Identification of Phosphorylation Sites Altering Pollen Soluble Inorganic Pyrophosphatase Activity

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One sentence summary: Phospho-regulation of key sites on pollen soluble inorganic pyrophosphatases inhibits their catalytic responsiveness in concert with key intracellular events.

Author Contributions: VEFT, JFH, SAW and FCHF conceived, designed and supervised the experiments; DJE, TH, YB, RT, BdeG and CGZ performed the experiments; NPJC provided technical assistance; HJC contributed new reagents/analytic tools; DJE, TH, NPJC and CGZ analyzed the data; DJE and SAW prepared the final figures; VEF-T wrote the paper with major contributions from JFH and FCHF.

Funding information: VEF-T and SAW were funded by the Biotechnology and Biological Sciences Research Council (BBSRC). TH was funded by a Commonwealth Scholarship. HJC is funded by the Engineering and Physical Sciences Research Council (EPSRC; EP/023490/1). JFH was funded by National Science Foundation (DBI-0420033), US/Israel Binational Agriculture Research & Development Fund (IS-4652-13 R) and Hatch funds from UNR. Mass spectrometry at UNR was supported by the Idea Network of Biomedical Research Excellence grant from the National Institute of General Medical Sciences (P20GM103440). The Advion Triversa Nanomate and Thermo Fisher Orbitrap Velos mass spectrometer used in this research were funded through the Birmingham Science City Translational Medicine: Experimental Medicine Network of Excellence project, with support from Advantage West Midlands (AWM); HJC is funded by EPSRC (EP/L023490/1).

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ABSTRACT

Protein phosphorylation regulates numerous cellular processes. Identifying the substrates and protein kinases involved is vital to understand how these important post-translational modifications modulate biological function in eukaryotic cells. Pyrophosphatases catalyse the hydrolysis of PPi to P_i, driving biosynthetic reactions; they are essential for low cytosolic PPi. It was recently suggested that post-translational regulation of Family I soluble inorganic pyrophosphatases (sPPases) may affect their activity. We previously demonstrated that two pollen-expressed sPPases, Pr-p26.1a and Pr-p26.1b, from the flowering plant Papaver rhoeas, were inhibited by phosphorylation. Despite the potential significance, there is a paucity of data on sPPase phosphorylation and regulation. Here we have used liquid chromatographic tandem mass spectrometry (LC-MS/MS) to map phosphorylation sites to the otherwise divergent N-terminal extensions on these pollen sPPases. Despite the absence of reports in the literature on mapping phosphorylation sites on sPPases, a database survey of various proteomes identified a number of examples, suggesting that phosphorylation may be a more widely used mechanism to regulate these enzymes. Phosphomimetic mutants of Pr-p26.1a/b significantly and differentially reduced PPase activities by up to 2.5-fold at pH 6.8 and 52% in the presence of Ca^{2+} and H_2O_2 over unmodified proteins. This indicates that phospho-regulation of key sites can inhibit the catalytic responsiveness of these proteins in concert with key intracellular events. As sPPases are essential for many metabolic pathways in eukaryotic cells, our findings identify the phosphorylation of sPPases as a potential master regulatory mechanism that could be used to attenuate metabolism.

INTRODUCTION

Numerous cellular processes are regulated by reversible protein phosphorylation (Humphrey et al., 2015), including metabolism, cell-cycle progression, differentiation, biotic and abiotic
stress tolerance, and apoptosis. Many metabolic enzymes are regulated by phosphorylation. Identifying the targets, phosphorylation sites and protein kinases involved is vital to understanding how these important post-translational modifications affect biological functions. In flowering plants, there are more than 1000 protein kinases, of which 34 in Arabidopsis belong to a family of Calcium Dependent Protein Kinases (CPKs). CPK-related kinases have been implicated in regulating many aspects of plant biology, including pathogen defence, interactions with symbionts, abiotic stress responses, and pollen tube growth (Harper et al., 2004; Zulawski et al., 2014).

Pyrophosphatases (PPases) are ubiquitous, highly conserved phosphate-metabolizing enzymes that play a central role in cellular metabolism. Inorganic phosphate (PPi) is produced within cells as a by-product of anabolic processes, such as nucleic acid and protein biosynthesis, as well as carbohydrate synthesis (including that of cell wall materials required for pollen tube growth). Soluble inorganic PPases (sPPases) catalyse the hydrolysis of PPi into two molecules of inorganic phosphate (P_i). The net release of energy from this reaction provides a thermodynamic driving force for many biosynthetic reactions such as protein, polysaccharide and nucleotide synthesis. PPases are the key enzymes that keep cytosolic [PPi] low in cells. This is essential as high [PPi] is toxic (Cooperman et al., 1992). Removal of PPi is performed by two non-homologous PPase enzymes. In animal cells and yeast, Family I sPPases are solely responsible (Pérez-Castiñeira et al., 2002; Serrano-Bueno et al., 2013), but in plants the role of sPPases is disputed; it is hypothesized that their role has largely been taken over by the membrane-located proton-translocating PPases (H^+-PPases)(Buchanan et al., 2002). Nevertheless, several examples have shown the importance of sPPases in metabolically active plant tissues; photosynthetic carbon assimilation and metabolism are greatly affected by changes in the levels of sPPases (de Graaf et al., 2006; George et al., 2010; Sonnewald, 1992). The structure and catalytic
mechanisms of Family I sPPases are well characterised in bacteria and budding yeast, *Saccharomyces cerevisiae* (Cooperman et al., 1992).

Given their biochemical function, not surprisingly, the activity of sPPases is essential for cellular metabolism and growth. Knockouts of sPPase result in arrest of cell division in *E. coli* and inviability in budding yeast (Chen et al., 1990; Ogasawara, 2000; Pérez-Castiñeira et al., 2002), underlining the critical importance of PPi regulation. However, despite the cellular importance of [PPi] homeostasis, mechanisms regulating Family I sPPase activity have not been thoroughly studied, especially in eukaryotic cells. It is generally accepted until recently that a reduction in sPPase activity causes accumulation of PPi to toxic levels, causing cell death. In bacteria it has been suggested that post-translational regulation of catalytic activity may play an important role in regulating the activity of sPPases (Kukko-Kalske et al., 1989). However, evidence for this is scarce. Although two studies have reported phosphorylation of sPPases *in vitro* (Rajagopal et al., 2003; Vener et al., 1990), the sPPases from a flowering plant, *Papaver rhoeas* (de Graaf et al., 2006) appear to be the only example with *in vivo* evidence for phosphorylation of Family I eukaryotic sPPases modifying activity.

