVF]XRXXS/T [12], we identified three putative OST1 phosphorylation sites (Thr91, Ser97, Ser174) in the N-terminal region of AtrbohF. To test whether OST1 can phosphorylate AtrbohF, we carried out in vitro phosphorylation assays using a peptide from the region flanking Ser174 (LDRTRS^{174}SAQR) as a substrate for OST1. The peptide was phosphorylated by OST1 with a K_m of 8.3 ± 1.49 μM and a V_{max} of 2.4 ± 0.2 nmol/min/mg (Fig. 1A). We then examined whether the AtrbohF protein can be phosphorylated by OST1. Because of the very low expression level and solubility of the full-length AtrbohF protein when expressed in *E. coli*, we used an N-terminal cytosolic domain of AtrbohF (amino acid residues 1–378) in our analysis. Fig. 1B shows that the N-terminal domain of AtrbohF was also efficiently phosphorylated by OST1, indicating that AtrbohF is a substrate of OST1 in vitro.

To determine whether the predicted Ser174 is phosphorylated by OST1, we mutated Ser174 to Ala and compared the phosphorylation of AtrbohF and AtrbohF^{S174A}. Fig. 2 shows that the S174A mutation resulted in a ~40% reduction in phosphorylation in the mutant AtrbohF^{S174A}. This result suggests that either Ser174 is one of the amino acid residues that are phoshorylated by OST1 or the mutation changes the conformation of AtrbohF such that another residue in the protein cannot be as efficiently phosphorylated.

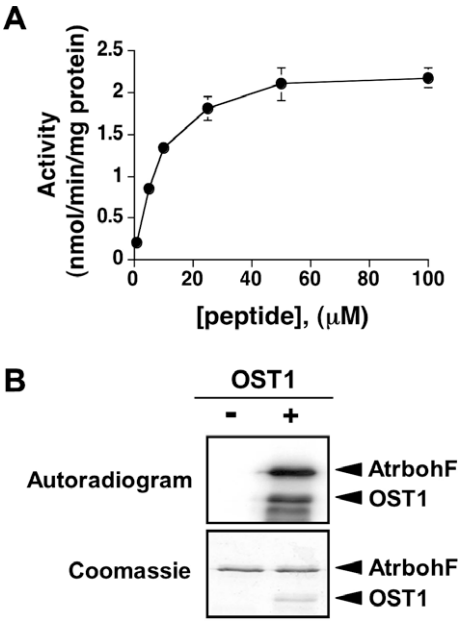

Fig. 1. AtrbohF is phosphorylated by OST1 in vitro. (A) The peptide containing Ser174 is phosphorylated by OST1 with a K_m value of 8.3 ± 1.5 μM. Error bars indicate S.E.M., $n = 3$ independent experiments. (B) OST1 phosphorylates an N-terminal cytosolic domain of AtrbohF. Autoradiogram shows the 75 kDa GST-AtrbohF fusion protein (AtrbohF) was phosphorylated by OST1. OST1 undergoes autophosphorylations as well.

