Self-incompatibility triggers irreversible oxidative modification of proteins in incompatible pollen

Haque, Tamanna; Eaves, Deborah; Lin, Zongcheng; Zampronio, Gleidiane; Cooper, Helen; Bosch, Maurice; Smirnoff, Nicholas; Franklin-Tong, Vernonica E.

Published in:
Plant Physiology

DOI:
10.1104/pp.20.00066

Publication date:
2020

Citation for published version (APA):
Haque, T., Eaves, D., Lin, Z., Zampronio, G., Cooper, H., Bosch, M., Smirnoff, N., & Franklin-Tong, V. E. (2020). Self-incompatibility triggers irreversible oxidative modification of proteins in incompatible pollen. *Plant Physiology*, *183*(2). https://doi.org/10.1104/pp.20.00066

Self-incompatibility triggers irreversible oxidative modification of proteins in incompatible pollen

Tamanna Haque[1,2], Deborah J. Eaves[1], Zongcheng Lin[1,3], Cleidiane G. Zampronio[1,4], Helen J. Cooper[1], Maurice Bosch[5], Nicholas Smirnoff [6*], and Vernonica E. Franklin-Tong[1*]

[1] School of Biosciences, College of Life and Environmental Sciences, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

[2] *Current address*: Department of Horticulture, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

[3] *Current address*: VIB Center for Plant Systems Biology, 9052 Ghent, Belgium

[4] *Current address*: School of Life Sciences, Gibbet Hill Road, University of Warwick, Coventry, CV4 7AL, UK.

[5] Institute of Biological, Environmental & Rural Sciences (IBERS), Aberystwyth University, Gogerddan, Aberystwyth, SY23 3EB, UK

[6] Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK.

*****Joint corresponding authors

Running title: **Mapping oxidative modifications in pollen triggered by SI**

Key words: irreversible oxidation, LC-MS/MS, mass spectrometry, nitrosylation, oxidative post-translational modifications (oxPTMs), *Papaver rhoeas,* pollen, self-incompatibility (SI)

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

Self-incompatibility (SI) is used by many angiosperms to prevent self-fertilization and inbreeding. In *Papaver rhoeas* interaction of cognate pollen and pistil *S*-determinants triggers programmed cell death (PCD) of incompatible pollen. We previously identified that reactive oxygen species (ROS) signals to SI-PCD. ROS induced oxidative post-translational modifications (oxPTMs) can regulate protein structure and function. Here we have identified and mapped oxPTMs triggered by SI in incompatible pollen. Notably, SI-induced pollen had numerous irreversible oxidative modifications; untreated pollen had virtually none. Our data provide the first analysis of the protein targets of ROS in the context of SI-induction and represent a milestone because currently there are few reports of irreversible oxPTMs in plants. Strikingly, cytoskeletal proteins and enzymes involved in energy metabolism are a prominent target. Oxidative modifications to a phosphomimic form of a pyrophosphatase result in a reduction of its activity. Therefore, our results demonstrate irreversible oxidation of pollen proteins during SI and show that this can affect protein function. We suggest that this reduction in cellular activity could lead to PCD.

Higher plants perform sexual reproduction using pollination, utilizing specific interactions between pollen (male) and pistil (female) tissues. Many angiosperms use self-incompatibility (SI) to prevent self-fertilization and inbreeding. These genetically controlled systems trigger rejection of “self” (incompatible) pollen. *Papaver rhoeas* uses a SI system involving the female *S*-determinant (PrsS) protein, a ligand secreted by the pistil (Foote et al., 1994) and the male *S*-determinant protein, PrpS(Wheeler et al., 2009). SI also triggers PCD, involving the activation of a DEVDase/caspase-3-like activity (Bosch and Franklin-Tong, 2007). A MAP kinase, p56, is involved in signalling to SI-PCD(Rudd, 2003; Li et al., 2007; Chai et al., 2017). The actin cytoskeleton is an early target of the SI signalling cascade in *Papaver* pollen (Geitmann et al., 2000; Snowman, 2002) beginning with actin depolymerization and formation of punctate F-actin foci (Geitmann et al., 2000; Snowman, 2002; Poulter et al., 2010). SI also triggers transient increases in reactive oxygen species (ROS) and nitric oxide (NO) (Wilkins et al., 2011). Live-cell imaging of ROS in growing *Papaver* pollen tubes, using chloromethyl- 2’7’-dichlorodihydrofluorescein oxidation, showed that SI induces relatively rapid and transient increases in ROS, as early as 2 min after SI in some incompatible pollen tubes. A link between SI-induced ROS and PCD was identified using ROS scavengers, which revealed alleviation of SI-induced events, including formation of actin punctate foci and the activation of a DEVDase/caspase-3-like activity(Wilkins et al., 2011). These data provided evidence that ROS increases are upstream of these key SI markers and are

63 required for SI-PCD(Wilkins et al., 2011) and represented the first steps in understanding
64 ROS signalling in this system.

65 Exactly how ROS mediate SI-induced events is an important question that needs to be
66 addressed. One possibility is that oxidative post-translational modifications to proteins
67 (oxPTMs) are involved. These include reversible modifications to cysteine (e.g. sulfenylation,
68 disulphide bonds, S-glutathionylation) and methionine (methionine sulfoxide) as well as a
69 range of irreversible oxPTMs (Møller et al., 2007). In the case of cysteine, reversible
70 oxPTMs mediate signalling or changes in protein function(Waszczak et al., 2014; Akter et
71 al., 2015a; Waszczak et al., 2015). NO produced during SI (Wilkins et al., 2011) also
72 provides the possibility of a role for cysteine S-nitrosylation. Although we had previously
73 identified ROS as a signal to SI-PCD(Wilkins et al., 2011), earlier studies did not extend to
74 identifying the protein targets of oxidation. Here investigations were aimed at identifying and
75 mapping oxPTMs on pollen proteins triggered by SI and H_2O_2 using LC tandem mass
76 spectrometry (LC-MS/MS). This is the first study to analyse the protein targets of ROS in the
77 context of SI-induction and identification and mapping of specific modifications. Our data
78 reveal that irreversible oxidation is likely to be an important mechanism involved in SI events
79 in incompatible *Papaver* pollen and provide the first link between irreversible oxPTMs and a
80 ROS-mediated physiological process.