Cellular responses require an integrated signal perception and signal transduction network. During pollination higher plants use specific interactions between male (pollen) and female (pistil) tissues. Many flowering plants utilize genetically controlled "self" rejection systems: self-incompatibility (SI), to prevent self-fertilization and inbreeding. *Papaver rhoeas* uses a SI system involving the female S-determinant (PrsS) protein, which is a ligand secreted by the pistil (Foote et al., 1994) and the male S-determinant protein, PrpS (Wheeler et al., 2009). In this SI system, interaction of pollen with 'self' (incompatible) PrsS protein induces an influx of Ca$^{2+}$ and K$^+$ (Wu et al., 2011) and transient increases in cytosolic free Ca$^{2+}$ concentrations ([Ca$^{2+}$]$_{cyt}$) in incompatible pollen (Franklin-Tong et al., 1997). Downstream, SI triggers...
Transient increases in Reactive Oxygen Species (ROS) and Nitric Oxide (NO) which participate in signalling alterations to the actin cytoskeleton and programmed cell death (PCD) (Wilkins et al., 2011; Wilkins et al., 2014). SI also triggers dramatic acidification of the cytosol, which is necessary and sufficient to trigger PCD, involving the activation of a DEVDase/caspase-3-like activity (Bosch and Franklin-Tong, 2007; Wilkins et al., 2015).

However, one of the earliest targets of the SI-mediated Ca\(^{2+}\) signals are a pair of pollen-expressed Family I soluble inorganic pyrophosphatases (sPPases), Pr-p26.1a and Pr-p26.1b (hereafter called p26a and p26b), that are rapidly phosphorylated in a Ca\(^{2+}\)-dependent manner in incompatible pollen after SI (Rudd et al., 1996). Increases in [Ca\(^{2+}\)]\(_{cyt}\) and phosphorylation of these sPPases resulted in a reduction in their activity (de Graaf et al., 2006).

Here we have mapped phosphorylation sites on p26a/b catalysed by endogenous pollen kinases and recombinant CPKs. Using recombinant phosphomimetic p26a/b proteins, we provide evidence that phosphorylation status on these sites is an important factor which differentially modulates the catalytic responsiveness of the two proteins in relation to key intracellular events that are triggered during the inhibition of pollen tube growth by SI. As the activity of PPases is crucial for all living eukaryotic cells, our findings provide an important conceptual advance in our general knowledge about the modulation of a major group these essential housekeeping enzyme.
RESULTS

p26a/b have activities characteristic of Family 1 sPPases

The two *Papaver* p26 sPPase sequences are highly conserved, with 79.5% amino acid identity between their core enzymatic regions. However major variation occurs in their N-terminal regions (31.6% identity between 36 and 57 amino acid residues in p26a and p26b respectively, Fig S1). The substrate specificities for recombinant p26a and p26b are virtually identical, with preference for pyrophosphate (Fig S2, Table S1). Both p26a and p26b have classic Mg$^{2+}$-dependent sPPase activities with identical requirements for Mg$^{2+}$ (NS, p=0.994; Fig S2Ai). Although divalent cations affect prokaryotic sPPase activities, there was no significant difference between p26a and p26b PPase activities at any concentrations tested for ZnCl$_2$ (NS, p=0.890), Co$^{2+}$ (NS, p=0.809) or Mn$^{2+}$ (NS, p=0.573) tested (Fig S2Aii-iv). Ca$^{2+}$ is a competitive inhibitor to Family I sPPases (Cooperman et al., 1992) and increasing [CaCl$_2$] resulted in loss of Mg$^{2+}$-dependent pyrophosphatase activity (IC$_{50}$ < 100µM, Fig S2Bi). The p26a/b PPase activities were strongly inhibited by F$^-$ (like other eukaryotic Family I sPPases), but there was no differential response (NS, P=0.238, Fig S2Bii). As a large influx of K$^+$ is triggered by SI in incompatible pollen (Wu et al., 2011), we examined if K$^+$ affected the p26 PPase activities. K$^+$ did not inhibit their PPase activities and they both behaved similarly in the presence of K$^+$ (NS, P=0.172; Fig S2Biii). Thus, p26a and p26b without any phospho-modifications, exhibited virtually identical sPPase activities under various biologically relevant conditions.

In vitro phosphorylation of p26 using pollen extracts

We previously demonstrated Ca$^{2+}$-dependent phosphorylation of pollen-expressed sPPases, p26a and p26b in living pollen tubes undergoing the SI response and a corresponding decrease in their PPases activity (de Graaf et al., 2006; Rudd et al., 1996). Here we determined the sites phosphorylated on the His-tagged recombinant p26a/b proteins to gain
insights into the functional significance of the phosphorylation. We performed liquid chromatographic tandem mass spectrometry (LC-MS/MS) using electron transfer dissociation (ETD) to map amino-acid residues modified on p26a and p26b after phosphorylation by native *P. rhoeas* pollen kinases in pollen extracts. This analysis identified phosphorylation on Ser13, Thr18 and Ser27 for p26a and phosphorylation on Thr25, Ser41 and Ser51 for p26b (Fig 1, Fig S1, Table S2A,B). Notably, all of the detectable phosphorylation sites were located in the N-terminal extensions.

**Identification of CPKs that can phosphorylate p26 sPPases**

As we previously showed that p26a/b were phosphorylated by a Ca\(^{2+}\)-dependent protein kinase (CPK) type activity (Rudd et al., 1996), we tested to see if p26a/b was a substrate for AtCPK34, a CPK that is highly expressed in *Arabidopsis* pollen and required for pollen fitness (Harper et al., 2004; Myers et al., 2009). Using *in vitro* kinase assays, AtCPK34 phosphorylated p26a and p26b at levels several-fold greater than casein kinase II (CKII) and protein kinase A (PKA) (Fig 2A, B). This confirms p26 as a potential target for CPK-
mediated phosphorylation. We therefore initiated cloning of *Papaver* pollen-expressed orthologs and obtained cDNA clones for 3 *Papaver* CPKs: PrCPK14, PrCPK6/26 and PrCPK17/34, named after their homology to three distinct groups of CPKs from *Arabidopsis* (SFig 3A,B). These CPKs were engineered as recombinant His-tagged versions and used with *in vitro* kinase assays to assess whether the p26 proteins were substrates.