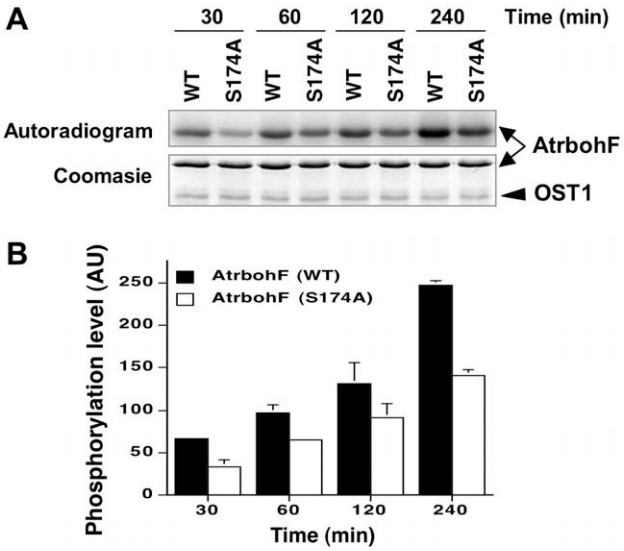

Fig. 2. S174A point mutation leads to ~40% reduction in the AtrbohF phosphorylation by OST1. (A) A point mutation of Ser174 to Ala leads to reduction in the OST1 phosphorylation of AtrbohF. The N-terminal domain of AtrbohF and AtrbohF^{S174A} was incubated with OST1 for 30, 60, 120, and 240 min. (B) Quantification of AtrbohF phosphorylation levels. Phosphorylation levels on the autoradiogram were normalized to the protein level on Coomassie-stained gel. Error bars in the graph represent S.D., $n = 2$ independent experiments.

3.2. Mass spectrometry reveals that Ser13 on AtrbohF is also phosphorylated by OST1

To verify the phosphorylation of Ser174 and to identify additional amino acid residues that are phosphorylated by OST1, AtrbohF and AtrbohF^{S174A} were phosphorylated by OST1, digested with trypsin, proteinase K and chymotrypsin, and then subjected to LC-MS/MS analysis. The LC-MS/MS analysis covered 83% of the protein sequence (Fig. S1). As anticipated, Ser174 was found to