81
82 ## RESULTS
83
84 ### SI causes oxidative modifications to proteins in incompatible pollen
85 As we had previously shown that ROS and NO increased during the SI response and played
86 a role in mediating actin alterations and PCD (Wilkins et al., 2011), we wished to examine
87 whether pollen proteins were oxidatively modified after SI. We used LC-MS/MS to examine
88 the extent and type of oxPTMs to pollen proteins during early SI, taking samples 12 min after
89 SI induction. We compared the SI response with H_2O_2 treatment to determine which of these
90 modifications were also induced by artificially generated oxidative stress. A number of
91 oxidative modifications were detected following both treatments (**Tables S1-S3**). SI pollen
92 proteins had far more oxPTMs than untreated pollen. We identified 181 uniquely modified
93 oxPTM peptides containing 251 different oxidatively modified amino acids in SI-induced
94 pollen (**Table S1**), while untreated pollen analysed in an identical manner side by side had
95 104 uniquely modified peptides with 110 different oxidatively modified amino acids (**Table**
96 **S2**). 262 unique oxPTMs were identified in H_2O_2-treated pollen (**Table S3**). Notably, proteins

which in control conditions contained methionine sulfoxide modification often showed increased oxidation to the sulfone form following SI induction and H_2O_2 treatment (**Table S1-S3**).

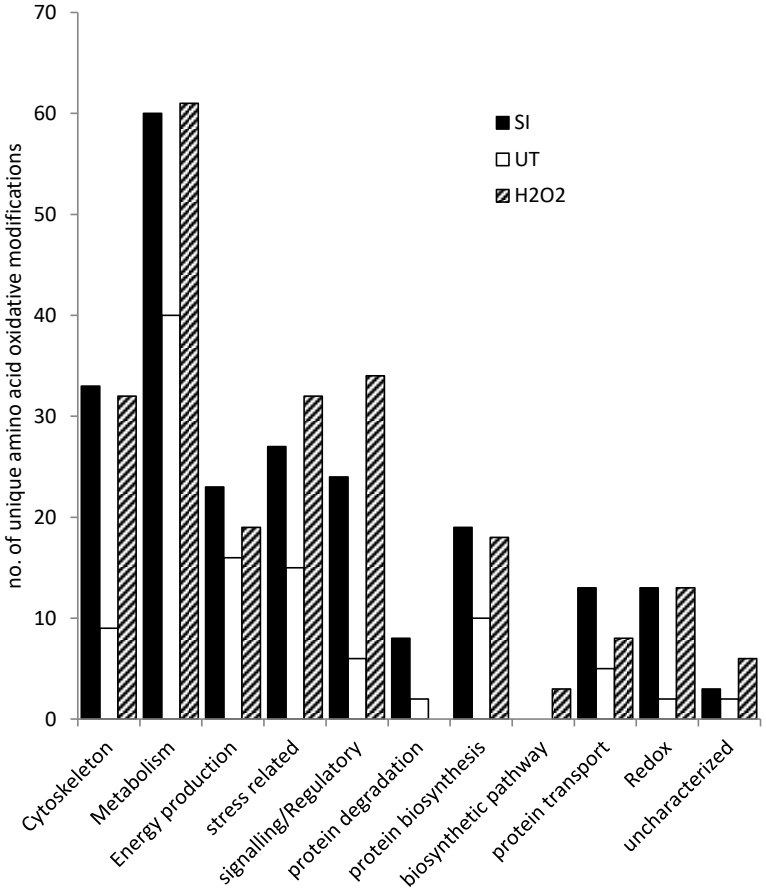

Figure 1. **Distribution of the number of unique oxidative modifications to amino acids on pollen proteins according to function after different treatments.** Each unique oxidatively modified amino acid was counted and categorized according to its function for each pollen treatment: SI induction (SI), H_2O_2 or untreated (UT).

Proteins with oxPTMs were categorised according to their general functions (**Figure 1**). For all of the functional groups, the SI samples had increased numbers of unique amino acids modified by oxidation compared to untreated pollen. The largest difference in numbers of oxidatively modified amino acids between SI-induced pollen and untreated pollen was found in the general functional grouping of cytoskeleton (33 vs 9), signalling/regulatory (24 vs 6), stress related (27 vs 15) and metabolism (60 vs 40), which together comprised 69% of the modified proteins in SI-induced pollen. However, even in functional groupings where fewer modifications were found in SI pollen proteins, proportionally the difference compared to

untreated pollen was large (e.g. for proteins involved in redox, SI had 13 differently modified amino acids, compared to 2 in untreated). The oxidatively modified proteins identified from the H_2O_2-treated pollen were also categorised based on their general functions. Like SI treatment, proteins involved in metabolism, signalling/regulation, stress and cytoskeleton comprised the majority (70%) of those with oxPTMs after H_2O_2 treatment (**Figure 1**). Although the frequency of oxPTMs in the dataset will be influenced by protein abundance, it is striking that cytoskeletal proteins and enzymes involved in energy metabolism represent a prominent target during the SI response (**Table S1, S3**). In relation to energy metabolism, a large proportion of enzymes associated with glycolysis (phosphoglucomutase, pyrophosphate-dependent phosphofructokinase, glyceraldehyde 3-P dehydrogenase, enolase, pyruvate kinase, inorganic pyrophosphatase), organic acid metabolism (aconitase, citrate synthase, citrate lyase, isocitrate dehydrogenase, malate dehydrogenase, phosphoenolpyruvate carboxylase) and ATP synthesis/use (ATP synthase, ATPases) have oxPTMs.

Proteins with oxPTM common to SI and H_2O_2 treatments

To gain a better idea of the overlap between SI and H_2O_2- treated samples, we identified peptides with identical oxPTMs in the SI-induced and the H_2O_2- treated samples, but not in untreated pollen (**Table 1, S1-S3**). 32 peptides shared 44 oxidatively modified amino acids, with identical modifications found in both SI-induced and H_2O_2 treated samples. This overlap gives confidence that the modifications triggered in incompatible pollen tubes are authentic ROS-mediated events and that these proteins are rapidly oxidatively modified by ROS formed during SI. There was no overlap between proteins/peptides with S-nitrosocysteine modifications in SI and H_2O_2 treated samples, suggesting that those modified during SI might be specific.

The proteins identified with identical oxPTMs after SI and H_2O_2 (**Table, 1**), suggest that some key common events are triggered. Actin and tubulin are shared targets, with 10 identical peptides containing 14 shared oxidatively modified amino acids. Other proteins known to be involved in tip growth, e.g. soluble inorganic pyrophosphatases, Rab GTPases and several elongation factor subunit peptides were also oxidatively modified in both SI and H_2O_2 treated pollen. These modified targets could contribute to inhibition of pollen tube growth. These data further suggest that protein synthesis and energy metabolism is altered by ROS during SI.