**p26a/b are substrates of *Papaver* pollen-expressed CPKs**

All three His-tag versions of the PrCPKs were able to phosphorylate syntide-2 (Table S3A) and p26a/b proteins (Fig 2C-D). Under Michaelis-Menten conditions, all three *Papaver* CPKs had comparable $K_M$ values for p26a/b, though the reaction rates ($k_{cat}$) varied (Table S3B, C). A LC-MS/MS analysis was used to ascertain which residues were phosphorylated after *in vitro* kinase assays(Fig 3, Table S2A). The overall protein sequence coverage of p26a and p26b peptides was virtually complete, at 93.5-95.3% coverage. No single CPK phosphorylated all sites identified. For p26a, ETD mass spectra revealed phosphorylation on Ser11, Ser12, Ser13, Thr17, Ser27, Ser28, Ser30 and Ser33. PrCPK17/34 showed broadest specificity and recognized all but Ser 33. While Ser33 was phosphorylated by PrCPK14, this kinase did not appear to recognize Ser11, Thr17, Ser27, Ser28. While a location in the N-terminal extension was observed for all phosphorylation sites in both p26a and p26b, the specific sequences surrounding the sites were different (Fig 3, Table S2B).
Several of the sPPase amino acids phosphorylated by these PrCPKs were identical to those phosphorylated by the endogenous pollen kinases (Ser13, Thr18 and Ser27 in p26a; Thr25,
Ser41, Ser51 in p26b). Notably, the phosphorylated sites mapped on p26a and p26b using the various recombinant CPKs were different.

Phosphomimic substitutions exhibit unchanged sPPase activities under standard conditions

To attempt to ascertain whether the mapped phosphorylation sites in p26a/b had any biological significance, we examined whether phosphomimic substitutions of key amino acids had any effect on the p26a/b PPase activities. To determine whether phosphorylation of these amino acid residues could attenuate PPase activity, we constructed phosphomimic (glutamic acid [E] substitution) and corresponding phosphonull (alanine [A] substitution) His-tagged mutant p26a/b proteins (Table S3A,B). The triple substitution phosphomimic mutants for p26a [S13E, T18E and S27E, named p26a(3E)] and p26b [T25E, S41E and S51E, named p26b(3'E)] and their corresponding phosphonull versions comprised the sites phosphorylated by the endogenous pollen kinases. We measured their PPase activities and kinetic parameters for hydrolysis of PPi, using recombinant p26 proteins. The $K_M$, turnover number ($k_{cat}$) and catalytic efficiencies of recombinant p26a and p26b were not significantly different (P= 0.499, P=0.991 and P=0.448 respectively; Table S3C,D). The kinetic parameters for PPi hydrolysis for the triple phosphomimic and phosphonull p26a and p26b mutants were also not significantly different from those for unmodified p26 sPPases (Table S3C,D), suggesting that phosphorylation of these residues alone are insufficient to alter the kinetics of these sPPases in vitro under these standard in vitro conditions. We constructed further phospho-mutants with five and seven substituted residues mapped from in vitro phosphorylations with recombinant CPKs (Table S3A,B): p26a(5E) and p26b(5'E) and p26a(7E) and p26b(7'E). There was no difference in activity between the wild type p26a/b and phosphomimic/null mutants at pH 7 (Fig 4), [p26a (NS, P= 0.772), p26b (NS, p= 0.109)].
pH sensitivity is enhanced in p26 phosphomimic mutants

We previously demonstrated that SI triggers rapid, dramatic cytosolic acidification of incompatible pollen, with cytosolic pH ([pH]_cyt) decreasing from pH 7.0 to pH 6.8 within 10 minutes. 

**Figure 4.** Effect of pH on PPase activities of p26a/b and their phosphonull and phosphomimic mutant versions. Recombinant p26 and mutant versions were assayed for PPase activity at variable pH; mean ± SEM (n=4). (A) p26a, (B) p26b. **pH sensitivity is enhanced in p26 phosphomimic mutants**
min, and to pH 5.5 within 60 min (Wilkins et al., 2015). Both p26a and p26b had identical pH profiles and displayed a pH-dependent attenuation typical of other Family I sPPases (Cooperman et al., 1992; Wilkins et al., 2015). Examining if pH affected the PPase activity of the phosphomimic mutants, we found that their activities were more sensitive to decreases in pH (Fig 4). In contrast to activities at pH 7, at pH 6.8 a large drop in PPase activity of all the phosphomimic mutants was observed, significantly lower than wild type and phosphonull mutants (p=0.000***; Fig 4A-B); the drop in activity of the 5E/5’E and 7E/7’E mutants was much larger than that of the 3E/3E’ mutants (~42.5-37.4% compared to 75.6-69.5%, Fig 4A-B). The PPase activity of the phosphomimic mutants were completely inhibited below pH 5.5; significantly lower than wild-type and phosphonull mutant enzymes (p=0.002**, p=0.000***, respectively), which retained residual activity at this pH. Thus, although these two pollen-expressed sPPases exhibited indistinguishable PPase activities under normal physiological (~pH 7) conditions and phosphomimic substitutions did not alter their kinetic parameters, at lowered pH the phosphomimic mutants exhibited differential reductions in sPPase activity. This implicates phosphorylation as having an effect on p26 PPase activity when physiological pH drops and provides strong evidence that phosphorylation at several amino acid residues in the N-terminal region affects p26 PPase activity at low pH.

**Ca**^{2+} and **H**_{2}O_{2} additively inhibit p26 phosphomimic/null activities differentially

The inhibitory effect of Ca^{2+} on PPases is well known (Cooperman et al., 1992); this is also the case for the Papaver sPPases (de Graaf et al., 2006; Rudd et al., 1996). As SI triggers increases in [Ca^{2+}]_{cyt} and phosphorylation of p26 in incompatible pollen preceding a ROS burst (Wilkins et al., 2011), we investigated the effect of H_{2}O_{2} combined with Ca^{2+} on the recombinant p26 enzymes and their triple phosphorylation site substitution mutants at pH 7. This pH was chosen, as the PPase activity of the wildtype enzyme is negligible at pH 5.5, making it difficult to quantify any additional negative contributions from the phospho-mimic mutants. Moreover, we thought that the physiologically most important question was to
evaluate the enzymes under the conditions that would exist at the start of the SI response when the pH was ~pH 7.0.