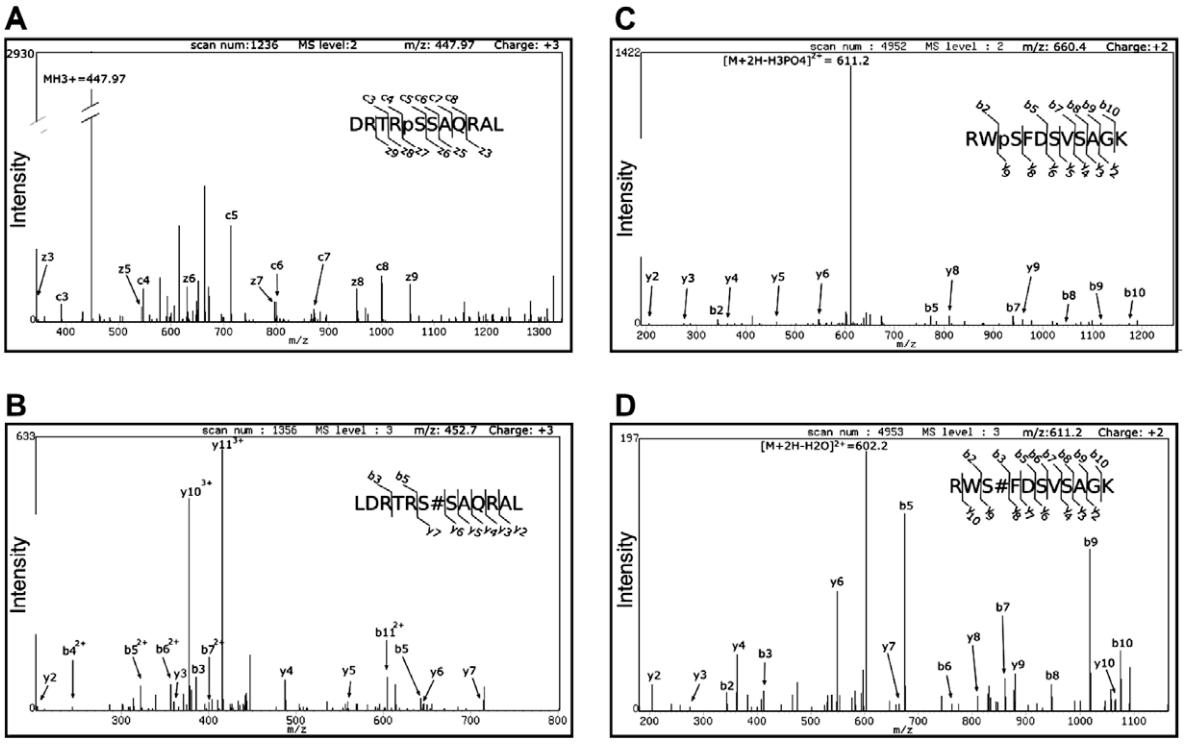

Fig. 3. LC-MS/MS analysis reveals that Ser174 (A, B) and Ser13 (C, D) are phosphorylated by OST1. (A) MS2 spectra showing the neutral loss of phosphoric acid (H_3PO_4) after CID fragmentation of the charged peptide LDRTRpSSAQRAL at m/z 485.4. This peptide was generated from the proteolysis of AtrbohF by proteinase K. (B) Phosphorylation of Ser174 was confirmed in MS3 spectra of the charged [MH^{2+}-H_3PO_4] peptide ion at m/z 452.7. S# indicates dehydroalanine. (C) MS2 spectra showing the neutral loss of phosphoric acid (H_3PO_4) after CID fragmentation of the charged RWpSFDSVSAGK precursor ion at m/z 660.4. This peptide was generated from the proteolysis of AtrbohF by trypsin. (D) Phosphorylation of S13 was confirmed in MS3 spectra of the charged [MH^{2+}-H_3PO_4] peptide ion at m/z 611.2.

be phosphorylated (Fig. 3A and B). We also found that Ser13 was phosphorylated by OST1 (Fig. 3C and D), which was not predicted because of aspartic acid at the −5 position instead of a hydrophobic amino acid. We did not identify other phosphorylated amino acid residues on AtrbohF or AtrbohF^{S174A}.

3.3. OST1 physically interacts with AtrbohF

To investigate whether OST1 can physically interact with AtrbohF in plant cells, we conducted the bimolecular fluorescence complementation (BiFC) assay [13,14]. *OST1* and *AtrbohF* in the BiFC vectors were transiently co-expressed in onion epidermal cells, and reconstituted YFP fluorescence was observed at the periphery of several examined cells when *YFP^N-OST1* was co-expressed with *YFP^C-AtrbohF* (Fig. 4). In contrast, onion epidermal cells expressing either of these constructs in conjunction with the empty vector or a control membrane protein that does not interact with OST1, showed no detectable fluorescent signals (Fig. 4). This result indicates that OST1 is able to interact with AtrbohF in vivo and further suggests that the interaction occurs near the plasma membrane where NADPH oxidases are localized [15].

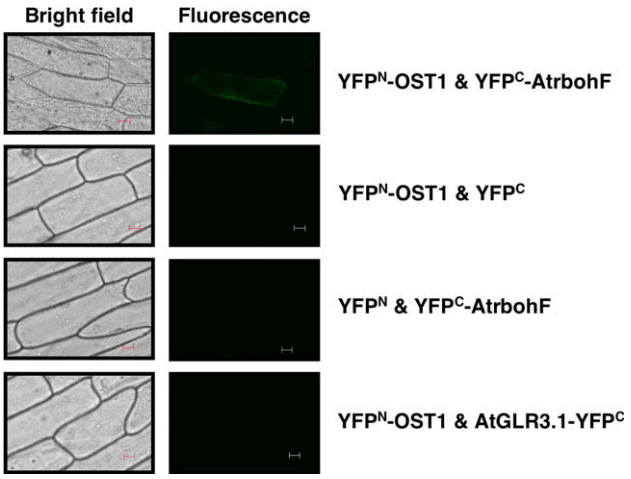

Fig. 4. Reconstitution of YFP from YFP^N-OST1 and YFP^C-AtrbohF in transiently transformed onion epidermal cells. Co-expression of *P35S:YFP^N-OST1* and *P35S:YFP^C*, *P35S:YFP^C-AtrbohF* and *P35S:YFP^N*, or *P35S:YFP^N-OST1* and *P35S:AtGLR3.1-YFP^C* does not reconstitute fluorescence. Scale bars = 100 μm.

We then examined the sub-cellular localization of OST1 in Arabidopsis protoplasts. GFP-OST1 is localized in the cytoplasm, the nucleus, and in close proximity to the plasma membrane (Fig. 5). ABA treatment did not affect OST1 localization, suggesting that the activity of OST1 [10] but not its sub-cellular localization is regulated by ABA.