Types of oxidative modifications induced by SI and H_2O_2

Identifying the nature of the oxPTMs on individual proteins is an important step to understanding how cells interpret oxidative signals and translate them into a response. The types of oxidative modifications identified on peptides from SI-induced pollen proteins were

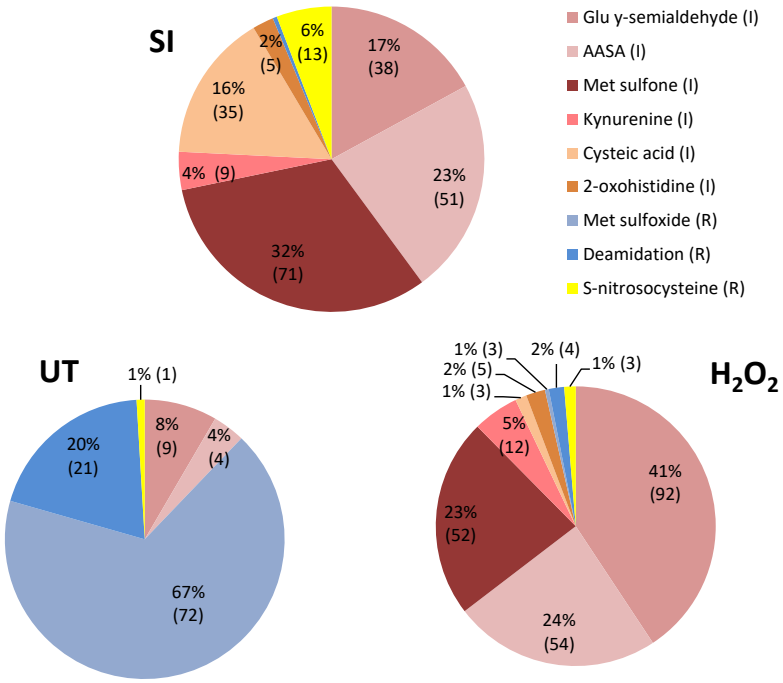

Figure 2. Distribution of types of oxidative modifications of pollen proteins after different treatments. Each unique oxidative modification identified on a unique peptide for each type of pollen treatment: SI induction (SI), H_2O_2 or untreated (UT) was categorized according to its type of modification and counted. These were represented proportionally in pie charts and are shown as a percentage of total counts, with the actual number of modifications identified in brackets.

quite different from those identified on peptides from untreated pollen (**Figure 2**). Notably, we found that the majority (94%) of the oxidatively modified amino acids in the SI sample were irreversibly modified (209/223), compared to only 13/107 (12%) in the untreated pollen sample. Irreversible modifications identified in SI-induced samples included 71 methionine to Met sulfone, 51 aminoadipic semialdehyde (AASA) on lysine, 38 proline to Glu γ-semialdehyde, and 35 cysteine to cysteic acid; other modifications were kyneurine on tryptophan (9) and 2-oxohistidine (5; **Figure 2**). Most of these modifications are to the highest level of oxidation and irreversible. Few reports of such irreversible oxidative modifications exist. Cysteines are generally irreversibly oxidized to cysteine sulfonic acid or cysteic acid in response to severe oxidative stress, which generally leads to protein

inactivation and degradation (Møller et al., 2007). Modification of lysine to aminoadipic semialdehyde (AASA) is a carbonylation modification which is the most common type of irreversible oxidative modification to a protein which generally inhibits the function of proteins. Together these data demonstrate that during early SI, many proteins are permanently modified.

In contrast, the majority of the oxPTMs on untreated pollen proteins were of a reversible nature (94 out of 107 modifications identified; **Figure 2**). These mainly comprised 72 methionines modified to Met sulfoxide. Untreated samples also had 21 deamidated amino acids. In contrast, the SI-induced pollen had no Met sulfoxide modifications; only one deamidation was identified. H_2O_2-treatment of pollen also resulted in a majority of irreversible oxidative modifications (**Figure 2**), with 218 irreversibly modified amino acids over 155 different peptides; the remaining 8 oxPTMs were reversible. Irreversible modifications identified in H_2O_2 treated samples included 92 proline to Glu γ-semialdehyde, 54 aminoadipic semialdehyde (AASA) on lysine and 52 methionine to Met sulfone.

Pollen proteins are modified by *S*-nitrosylation after SI

We previously showed that increases in NO were observed after SI-induction in incompatible pollen (Wilkins et al., 2011). NO, *via S*-nitrosoglutathione (GSNO) production could induce protein S-nitrosylation. Here we directly examined if SI stimulated *S*-nitrosylation by analysing protein extracts from pollen after SI induction using LC-MS/MS. First, we examined pollen protein extracts for S-nitrosylation using western blotting, treating germinated pollen with GSNO as a comparison. Pollen extracts were selectively labelled for proteins containing an S-nitrosylated cysteine using iodoTMTzero™, then visualised after western blotting using an anti-TMT antibody. Both SI-induced and GSNO-treated pollen had high levels of *S*-nitrosylation, whereas little staining of S-nitrosylated proteins was detectable in the untreated pollen (**Figure 3**). Addition of the reducing agent DTT during protein extraction resulted in the almost complete loss of staining, verifying that the staining was detecting oxidised proteins. Thus, SI treated pollen has more S-nitrosylated proteins than untreated pollen. LC-MS/MS identified 13 S-nitrosocysteine (CySNO) modifications in the SI-induced pollen samples (**Table S1, Fig 1**). In comparison, only one and three CySNO modified peptides were identified in untreated and H_2O_2 treated pollen, respectively. This provides good evidence for authentic S-nitrosylation of proteins triggered in pollen by SI.