As expected, Ca\(^{2+}\) significantly inhibited activity of all forms of p26 (p=0.000***). Notably, PPase activity in the phosphomimic mutants was more strongly inhibited by Ca\(^{2+}\) than their corresponding phosphonull mutants (p=0.000***, Fig 5). Similarly, all the p26 phosphomimic mutants were significantly more sensitive to an H\(_2\)O\(_2\) treatment ((p=0.038* for 3A vs 3E); (p=0.030* for 5A versus 5E); (p=0.022* for 7A vs 7E); Fig 5A] and similarly for p26b (Fig 5B). Combined Ca\(^{2+}\) and H\(_2\)O\(_2\) had a much greater effect with PPase activity <20% activity compared to untreated (Fig 5A-B). Thus under these conditions we established key phosphorylation sites that are important for modulating their activity.

Maximal reduction in activity was achieved in the phosphomimic forms of p26a(3E)/b(3'E); additional phosphorylation site substitutions (5E and 7E) had no further effect. Thus, the triple phosphomimic forms of p26a/b (phosphorylation sites attributed to the endogenous pollen kinases) contributed to enhanced inhibition by Ca\(^{2+}\) and H\(_2\)O\(_2\).

\textbf{Ca}^{2+}, \textbf{H}_2\textbf{O}_2 and \textbf{pH all contribute to inhibit p26 activity}

Having established that combining Ca\(^{2+}\) and H\(_2\)O\(_2\) resulted in further reduced PPase activity prompted a related, but subtly different question: if these conditions are combined with reduced pH (also triggered by SI, but slightly later), does the sPPase activity go down even further? We therefore measured the recombinant p26a/b sPPase activities with Ca\(^{2+}\) and H\(_2\)O\(_2\) combined with several pH points relevant to SI. As shown earlier, we found that the PPase activities of both p26a and p26b were significantly reduced by Ca\(^{2+}\) and further reduced by Ca\(^{2+}\) combined with H\(_2\)O\(_2\) (Fig 6A-B). As expected, the PPase activity for all of these observations was substantially reduced further by a reduction in pH, and by pH 5.5, activity was \(~10\%\) (Fig 6A-B). The PPase activities of p26a treated with Ca\(^{2+}\), H\(_2\)O\(_2\) or Ca\(^{2+}\) and H\(_2\)O\(_2\) treatments were reduced and significantly different at each pH (Fig 6A, P= 0.005,
p26b behaved in a similar manner (Fig 6B). At pH 5 the PPase activity for all the treatments was ~zero and none of the treatments were significantly different (P= 0.660, ANOVA). These data demonstrate that all three SI-induced events can contribute to reduce sPPase activity.

Mapping of phosphorylation sites on CPKs

We also mapped several phosphorylation sites on recombinant forms of the three PrCPKs and AtCPK34 (Table S5A-C, Fig S4). This information is important for the plant CPK field. Notably, we mapped a phosphorylated tyrosine (Tyr82) on peptide VKSIYTIGKE in PrCPK17/34 which aligns to the same conserved Tyr residue within the kinase catalytic domain as identified in soybean CPKβ (Tyr24). This confirms the finding that CPKs can phosphorylate Tyr in plants (Oh et al., 2012) and is of interest, as very few Tyr phosphorylation sites have been identified for plant proteins and no bona fide Tyr kinases have been found (Sugiyama et al., 2008).
**DISCUSSION**

**Identification of phosphorylation sites identified on sPPases**

**Figure 6.** pH further reduces PPase activity in the presence of Ca$^{2+}$ and H$_2$O$_2$. Recombinant sPPase p26a (A) and p26b (B) enzymes assayed for PPase activity (diamonds) and in the presence of CaCl$_2$ (circles), H$_2$O$_2$ (squares) and CaCl$_2$ + H$_2$O$_2$ (triangles) at several pHs. Activity for each was reduced further by lowered pH. Mean ± SEM (n=5 for p26a and n=3 for p26b).
It was recently suggested that post-translational processes may play an important role in regulating the activity of Family I sPPases (Serrano-Bueno et al., 2013). However, studies reporting phosphorylation of sPPases have been scarce. We previously identified phosphorylation of two pollen-expressed Family I sPPases (de Graaf et al., 2006; Rudd et al., 1996). Here we have mapped several phosphorylation sites on these proteins, using both endogenous pollen kinases and recombinant CPKs and identified multiple (and different) phosphorylation sites on p26a and b in their N-termini. We queried the P3DB plant phosphorylation database (www.p3db.org) for other phosphorylated sPPases using BLAST analyses with p26a/b sequences. This identified phosphorylated sites on several eukaryotic sPPases. The plant sPPases, including Arabidopsis thaliana, maize (Zea mays), rice (Oryza sativa) and grape (Vitis vinifera) had the majority of phosphorylation sites located within their N-termini (Fig S5). Although there are now several large-scale phosphoproteomic analyses, to our knowledge, no reports in the literature have discussed the direct mapping of phosphorylation sites on sPPases from any organism. Many of the phosphorylation sites on sPPases are clustered within the variable N- and C-terminal extensions that appear to be a feature of eukaryotic sPPases, rather than the conserved catalytic region; we discuss these below. As no phospho-modifications were identified at or near the catalytic active site, it is currently difficult to build a model of how phosphorylation of eukaryotic sPPases might affect their enzymatic activity.

We also identified a tyrosine residue that is phosphorylated in human PPA1 (Tyr143), mouse and E. coli sPPases. This site in E. coli (Tyr142) has a role in binding to one of the two phosphates in the substrate. Our finding suggests that phosphorylation at this site may play a role in regulating PPase activity in some eukaryotes, as the active site and this tyrosine are highly conserved across all Family I PPases. The phospho-tyrosine would both sterically clash and electrostatically repel PPI binding at the active site, affecting substrate binding and enzyme catalytic activity. The identification of phosphorylated amino acid residues in a number of species provides an important foundation for exploring the basis of these post-
translational modifications of these important enzymes and their biological consequences in the future. Whether there is an analogous phosphotyrosine modification during an SI response is uncertain. However, we did obtain evidence that the three PrCPKs and AtCPK34 tested here for in vitro activities are dual specificity kinases (Oh et al., 2012) (Table S5A-C, Fig S4), which leaves open their potential to function in planta as a tyrosine kinase under certain conditions.