4. Discussion

In Arabidopsis guard cells, AtrbohF and AtrbohD have been shown to be responsible for ABA-triggered ROS production and stomatal closure [2]. A previous study showed that OST1 acts upstream of ROS in guard cell ABA signaling [10], but functional relationship between OST1 and the two NADPH oxidases remained to be addressed. Here, we show the physical interaction of OST1 with AtrbohF and the phosphorylation of Ser13 and Ser174 on AtrbohF by OST1. Our results provide biochemical evidence that OST1 directly phosphorylates the AtrbohF NADPH oxidase, probably as a way to regulate their activity in response to ABA in guard cells.

Recent studies show that plant NADPH oxidases are phosphorylated [4–6]. Potato StCDPK4 and StCDPK5 were shown to phosphorylate Ser82 and Ser97 in the N-terminus of StrbohB [4] (see

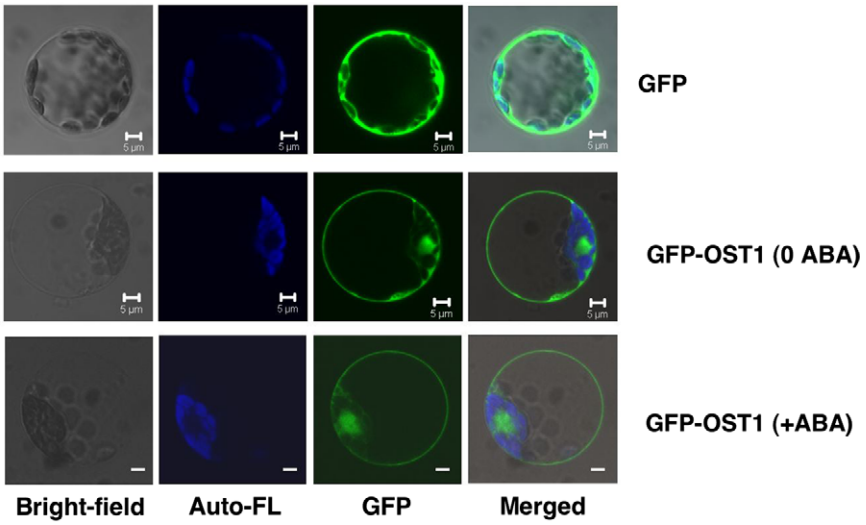

Fig. 5. Sub-cellular localization of GFP-OST1 in Arabidopsis protoplasts before (0 ABA) and after (+ ABA) 100 μM ABA treatment. Localization of GFP alone is shown (top row). Scale bars = 5 μm.

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StrbohB     1 ------MEIEN----------TRDSDSMRGSRVGFSG----------------SLVSGKKS  29
AtrbohD     1 -MKMRRGNSSNDHELGILRGANSDTNSDTESIASDRGAFSGPLGRPKRASKKNARFADDLP  60
AtrbohF     1 MKPFSKND-RRR--------WSFDSVSAGKTAVG-----------------SASTSPGTE  34

StrbohB    30 ARFK-------DDESYVEITLDVRDD-SVSVQNIKGADHEA--------------------  62
AtrbohD    61 KRSNSVAGGRGDDDEYVEITLDIRDD-SVAVHSVQQAAGGGG------------------- 101
AtrbohF    35 YSIN-------GDQEFVEVTIDLQDDDTIVLRSVEPATAINVIGDISDDNTGIMTPVSISR  88

StrbohB    63 ------------ALLASRLEKRPNNTLGSQLSF--HLRQVSKELKRMTSSN----------  99
AtrbohD   102 ------------HLEDPELALLTKKTLESSLNNTTSLSFFRSTSSRIKNASRELRR-----  145
AtrbohF    89 SPTMKRTSSNRFRQFSQELKAEAVAKAKQLSQELKRFSWSRSFSGNLTTTSTAANQSGGAG  159
              *     *

StrbohB   100 ---------------KFQKIDRSKSGAARALRGLQFMN-KNVGTEGWSEVESRFDQLAVN-  143
AtrbohD   146 -------VFSRRPSPAVRRFDRTSSAAIHALKGLKFIA-TKT--AAWPAVDQRFDKLSADS  196
AtrbohF   160 GGLVNSALEARALRKQRAQLDRTRSSAQRALRGLRFISNKQKNVDGWNDVQSNFEKFEKN-  219