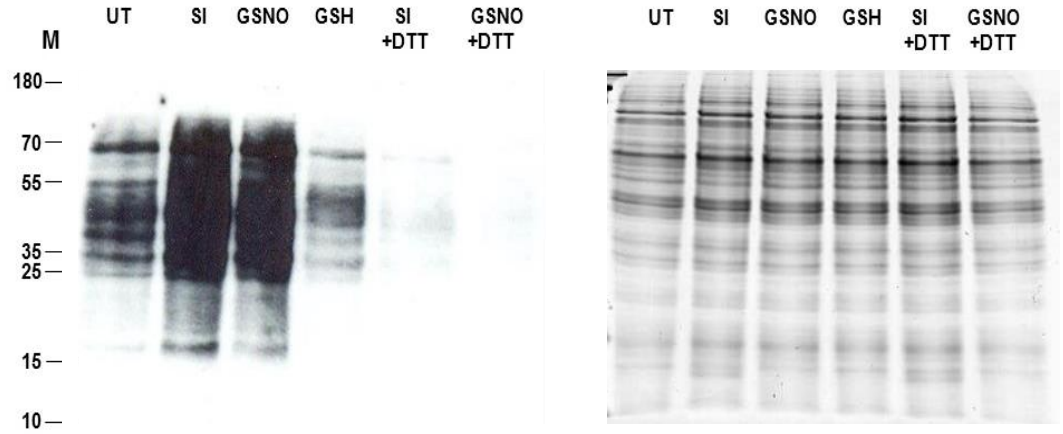

Figure 3. Detection of S-nitrosylated proteins from pollen tubes by Western blot analysis. Western blot of S-nitrosylated proteins detected with Pierce™ S-nitrosylation western blot kit. UT=Untreated sample, SI=SI-induced sample, GSNO=NO donor S-nitrosoglutathione, GSH= Reducing agent glutathione, SI+DTT=SI induced S-nitrosylated proteins were reduced by addition of DDT, GSNO+DTT= NO donor treated S-nitrosylated proteins reduced by addition of DTT. M= Molecular marker (kDa). Right-hand panel: coomassie blue staining of these S-nitrosylated proteins on SDS-PAGE showing equal loading of proteins.

Soluble inorganic pyrophosphatases are targets of ROS-mediated irreversible modification during SI & H_2O_2 treatment

Two proteins that were identified as having oxPTMs after SI-induction by LC-MS/MS were the soluble inorganic pyrophosphatases (sPPases) p26.1a/b (referred to here as p26a/p26b). These were previously identified as targets for SI-induced phosphorylation (Rudd et al., 1996; de Graaf et al., 2006; Eaves et al., 2017). Three oxidatively modified peptides from p26a, comprising 6 oxPTMs, and three from p26b, also comprising 6 oxPTMs, were identified in SI-induced pollen samples (**Table S1, Figure 4A**). Most of the modifications observed in the SI-induced pollen were irreversible; for p26a, Met129 was irreversibly modified to Met sulfone; Pro38 and Pro130 were both irreversibly modified to Glu γ-semialdehyde; Trp39 was modified to kynurenine, His40 to 2-oxohistidine, and Lys60 was modified to AASA. Five irreversible oxPTMs were identified on p26b (His37, 2-oxohistidine; Met150, met sulfone; Pro151, glu γ-semialdehyde; Lys202 and Lys217, AASA) and one reversible modification (Asp43, deamidation) (**Table S1, Figure 4A**). All the modifications identified in the SI-induced samples of p26a were identical to those identified in samples from H_2O_2-treated pollen, suggesting that they are authentic ROS-stimulated modifications.

In untreated pollen, only reversible Met sulfoxide oxPTMs were identified. These data provide good evidence for these p26 sPPases (which play a critical role in modulation of pollen tube growth) as a target of largely irreversible oxidation after SI-induction.

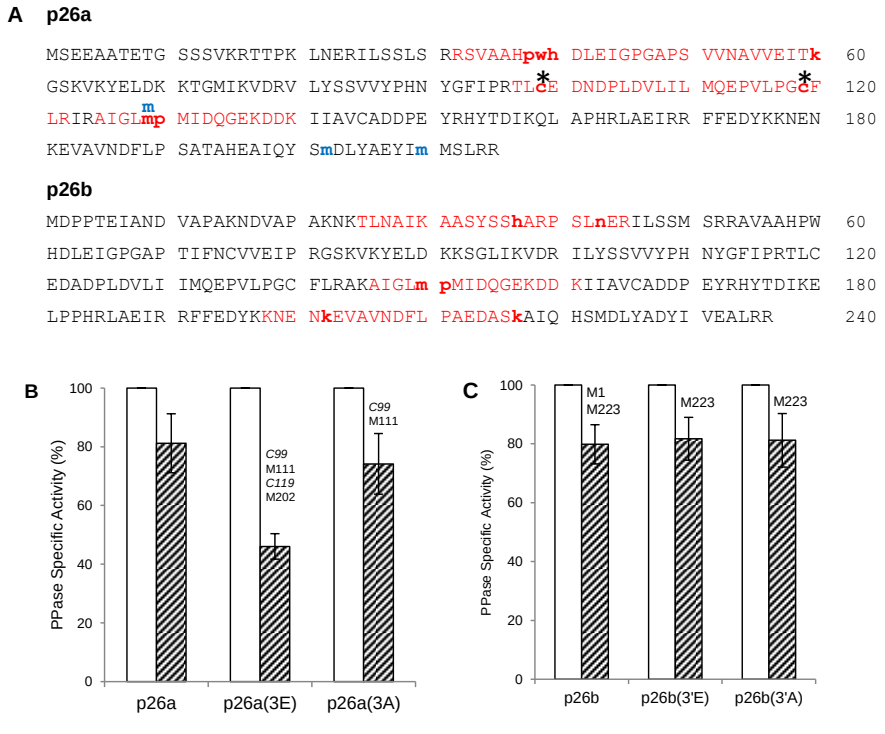

Figure 4. Oxidative modifications identified on the sPPase, Prp26.1a/b and alterations to PPase activity in the p26(3E) mutant recombinant protein. A. Sequence of the sPPase p26a and p26b from *Papaver rhoeas* showing the peptides identified from pollen after SI induction (red), with the oxidatively modified amino acids in bold (small letters); notably all 8 were also identified in H_2O_2-treated samples. Modifications indicated in blue were found in untreated samples. B. PPase activities in recombinant p26a/b and its phosphomimic/null (3E/A) mutant proteins after treatment with H_2O_2. Recombinant p26 enzymes were assayed for PPase activity at pH7.2 (white bars) and supplemented with H_2O_2 (hatched bars). Values for PPase activity are mean $\pm$ SE ($n \geq 3$). The oxidative modifications identified on each of these proteins are indicated above the bars.