Clustering of phosphorylation sites in the N-terminal region

Notably, the most phosphorylated Ser/Thr residues identified in this study on pollen occur in the poorly conserved N-terminal region. Many of the other plant sPPase phosphorylation sites are clustered in the 36-57 amino acid N-terminal region of low homology. A feature of sPPases from eukaryotes is that they have variable N or C-terminal extensions, which are absent from well-characterized prokaryotic sPPases (Fig S5); however, their function is unknown. As the E. coli sPPase is fully active and these N-terminal and C-terminal extensions are absent, it is assumed that they are non-essential for catalytic activity. However, clearly phosphorylation in these regions can affect sPPase activity. For plant sPPases, these N-terminal extensions have been modelled and postulated to be involved in regulation of enzyme activity (Rosales-León et al., 2012). Although a crystal structure for a Family I sPPase has recently been solved for plants (Grzechowiak, 2013), the structure does not include an N-terminal extension. Nevertheless, our results and a survey of public phospho-proteomic databases provide many examples of in which eukaryotic sPPase have been observed with phosphorylation sites clustered in their variable N-terminal regions. From primary sequence comparisons of p26a/b with other sPPases in combination with our data, we tentatively suggest that phospho-modification of the N-and C-termini may affect eukaryotic PPase activity. While it is not yet clear how these regions might regulate enzyme activity, evidence provided here provides an example in which phosphomimic changes in N-terminal regions can make sPPases more sensitive to inhibitory conditions. It is also
possible that these phospho-modifications could have synergistic impacts in vivo, for example by promoting or inhibiting protein-protein interactions. This merits investigation in the future.

**CPKs are involved in phosphorylation of plant sPPases**

Our identification of CPKs as capable of phosphorylating these sPPases is a milestone, as to date few kinases responsible for phosphorylating eukaryotic sPPases have been identified. Not only do we identify CPK-dependent phosphorylation sites on p26a/b, but we also identified in the phosphoproteomic databases several other plant sPPases that were phosphorylated at sites in common with p26a/b, including several consensus CPK sites [basic-xxS/T and S-x-basic (Curran et al., 2011; Harper and Harmon, 2005; Neumann et al., 1996; Roberts and Harmon, 1992)]. For example, the region RILS$_{27}$S$_{28}$LS$_{30}$RR is phosphorylated in p26a, and appears to be conserved in several plant sPPases. Within this region is an RxxSxx substrate motif for CPKs and Ca$^{2+}$/CaM-dependent kinase (Mayank et al., 2012). While these data demonstrate that sPPases can be phosphorylated by CPKs, it is also possible that in planta regulation of sPPases could be mediated by other kinases that phosphorylate the same or different sites.

**What is the biological significance of sPPase phosphorylation?**

Although PPI activity in eukaryotic cells is critically important (Chen et al., 1990; de Graaf et al., 2006; George et al., 2010; Sonnewald, 1992), to date, very few studies of the mechanisms underlying the regulation of PPI homeostasis (in eukaryotes in particular) have been reported. Our findings substantially improve knowledge of how sPPases can be post-translationally modified to affect their activity. With the exception of a single paper, reporting in vitro phosphorylation of rat liver sPPase by cAMP-dependent protein kinase (increasing its activity (Vener et al., 1990)), to our knowledge, there have been no other reports of
phosphorylation affecting Family I sPPase activity. Our findings here identify and suggest that multiple phosphorylation sites play a role in modulating the sensitivity of these sPPases to inhibition by several physiologically relevant cellular conditions. We showed that the phosphomimic forms of p26\(a/b\) are more responsive to inhibition by Ca\(^{2+}\), even more so to Ca\(^{2+}\) combined with H\(_2\)O\(_2\) and that PPase activity can be modulated by lowered [pH\(_{\text{cyt}}\)]. Thus, these conditions allowed us to establish which phosphorylation sites are important for modulating their PPase activity.

As both Ca\(^{2+}\) and ROS are stimulated very rapidly by SI, this suggests that SI not only stimulates phosphorylation of p26, but that cellular conditions stimulated by SI contribute to further reduction of PPase activity (and have a greater effect on the activity of the phosphorylated forms). Moreover, our data suggest that the slightly later pH drop stimulated by SI, can further reduce the p26 sPPase activities. Together, our data implicate that all of these SI-triggered intracellular events are likely to contribute to the reduction in PPase activity. Thus, our findings have high biological relevance in the SI system. Whether the PPase phosphorylation is absolutely required for SI is difficult to assess; however it clearly occurs and this can alter activity under certain conditions. It would be of interest to examine whether other plant sPPases might be regulated in this manner. This is something that might be explored in the future.

As ROS plays a pivotal role in many stress pathways, including apoptosis and PCD (Circu and Aw, 2010; Gadjev et al., 2008; Van Breusegem et al., 2008), this provides a significant advance in our understanding of mechanisms regulating this important class of enzymes. Not only are our observations placed in a physiologically relevant context for SI in pollen, but it also provides, to our knowledge, the first demonstration of pH affecting the sensitivity of sPPase activity in combination with phosphorylation. The role of cytosolic pH in modulating...
physiological processes in eukaryotic cells has been rather ignored until recently, when two
studies showed that shifting $[\text{pH}]_{\text{cyt}}$ is pivotal in triggering PCD (Fendrych et al., 2014; Wilkins
et al., 2015). It was suggested that the gateway to PCD could be cytosolic acidification,
when the drop in $[\text{pH}]_{\text{cyt}}$ passes a threshold and this activates caspase-3-like/DEVDase
(which is normally inactive at pH 7). The current study suggests that phosphorylation of
sPPases can also be modulated by lowered $[\text{pH}]_{\text{cyt}}$ and identifies several amino acids as
being involved in modulation of PPase activity, by amplifying pH sensitivity.

Many cellular processes are regulated by reversible protein phosphorylation and identifying
the targets, sites and protein kinases involved is crucial to understand how these important
post-translational modifications affect biological function (Humphrey et al., 2015). Although
many target kinases are known to be phosphorylated, rather few non-signaling proteins have
been highlighted (Mayank et al., 2012). The finding that pollen sPPases are targets of
phosphorylation and that this can affect activity suggests that this might be an important way
to simultaneously inhibit a large number of metabolic processes that require sPPase activity
as part of a thermodynamically coupled reaction. As PPases can regulate many pathways,
this suggests that they might act as a master-regulator. Future studies should investigate
these aspects further, testing the possibility that sPPases could be a regulatory hub where
their phospho-inhibition might trigger global changes in cellular metabolism in other cell
types and responses other than pollen SI.