StrbohB   273 QAPSHSMNLSTN-SRVLSRMISQKLKPTKERNPFKR 307
AtrbohD   328 QAPNQ--SVRMGDSRILSQMLSQKLRPAKESNPLLR 361
AtrbohF   341 QKDTY--LNYSQALSYTSQALSQNLQGLRGKSRIHR 372
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Fig. 6. Phosphorylation sites in AtrbohF, AtrbohD, StrbohB NADPH oxidases. Serine residues on AtrbohF that are phosphorylated by OST1 are highlighted in bold and indicated with an arrowhead. Predicted but unverified OST1 phosphorylation sites (Thr91, Ser97) on AtrbohF are underlined and indicated with an asterisk. Ser82 and Ser97 on StrbohB phosphorylated by StCDPK4 and StCDPK5 are highlighted in bold. Six serine residues on AtrbohD identified by a phosphoproteomics approach are highlighted in bold. Identical amino acid residues in all three protein sequences are shown in green. Protein sequences of four Atrboh proteins (AtrbohC, AtrbohD, AtrbohF, AtrbohI) and two Strboh proteins (StrbohB, StrbohD) were initially aligned using the ClustalW program to obtain a comprehensive alignment. Three protein sequences (AtrbohC, AtrbohI, StrbohD) were removed from the alignment for clarity.

Fig. 6). A phosphoproteomics study revealed that phosphorylation of Ser343 and Ser347 is necessary for activation of AtrbohD [6]. Ser343 but not Ser347 on AtrbohD was also found to be phosphorylated in flg22-treated Arabidopsis suspension cells [7]. Another study showed that phosphorylation of AtrbohD and calcium-induced ROS production by AtrbohD were enhanced by the protein phosphatase inhibitor calyculin A [5]. However, it remains unknown what protein kinase(s) is responsible for phosphorylating these residues on AtrbohD.

Our results show that Ser13 and Ser174 on AtrbohF are phosphorylated by OST1 (Figs. 1–3 and 6). Ser174 on AtrbohF is conserved in AtrbohD (Ser163) and other NADPH oxidases. The conserved serine residues on other NADPH oxidases also have Arg at the −3 position, suggesting that they can be phosphorylated by OST1 and/or other members of the SnRK2 family. Ser163 on AtrbohD was shown to be phosphorylated in response to flg22 [7]. Interestingly, flg22 has been shown to induce stomatal closure in WT plants but not in the *ost1* mutant [16], indicating that OST1 might integrate both ABA and pathogen signaling to induce stomatal closure through the activation of NADPH oxidases. In contrast, AtrbohD Ser8, Ser39 and Ser152 that were found to be phosphorylated are not conserved in AtrbohF [6,7]. The differences

in environments of these serine residues suggest that AtrbohD and AtrbohF might be differentially regulated in response to abiotic/biotic stimuli. In fact, AtrbohD is thought to play a more important role in plant–pathogen interactions while AtrbohF is thought to be more important in ABA signaling, although these two NADPH oxidases have functional redundancy [2,17].

The results from the phosphorylation assays with AtrbohF and AtrbohF^{S174A} indicate that Ser174 accounts for ~40% of the phosphorylation by OST1 (Fig. 2). Although we found that Ser13 is also phosphorylated (Fig. 3), it is still possible that there are additional Thr and/or Ser residues on AtrbohF that are phosphorylated by OST1. Our phosphorylation motif analysis predicted that Thr91 and Ser97 on AtrbohF are likely to be phosphorylated by OST1 although it is not clear whether these two residues are phosphorylated by OST1 due to the poor LC-MS/MS coverage on this region and also because of the low ionization efficiency of phosphopeptides.

A recent study with an Arabidopsis mutant in which *OST1*, *SnRK2.2*, and *SnRK2.3* were disrupted showed that these three kinases play a central role in ABA signaling [18]. It will be interesting to test whether phosphorylation of AtrbohF by these ABA-activated SnRK2 kinases is required for activation of the NADPH oxidase in response to ABA in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.08.033.

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