We examined the possible effects of ROS on p26a/b further, to see if PPase activity might be affected. We had previously made triple phosphomimic mutant recombinant proteins [p26a(3E) and p26b(3'E)], which mimic the three sites phosphorylated during SI and their corresponding phosphonull mutants [p26a(3A) and p26b(3'A)]. These phosphomimic mutant proteins exhibited significantly reduced PPase activity in the presence of Ca^{2+} and/or H_2O_2 (Eaves et al., 2017). We treated recombinant p26a/b proteins and their mutant forms with H_2O_2 and then analysed them for both PPase activity and oxPTMs using LC-MS/MS. The phosphomimic recombinant p26a(3E) protein had reduced PPase activity and contained two

unique irreversible oxidative modifications on Cys119 (cysteic acid) and Met202 (met sulfone) that were not found in p26a or the phosphonull p26a(3A) treated with H_2O_2 (**Table 2**, **Figure 4B**). Two further irreversible oxPTMs were identified (cysteic acid on Cys99 and S-nitrosocysteine on Met111) which were also present on the phosphonull mutant p26a(3A) protein and did not have significantly different PPase activity from the phosphomimic (3E). However, it is plausible that these, when modified in combination with the other oxidised amino acids, Cys119 and Met202, may alter function, as Cys99 and Cys119 are adjacent to the active site (Cooperman et al., 1992). Moreover, Cys119 is also additionally modified by nitrosylation. The phosphomimic protein p26a(3E) was much more sensitive to H_2O_2 than the wild-type enzyme, displaying significantly lower PPase activity (P = 0.0064; **Figure 4B**). In contrast, the phosphonull recombinant p26a(3A) protein did not have significantly different PPase activity from p26a (P = 0.650; **Figure 4B**). Irreversible oxidative modifications were also found on the p26b recombinant protein (Met1 and Met223, met sulfone), but no significant alteration in PPase activity was detected in the phosphomimic mutant p26b(3'E) compared to that exhibited by p26b and p26b(3'A) after treatment with H_2O_2 (NS, P = 0.852 and 0.966 respectively; (**Figure 4B**)), so these also are unlikely to be involved in modulating PPase activity. These data suggest that the oxidative modifications on the phosphomimic p26a(3E) protein contribute to the reduction in PPase activity.

Cytoskeletal proteins are oxidatively modified after SI-induction

We identified thirty unique oxidatively modified cytoskeletal protein peptides with 36 different oxidative modifications after SI-induction compared to eight peptides with 9 different oxPTMs identified in untreated pollen. Notably, these peptides from the SI-induced pollen contained many more irreversible modifications (31/39, **Table S1**) than untreated pollen (1/8, **Table S2**). It is of interest that the H_2O_2-treated pollen contained 13 identically modified amino acids on actin and tubulin as the SI-induced pollen (**Table 1**). These data confirm that SI induces a similar ROS response as H_2O_2 treatment, suggesting these are authentic ROS-mediated events. In addition, three actin binding proteins (ABPs; one profilin and two fimbrins), identified by 6 different modified peptides containing 8 irreversibly modified oxPTMs, were found in the SI induced sample (**Table S1**). Modification of profilin might alter its affinity for binding to actin filaments or could affect its actin sequestering property. Similarly, modifications to fimbrin could potentially affect its binding to actin and consequently affect actin filament bundling. Thus, oxPTMs to these proteins could potentially impact on the organization of the actin cytoskeleton in incompatible pollen. Although

previous studies showed that the actin cytoskeleton is a target for ROS signals(Wilkins et al., 2011), these studies were indirect, using ROS scavengers, and we had not previously shown a direct link between increases in H_2O_2 and formation of actin punctate foci. Having identified many oxPTMs on actin in the current study, we examined whether addition of H_2O_2 might trigger alterations to pollen tube F-actin configuration.

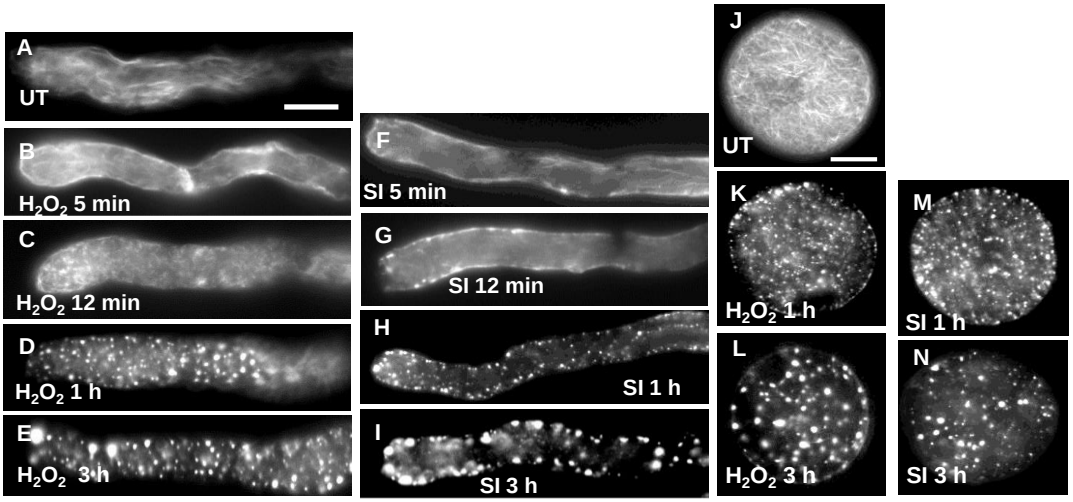

Figure 5. F-actin alterations in pollen induced by H_2O_2 in *Papaver* pollen tubes. F-actin was visualized with rhodamine-phalloidin using fluorescence microscopy. (A) F-actin organization in a representative untreated pollen tube, (B-C) H_2O_2 treated pollen tubes after 5 min, 12 min, 1 h and 3 h of treatment. Alterations were observed as early as 5 min after treatment. At 1 and 3h large punctate foci of actin were formed. (F-I) Pollen tubes at 5 min, 12 min, 1 h and 3 h after SI-induction showed similar alterations to F-actin. (J-N) Pollen grains showed similar alterations. (J) Untreated pollen grain with F-actin filament bundles (K-L) H_2O_2 treated pollen grains and (M-N) Scale bar = 10 µm.