Materials and Methods

His-tag affinity purification of recombinant p26 proteins

C-terminal His-tagged recombinant p26 proteins (de Graaf et al., 2006) were expressed from
pET21b (Novagen) in LB medium (100 µg.mL$^{-1}$ ampicillin) supplemented with 2 mM MgCl$_2$ to
allow optimal activity after purification. Protein expression was induced in *E. coli* BL21 using 1 mM IPTG at 22°C. Proteins were purified using a Ni-NTA agarose FPLC column or Ni-NTA spin columns (Qiagen).

**Cloning of PrCPKs**

cDNA was synthesized using Invitrogen™ SuperScript™ II Reverse Transcriptase kit using total RNA extracted from *P. rhoeas* pollen (RNAeasy Plant Mini Kit, QIAGEN). Degenerate primers (CPK-F and CPK-R) based on Arabidopsis CPKs were then used to obtain partial pollen-expressed *P. rhoeas* CPK cDNAs. Full-length cDNAs of PrCPK14, PrCPK6/26 and PrCPK17/34 were then obtained using a combination of 3’ and 5’ RACE PCR. 3’RACE amplification was carried out on *P. rhoeas* pollen cDNA using a 3’RACE (3’RACE 17AP) and 5’ gene specific primers (3’RACE1 primers). The product was PCR-purified and re-amplified with a 3’RACE primer (3’RACE UAP) and nested gene-specific 5’ primer (3’RACE2 primers).

For 5’RACE, cDNA was synthesised using a primer specific to the 3’ end of the gene of interest (5’RACE1 primers) instead of the oligo dT primer. cDNA was cleaned-up using a QIAquick PCR Purification Kit (Qiagen). A tailing reaction was then performed to add a poly-C tail at the 5’ end. PCR amplification was then carried out with a 5’RACE abridged primer (5’RACE AAP) and a second gene specific 3’ primer (5’RACE2 primers). This amplification product was PCR purified and then re-amplified with a 5’RACE unabridged (5’RACE UAP) and third nested gene specific 3’ primer (5’RACE3 primers).

**Primers used for CPK cloning**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5’→3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK-F</td>
<td>GAGGATGTAGGAGGGAGGTGATGTATG</td>
</tr>
<tr>
<td>CPK-R</td>
<td>TCACTCTCTGCCCAATGGGCGGAACACCAC</td>
</tr>
<tr>
<td>3’RACE 17AP</td>
<td>GACTCGAGTGCAGACTGATTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
</tr>
<tr>
<td>3’RACEUAP</td>
<td>GACTCGAGTGCACATCGA</td>
</tr>
<tr>
<td>5’RACE AAP</td>
<td>GGCACACGCCTCGACTAGTACGGG55GG55GG55GG55GG55GG55GG55</td>
</tr>
<tr>
<td>5’RACE UAP</td>
<td>GGCACACGCCTCGACTAGTAC</td>
</tr>
</tbody>
</table>
Phylogenetic analysis of *Arabidopsis thaliana* and *Papaver rhoeas* CPKs were performed using PhyML (www.phylogeny.lirmm.fr).

**His-tag affinity purification of recombinant CPK proteins**

His-tagged recombinant calcium-dependent protein kinases (CPKs) were made using pET21b (Novagen). Protein expression was induced in *E. coli* BL21 Rosetta™ (Novagen) using 1 mM IPTG at 22°C for 16 h. Cell pellets were resuspended and lysed in 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 5 mM EDTA, 1 mM EGTA, 0.5 mM AEBSF (Calbiochem), cOmplete™ EDTA-free protease inhibitor cocktail (Roche Diagnostics), 0.5 mg mL⁻¹ lysozyme. Cleared lysate was dialysed against 20 mM Tris-HCl (pH 8.0), 0.1 M NaCl to remove trace calcium. CPKs were isolated using 1.0 mL Ni-NTA agarose resin (Qiagen) at 4°C and eluted with 50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 250 mM imidazole. Pooled eluate fractions were precipitated using 0.5 M ammonium sulphate and cleared supernatant applied to a HiTrap Phenyl HP column (GE Healthcare, UK), washed with 20 mM Tris-HCl pH 8.0, 0.5 M ammonium sulphate and eluted with a reverse gradient to 20 mM Tris-HCl pH 8.0 (0.5 mL min⁻¹). Fractions were concentrated using a Sartorius™ Vivaspin™ 500 (10K MWCO) and applied to Superdex 200 26/60 (GE Healthcare, UK) equilibrated with 20 mM Tris-HCl pH 8.0, 0.1 M NaCl.
Site-directed mutagenesis

Triple, quintuplet and septuplet phosphomimic and phosphonull phosphorylation site substitution mutants of p26a (3E/3A, 5E/5A and 7E/7A respectively) and p26b (3'E/3'A, 5'E/5'A and 7'E/7'A respectively) were constructed using Quikchange II Site Directed Mutagenesis Kit (Agilent Technologies). Mutagenic primers were designed according to manufacturer's instructions using GenBank sequences for p26.1a (AM162550.1) and p26.1b (AM162551.1). The location of these amino acid substitutions is shown in SFig 7; mutants are listed in S6 Table.

PPase assays

Recombinant His-tagged p26 proteins or their substitution mutant versions were diluted to 10 µM in 50 mM Hepes-KOH, pH 8.0, 50 µM EGTA, 2 mM MgCl₂. 250 ng aliquots were assayed for free phosphate using a discontinuous PPase assay, adapted from the Fiske-Subbarow method (Fiske, 1925) using 2 mM sodium pyrophosphate (or other substrate as appropriate). The assay buffer was supplemented with 2 mM MgCl₂ (or other metal salts/inhibitors as appropriate) or 0.1 mM CaCl₂ and/or 10 mM H₂O₂. Assays requiring a pH range used 50 mM propionic acid pH 5.0-7.0. To measure the effect of divalent cations other than Mg²⁺, His-tagged p26 proteins were incubated with the appropriate metal salts for 2 hours prior to assay.

The continuous method for monitoring orthophosphate was adapted from (Baykov and Avaeva, 1981). An aliquot p26 was injected into a continuous flow system containing a 15 mL mixing chamber equilibrated with 40 mM HEPES pH 7.2, 50 µM EGTA, 5 mM MgCl₂ and 0 to 500 µM sodium pyrophosphate. PPase activity was measured continuously at pH 7.2, 30°C by the addition of 0.0096% (w/v) methyl Green in 0.18 % (v/v) Triton X305 and 5.7%
(w/v) ammonium molybdate in 18.3% H₂SO₄ and monitored at 650nm using a UA-6 ISCO flow through detector (Ilias, 2004). Duplicate data were prepared from independent protein preps and solved for $K_M$ and $k_{cat}$ using curve-fit program SigmaPlot™ (Systat Software Inc).

Kinase assays for mapping phosphorylation sites

*In vitro* kinase assays using pollen protein were performed according to (Rudd et al., 1996). Briefly, pollen was homogenized on ice 15 min in 50 mM Tris-HCL pH 7.5, 0.1% Triton X-100 containing cOmplete™ EDTA-free protease inhibitor cocktail (Roche Diagnostics), cell debris removed by centrifugation and supernatants stored at -20°C until use. Recombinant His-tagged p26 was incubated with 100 µg crude pollen extract in 50 mM Tris-HCL pH 7.5, 1 mM ATP, 1 mM MgCl₂, 0.25 mM calyculin A for 15 min at 30°C. The p26 was isolated using Ni-NTA Spin Column (Qiagen) and digested with trypsin and analysed using LC-MS/MS (see later). Recombinant His-tagged p26 proteins were also incubated with recombinant His-tagged CPKs in kinase buffer (50 mM Tris-HCL pH7.5, 1 mM ATP, 10 mM MgCl₂, 0.1 mM CaCl₂) for 30 min. Aliquots were digested with either trypsin or endoproteinase Glu-C and analysed using LC-MS/MS.

In vitro kinase activity assays

Incorporation of $^{32}$P into recombinant His-tag purified p26 proteins (de Graaf et al., 2006) in the presence/absence of 50 ng recombinant CPKs (Harper et al., 1994) was determined after 10 min reaction at 30°C. 1 µl of 4000 Ci mmol⁻¹ [γ-$^{32}$P]ATP was added to 50 µl reaction mix on ice: 50 mM HEPES-KOH pH 7.7, 5 mM MgCl₂, 0.5 mM DTT, 300 µM ATP and 1.1 mM CaCl₂ and transferred to 30°C after 1 min. The reaction was stopped by adding protein loading dye. Phosphorylation of p26a/b was analysed using SDS-PAGE followed by autoradiography and scanning for relative labelling or scintillation counting (Liquid Scintillation Analyzer Tri-Carb 2810 TR, PerkinElmer).
For the kinetic kinase assays, purified recombinant His-tagged CPKs were assayed (Curran et al., 2011) over a time course using standard filter-based kinase assays to measure total incorporation of $^{32}$P into p26. Briefly, 25 µL kinase reactions contained 1 ng µL$^{-1}$ CPK His-tag fusion protein in 20 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mM EGTA, 1.1 mM CaCl$_2$, 0.1 mg mL$^{-1}$ BSA with varied syntide-2 (Sigma-Aldrich) substrate concentrations. Reactions were initiated by addition of 50 µM ATP spiked with 0.375 µCi $^{32}$P[y-ATP] (3000 Ci mmol$^{-1}$; PerkinElmer) and incubated at room temperature for 10 min. Total $^{32}$P counts incorporated into the substrate were determined by Cerenkov counting on a Liquid Scintillation Analyzer Tri-Carb 2810 TR (PerkinElmer). Activities were calculated as nmol phosphate incorporated min$^{-1}$ mg$^{-1}$ CPK moiety$^{-1}$. $K_m$ and $k_{cat}$ were determined by curve fitting using SigmaPlot 11.0. $K_m$ and $k_{cat}$ were determined as the mean from two independent data curves for two independent preparations.

**Digestion and enrichment of proteins for mass spectrometry**

Proteins were digested using either Trypsin gold (Promega) or Endoproteinase GluC (New England Biolabs, UK) according to manufacturer’s instructions. Briefly, 10 mM dithiothreitol was added to the protein, which was incubated in 100 mM ammonium bicarbonate (pH 8) at 56 °C for 30 min. Samples were cooled to room temperature and cysteine residues alkylated by the addition of 50 mM iodoacetamide (50 µl) and incubated in the dark for 30 min. Digested samples were enriched for phosphopeptides using Titansphere™ Phos-TiO Kit (GL Sciences). Samples were dried down and re-suspended in 50 µL of buffer B, 25% lactic acid plus 75% buffer A (2% trifluoroacetic acid in 80% acetonitrile) and loaded onto prepared Spin Tips, centrifuged and rinsed in buffers B and A. Phosphopeptides were eluted in 5% ammonium hydroxide, dried and re-suspended in 2% trifluoroacetic acid for desalting. Samples were desalted using ZipTip$_{C18}$ (Merck Millipore, Germany) according to
manufacturer's instructions and peptides eluted with 10 µL of 50% acetonitrile/0.1%
trifluoroacetic acid, then dried down and re-suspended in 0.1% formic acid.

**LC-MS/MS**

Phosphorylated peptides enriched from the tryptic digest were concentrated and separated
using an UltiMate® 3000 nano HPLC series (Dionex, Sunnyvale, CA USA). Samples were
trapped on a µPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 µm, 100Å 300 µm i.d. x 5
mm (Dionex, Sunnyvale, CA USA) and separated in Nano Series™ Standard Columns 75
µm i.d. x 15 cm, packed with C18 PepMap100, 3 µm, 100Å (Dionex, Sunnyvale, CA USA)
using a 3.2% to 44% solvent B (0.1% formic acid in acetonitrile) gradient for 30 min.
Peptides were eluted directly (~ 350 nL.min⁻¹) via a TriVersa®NanoMate nanospray source
(Advion Biosciences) into the LTQ Velos with Orbitrap™ ETD mass spectrometer
(ThermoFisher Scientific). Data-dependent scanning acquisition was controlled by
Xcalibur™ 2.1 software (Thermo Fisher Scientific Inc).