H_2O_2 stimulates the formation of actin foci in pollen tubes

We treated pollen tubes with either H_2O_2 or recombinant PrsS to induce SI and used rhodamine phalloidin staining to observe the alterations in F-actin configuration. In the untreated pollen tubes (**Figure 5A**), F-actin filament bundles were visible. Pollen tubes treated with H_2O_2 displayed alterations to the F-actin organization as early as 5 min (**Figure 5B, C**); the typical F-actin filament bundles were significantly reduced, even at 5 and 12 min

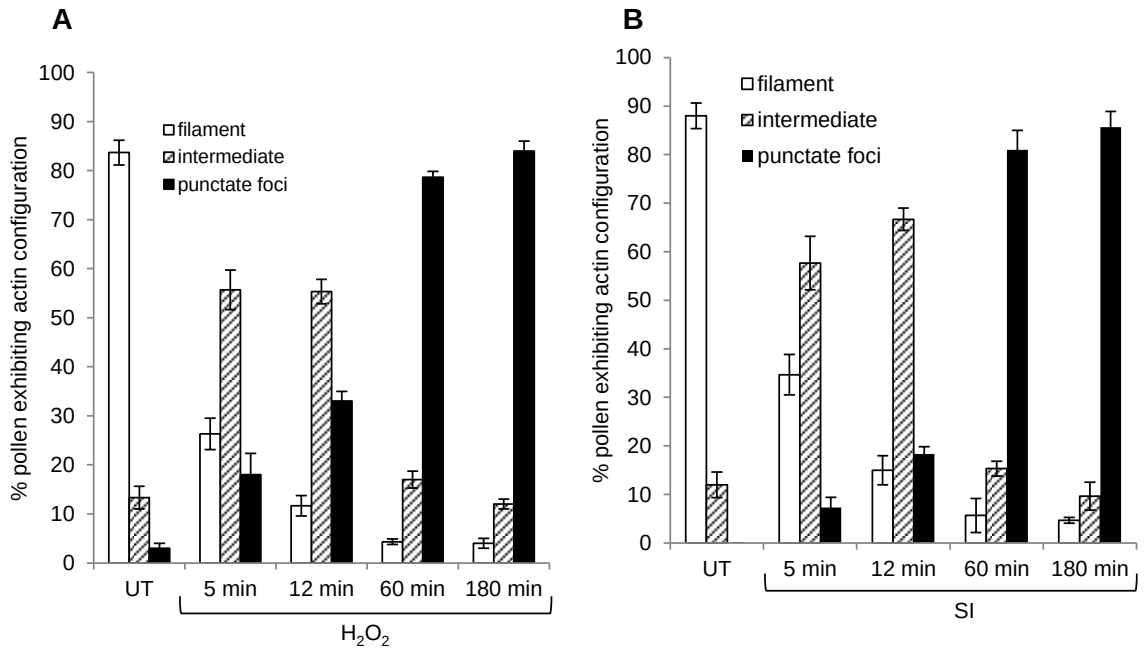

Figure 6. Quantitation of actin alterations stimulated by ROS and H_2O_2 in *Papaver* pollen. Pollen tubes were treated with SI induction or H_2O_2 and samples were fixed at different time points after treatment. F-actin was stained with rhodamine-phalloidin and examined using fluorescence microscopy. The actin configuration was evaluated by placing each pollen tubes into one of the three categories: according to Snowman et al (2002): Actin filaments only (Black bars), foci only (grey bars) or intermediate (i.e. filaments and foci; open bars). Three independent experiments scoring 100 pollen tubes for each treatment expressed as percentage of total. Data are mean ± SEM (n=100).

treatments (p=0.010*, (**Figure 6A**). After 1h small F-actin foci were present (**Figure 5D**) and large punctate foci were observed after 3h of treatment (**Figure 5E**); after 1-3h of treatment ~80% of pollen tubes contained punctate actin foci (**Figure 6A**). These alterations triggered by H_2O_2 appear very similar to those in SI induced pollen previously observed(Geitmann et al., 2000; Snowman, 2002; Poulter et al., 2010) (**Figure 5F-I, 6B**). Non-germinated pollen grains showed a similar response as the pollen tubes (**Fig. 5J-N**), showing that these can also respond to ROS. Our data show that ROS can stimulate major changes in actin configuration in pollen that are strikingly similar to those observed during SI. Together with the identification of oxPTMs to actin and associated proteins, this provides further evidence for the involvement of ROS in the formation of SI-stimulated F-actin punctate foci.

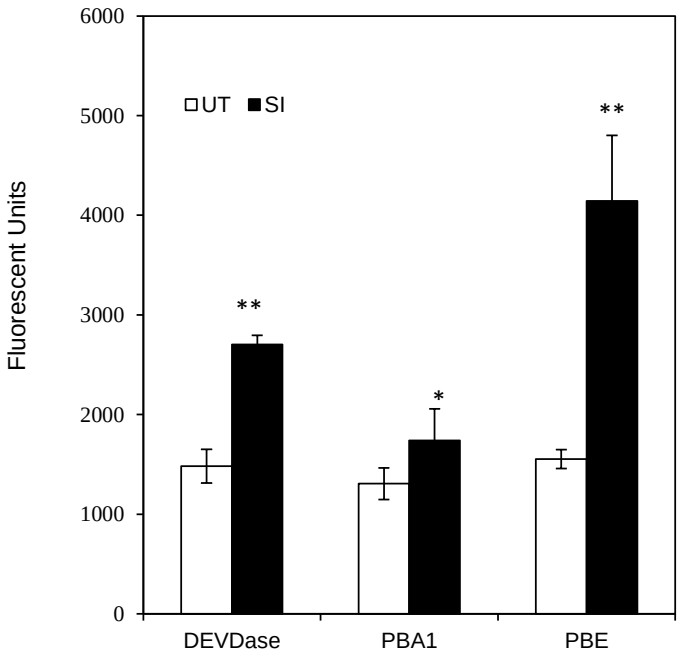

Figure 7. Measurement of various protease activities after SI in *Papaver* pollen extracts The 20S proteasomal activities in poppy SI response were measured using fluorogenic peptide substrates in pollen extracts 5h after SI induction (SI) or in untreated (UT) controls. DEVDase activity was measured as control. Significant increases of DEVDase, PBA1 and PBE activities were observed in the SI extracts. The actual values of DEVDase, PBA1 and PBE activities are not comparable, because different probes were used. Mean ±SD, n=4. *, $p<0.05$; **, $p<0.01$.

Increased 20S proteasomal activity is observed after SI

Irreversible oxidation damages proteins. As the 20S proteasome is implicated in removing oxidatively damaged proteins during apoptosis/PCD(Aiken et al., 2011), we investigated whether increased proteasomal activity might be triggered by SI. We characterized the activities of 20S proteasome β subunits β5 (PBE) and PBA1, during the SI-PCD response, using fluorogenic probes Z-GGL-amc and Ac-nLPnLD-amc as substrates. In the early phase (1h) of the SI response, there were no statistically significant changes in PBA1 and PBE activities (data not shown). However, later (5 h after SI), significant increases in both PBA1 and PBE activities were detected (**Figure 7**). This provides evidence that the 20S proteasome is activated by SI and could be involved in removal of irreversibly oxidised proteins in incompatible pollen.

DISCUSSION

Previously, we showed that SI-induced ROS and NO production are required for pollen tube PCD (Wilkins et al., 2011) but the mechanism was not determined. Both ROS and NO can modify proteins and we now show that the SI response involves rapid formation of many irreversible oxPTMs and provide evidence that this is linked to altered protein function. Critically, the pattern of oxPTM formation induced by SI overlaps with those induced by exogenous H_2O_2 and happens sufficiently rapidly (within 12 minutes) to strongly suggest it is not a consequence of PCD. Irreversible modifications found were Glu y-semialdehyde (from proline and arginine), aminoadipic acid (AASA from lysine), Met sulfone, Kynurenine (from tryptophan), Cysteic acid and 2-oxohistidine. Few reports of rapid irreversible oxidative modifications exist in plants. Moreover, little is known about the functional consequences of these irreversible oxPTMs(Møller et al., 2007; Rinalducci et al., 2008; Jacques et al., 2013; Jacques et al., 2015). The reversible modifications methionine sulfoxide(Jacques et al., 2013) and S-nitrosocysteine(Astier et al., 2011) were also detected but not sulfenylated cysteines, possibly because our method did not protect these reactive groups during extraction. Protein sulfenylation has been detected in plants following H_2O_2 treatment by trapping these groups(Waszczak et al., 2014; Akter et al., 2015b; Waszczak et al., 2015). While cysteine sulfenylation and S-nitrosocysteine formation have been implicated as mediators of H_2O_2 (Smirnoff and Arnaud, 2018) and NO(Astier et al., 2011) signalling, the extent to which irreversible oxPTMs represent damage or have a functional significance is less well understood. Our results provide the first evidence that rapid production of irreversible oxPTMs is involved in a physiological response in plants, rather than representing longer-term oxidative damage.

Irreversible modification of proteins is likely to inhibit function and they can be marked for proteolysis by the proteasome(Grune et al., 1996; Berlett and Stadtman, 1997). Irreversible protein oxidation is particularly detrimental in the cell, as this can render damaged proteins inactive or lead to functional abnormalities. Studies have implicated the 20S proteasome as important for the removal of damaged proteins, as (at least in animal cells) it is more resistant to oxidative stress than the 26S proteasome, maintaining activity even after treatment with moderate to high concentrations of H_2O_2 (Reinheckel et al., 1998; Aiken et al., 2011; Pajares et al., 2015). Moreover, 20S proteasomes can degrade oxidized proteins *in vitro*, independent of ubiquitin/ATP(Aiken et al., 2011). We measured a significant increase in 20S proteasomal activity in SI-induced poppy pollen. This is not inconsistent with the idea

385 that protein damage is triggered by SI and that the 20S proteasome may be recruited to
386 degrade oxidatively damaged proteins during the SI response.
387
388 It is striking that cytoskeletal proteins and enzymes involved in energy metabolism respond
389 prominently during the SI response. In relation to energy metabolism, a large proportion of
390 enzymes associated with glycolysis, organic acid metabolism and ATP synthesis/use have
391 oxPTMs. In animal cells one of the principle targets of protein oxidation is metabolism;
392 evidence suggests that oxidation of a few metabolic enzymes, especially those involved in
393 glycolysis, can dramatically affect the cellular energy status, thereby rapidly inducing cellular
394 dysfunction with a limited number of protein oxidation events. GAPDH is one of the best
395 examples of oxidation of a metabolic enzyme having direct control over apoptosis in animal
396 cells(Cecarini et al., 2007; Sirover, 2012; Villa and Ricci, 2016). In yeast, oxidative stress
397 inactivates GAPDH, enolase and aconitase(Cabiscol et al., 2000). Modulation of metabolism
398 resulting in inhibition of glycolysis leads to cell death *via* ROS-mediated cell death in
399 plants(Kunz et al., 2014). Thus, it is well established that inhibition of glycolysis leads to cell
400 death. It is noteworthy that cytosolic GAPDH from Arabidopsis was identified as a major
401 H_2O_2-oxidised protein; reversible cysteine oxidation resulted in inhibition of its activity
402 (Hancock et al., 2005; Yang and Zhai, 2017). In plants, there is increasing evidence
403 supporting the idea that plant cytoplasmic GAPDH has alternative, non-metabolic
404 “moonlighting” functions triggered by oxPTMs of the protein under stress conditions
405 (Zaffagnini et al., 2013). A study using Arabidopsis GAPDH knockout lines displayed
406 accelerated PCD in response to effector-triggered immunity(Henry et al., 2015). Our data
407 provide a mechanistic link between SI, which triggers PCD, and possible protein targets of
408 irreversible oxidation that could result in destruction of metabolism. In animal cells there is
409 good evidence that during apoptosis the loss of energy production contributes to the
410 dismantling of the cell. A decrease in ATP content during apoptosis has been shown to be
411 dependent on inhibition of glycolysis, leading to the impairment in the activity of two
412 glycolysis-limiting enzymes, phosphofructokinase and pyruvate kinase, (Pradelli et al.,
413 2014). While there is currently limited evidence that the oxPTMs modifications observed
414 here specifically cause SI-mediated PCD, the literature suggests that this may be the case
415 and this possibility should be investigated in future studies.
416
417 The soluble inorganic pyrophosphatase (sPPase, p26a/b) provides an example of an
418 enzyme involved in the SI response(de Graaf et al., 2006; Eaves et al., 2017) that is a target

of SI-ROS oxidation, displaying several oxPTMs within a few minutes of SI induction. Previously, we showed that p26a/b were phosphorylated following SI and this reduces PPase enzyme activity(de Graaf et al., 2006); phosphorylation together with Ca^{2+}, ROS and low pH further inhibited PPase activity(Eaves et al., 2017). Here we show that H_2O_2 treatment of the mutant recombinant enzyme p26a(3E) resulted in a reduction in PPase activity. Thus, the phosphomimic amino acid substitutions on this enzyme contribute to an increased susceptibility to oxidative modification, resulting in a reduction in PPase activity *in vitro*. Some of the oxidized residues (Met111 and Asp138) are located in regions of the protein that could potentially interfere with the enzyme's catalytic properties, based on 3D structures of *E. coli* sPPase(Cooperman et al., 1992). The irreversible modification of cysteine residues (Cys99 and Cys119) either side of conserved active site residues could affect function. sPPases are enzymes that hydrolyse inorganic pyrophosphate (PPi) to provide the driving force for many metabolic reactions. PPi is generated during biopolymer synthesis and hydrolysed to inorganic phosphate (2Pi); this reaction provides a thermodynamic pull favouring biosynthesis(Kornberg, 1962). In a biological context, phosphorylation of p26a during SI *in vivo* is rapidly followed by an increase in ROS; this oxidative modification could further reduce PPase activity, which will result in lowering of ATP levels and further impact on cellular energetics. Thus, our data provide insights into a novel mechanism whereby PPase activity can be inhibited. Here we not only show that ROS can contribute to SI by inhibiting a crucial enzyme for biosynthesis, but this provides a significant advance by providing an example of ROS modifying an enzyme to affect its activity. This finding could have implications for many biological systems that involve biosynthesis.