Phosphopeptides were analysed by CID neutral loss triggered Electron Transfer Dissociation
(ETD). A full FT-MS scan (m/z 380 – 1600) and subsequent collision-induced dissociation
(CID) MS/MS scans of the 20 most abundant ions was performed. Survey scans were
acquired in the Orbitrap™ with a resolution of 30 000 at m/z 400 and automatic gain control
(AGC) 1x10⁶. Precursor ions were isolated and subjected to CID in the linear ion trap with
AGC 1x10⁵. Collision activation for the experiment was performed in the linear trap using
helium gas at normalized collision energy to precursor m/z of 35% and activation Q 0.25. If
neutral loss of 98 Da from the precursor ion was observed in the CID MS/MS mass
spectrum, ETD from the same precursor ion was triggered in the linear ion trap. Isolation
width was 2 m/z, AGC target 1 x 10⁵ and maximum inject time 50 ms. ETD was performed
with fluoranthene ions, which the AGC target for fluoranthene ions was 1 x 10⁶ (maximum fill
Precursor ions were activated for 130 ms and supplemental activation was performed with normalized collision energy of 25%.

CID MS/MS and ETD MS/MS data were searched against NCBI-nr Green Plant database using the SEQUEST algorithm (Thermo Scientific). Modifications searched for were deamidation (N and Q), oxidation (M) and phosphorylation on serine (S), threonine (T) and tyrosine (Y). Two missed cleavages were allowed. The precursor mass tolerance was 5 ppm and the MS/MS mass tolerance was 0.8 Da with FDR 1%. Phosphorylation sites identified in sPPases were collated from BLAST search analyses, using the p26a and p26b sequences, in publically available databases: www.p3db.org, www.phosphat.uni-hohenheim.de, www.phosida.com, www.phosphosite.org, www.UniProtKB.org, www.phosphgrid.org, www.phosphopep.org. Sequence alignments were performed using Clustal Omega.

Accession numbers: Papaver rhoeas calcium dependent protein kinase (CPK) gene sequences are available at the European Nucleotide Archive at http://www.ebi.ac.uk/ena/data/view/LT605077-LT605079. Accession nos: LT605077 (CPK14); LT605078 (CPK17/34); LT605079 (CPK6/26).
Supplemental data:

Fig S1. Amino acids phosphorylated by endogenous pollen kinases in p26 sPPases
Fig S2. General properties of p26a/b sPPases
Fig S3. Sequence homology between Papaver rhoeas and Arabidopsis thaliana CPKs
Fig S4. Auto-phosphorylation sites mapped in CPKs
Fig S5. Phosphorylation sites identified on sPPases
Table S1. Substrate specificity of p26 sPPases
Table S2. Location of phosphorylated residues identified in recombinant p26a & p26b
Table S3. Phosphomimic/phosphonull mutants of p26a and p26b and kinetic parameters of pyrophosphate hydrolysis by p26a and p26b and their phosphomimic/phosphonull mutants
Table S4. Kinetic assessment of recombinant CPK activity using syntide-2 (PLARTLSVAGLPGKK) as substrate and of p26 proteins as substrates for CPKs in vitro
Table S5. Mapping of phosphopeptides in recombinant PrCPKs auto-phosphorylated during in vitro phosphorylation assays using LC-MS/MS

Additional supplementary data (raw data) supporting this research is openly available from the University of Birmingham data archive at http://findit.bham.ac.uk/

Abbreviations: CPK: calcium dependent protein kinase; LC-MS/MS: liquid chromatographic tandem mass spectrometry; PPi: inorganic pyrophosphate; PCD: programmed cell death; sPPase: soluble pyrophosphatase

Figure Legends

Fig 1. Phosphorylation sites identified by LC-MS/MS in recombinant p26 phosphorlated in vitro by endogenous pollen kinases
Sequence alignment of the N-terminal regions of the p26 sPPase proteins annotated to indicate amino acid residues identified as phosphorylated by endogenous pollen kinases by LC-MS/MS. (A)-(F) show ETD mass spectra detected from p26a (A-C) and p26b (D-F) after phosphorylation using pollen extracts.

(A) Ser13 phosphorylation recorded on +2 ions at m/z 809.85; (B) Thr18 phosphorylation recorded on +3 ions at m/z 399.19; (C) Ser27 phosphorylation detected on +2 ions at m/z 428.22; (D) Thr25 phosphorylation detected on +2 ions at m/z 491.25; (E) Ser41 phosphorylation detected on +3 ions at m/z 575.92; (F) Ser51 phosphorylation detected on +2 ions at m/z 523.24

Fig 2. Phosphorylation of recombinant p26 by CPKs in vitro
Incorporation of $^{32}$P into p26 after in vitro kinase assays using recombinant AtCPK34, CKII or PKA with recombinant p26a (A) and p26b (B). Top panels: autoradiographs showing $^{32}$P incorporation (total pmoles); lower panel: western blots to show equal loading of protein.

(C-D) Incorporation of $^{32}$P into p26 during in vitro phosphorylation using PrCPK17/34, PrCPK14, PrCPK6/26, AtCPK34; mean ± SEM (n=3). (C) p26a, (D) p26b.

Fig 3. Phosphorylation sites identified by LC-MS/MS in p26 sPPase proteins phosphorylated by CPKs. Sequence alignment of the N-terminal regions of the p26 sPPase proteins annotated to indicate phosphorylations by individual recombinant kinases and whole pollen extract. – phosphorylated residues.

Fig 4. Effect of pH on PPase activities of p26a/b and their phosphonull and phosphomimic mutant versions. Recombinant p26 and mutant versions were assayed for PPase activity at variable pH; mean ± SEM (n=4). (A) p26a, (B) p26b.

Fig 5. Ca$^{2+}$ and H$_2$O$_2$ additively affect PPase activities of p26a/b and differentially affect their phosphonull and phosphomimic mutants. Recombinant p26 enzymes were assayed for PPase activity at pH 7.0 and supplemented with CaCl$_2$ and/or H$_2$O$_2$, mean ± SEM (n=3).

Fig 6. pH further reduces PPase activity in the presence of Ca$^{2+}$ and H$_2$O$_2$. Recombinant sPPase p26a (A) and p26b (B) enzymes assayed for PPase activity (diamonds) and in the presence of CaCl$_2$ (squares), H$_2$O$_2$ (circles) and CaCl$_2$ + H$_2$O$_2$ (triangles) at several pHs. Activity for each was reduced further by lowered pH. Mean ± SEM (n=5 for p26a and n=3 for p26b).


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