We show that cytoskeletal proteins (both actin and tubulin) and the ABPs fimbrin and profilin, are targets of extensive irreversible oxidative modifications. Methionine residues in actin are commonly oxidised to the irreversible sulfone form, while oxidation of actin methionines has been reported previously(Dalle-Donne et al., 2001). Moreover, oxidation of key cysteine residues of actin results in cell death in yeast(Farah et al., 2007). The actin cytoskeleton plays an essential role in pollen tube growth(Gibbon et al., 1999; Vidali et al., 2001), and is implicated in mediating apoptosis in yeast. In yeast, during acute oxidative stress, F-actin forms oxidized actin bodies (OABs) that sequester actin into immobile, non-dynamic structures that regulate the oxidative stress response, playing a pivotal protective role in the decision whether to enter apoptosis(Farah et al., 2011). These OABs appear similar to the

highly stable F-actin foci that we observed in SI(Geitmann et al., 2000; Snowman, 2002; Poulter et al., 2010) and H_2O_2-treated pollen (Wilkins et al., 2011). We previously demonstrated that SI-induced ROS and NO production was required for the formation of these distinctive actin structures, which were concomitant with initiation of PCD (Wilkins et al., 2011). Here we show that H_2O_2 induces the formation of actin foci. In yeast, it is well established that a decrease in actin dynamics and accumulation of aggregates of stabilized F-actin can induce 'actin mediated apoptosis' (ActMAp) involving ROS-mediated apoptosis(Gourlay et al., 2004). The apparent underlying similarities in actin involvement in plant PCD have been commented upon (Franklin-Tong and Gourlay, 2008) and the current study reinforces this idea. Together, these data suggest that the oxidation of cytoskeletal proteins observed here may play a key role in SI-PCD in pollen. The role of oxidation in cytoskeletal function in plant cells requires further investigation. Clearly the cytoskeleton and its associated proteins are an important target during SI and we have shown for the first time that several are oxidatively modified. These modifications may affect cytoskeletal dynamics, as several irreversible modifications occur in the binding domain of actin which would restrict actin or ABPs to bind with actin and thus might alter actin dynamics.

We identified several *S*-nitrosylated proteins in the SI-induced pollen samples. The majority of NO affected proteins appear to be modified by *S*-nitrosylation of the thiol group of a single cysteine residue. To date, around 20 different *S*-nitrosylated proteins have been characterized in detail in plants and most of them have been reviewed recently with regard to their functional significance in NO signaling(Astier et al., 2011; Lamotte et al., 2015). The identified proteins from plant proteome-wide studies have been shown to take part in major cellular activities, notably primary and secondary metabolism, photosynthesis, protein folding, cellular architecture, and stress responses (Astier et al., 2011). It is thought that NO signalling in plants uses *S*-nitrosylation of cysteine residues of redox-sensitive proteins (Wang et al., 2006; Moreau et al., 2010), which can affect protein activity, and so has the potential to be important in regulating cellular events (Lindermayr et al., 2005; Couturier et al., 2013). The phosphomimic mutant recombinant protein p26a(3E) sPPase was not only irreversibly oxidised on Cys119 to cysteic acid but was also nitrosylated on this site. As this modified protein had significantly reduced PPase activity, it suggests oxidation may play a role.

In conclusion, we have shown that oxidation is an important mechanism triggered by the SI

response in *Papaver* pollen. Here we have shown that the SI response results in rapid and extensive oxidation of pollen proteins. Strikingly, many of these oxPTMs are irreversible. We provide evidence for increased proteasomal activation, which is consistent with the idea that following inactivation, oxidised proteins may be removed by the 20S proteasome. The observed oxidative modifications particularly impact enzymes associated with energy production and the cytoskeleton. In some cases (GAPDH and sPPase here) there is evidence that such irreversible modifications inhibit critical core metabolic enzyme activity. These modifications could therefore contribute to the very rapid growth inhibition and PCD following induction of SI. We also show that actin is a target for extensive irreversible oxidation and that oxidation stimulates the formation of stable actin foci in pollen. Actin dynamics have previously been implicated in the decision whether to enter PCD and this study further suggests that this is the case. Together, our data demonstrate irreversible oxidation of key pollen proteins and suggest that this triggers a catastrophic reduction in cellular activity that could lead to PCD.

MATERIALS AND METHODS

Pollen tube growth, SI-induction and other treatments

Papaver rhoeas pollen was hydrated then grown *in vitro* in liquid germination medium (GM) [0.01% H_3BO_3, 0.01% KNO_3, 0.01% $Mg(NO_3)_2.6H_2O$, 0.036% $CaCl_2$-$2H_2O$, and 13.5% Sucrose] at 25°C for 1 h (Snowman, 2002). SI was induced by adding incompatible recombinant S proteins (final concentration 10 μg mL^{-1}) as described previously (Snowman, 2002). Samples were taken at 12 min after SI-induction. For each SI-induced sample, a non-induced control was prepared by adding only GM to the pollen. For H_2O_2 treatments, germinated pollen tubes were treated with H_2O_2 (2.5 mM) for 12 min, as this was when ROS increases were detected in incompatible pollen tubes (Wilkins et al., 2011). Pollen was harvested by centrifuging, resuspended in HEN buffer (250 mM HEPES/pH7.7, 1 mM EDTA, 0.1mM neocuproine), homogenised on ice and clarified by centrifugation. The protein content of the supernatant was determined using by Bradford assay (Bradford, 1976), which was stored at -20ºC until required.

To generate S-nitrosylated proteins for western blots, germinated pollen was treated with 500 µM NO donor *S*-nitrosoglutathione (GSNO) for ~30 min. Proteins (60 µg) were extracted as described above except Trypsin digests were performed without DTT. Peptides were

adjusted to 3 µg.µL^{-1} in HEN buffer. Thiols were blocked using 0.2 % *S*-methyl methane thiosulfonate (MMTS) and 2.5% SDS and proteins peptides incubated for 20 min at 50°C then removed using Spin 6 columns (BioRad) and equilibrated in HEN buffer according to manufacturer's instructions.

Detection of *S*-nitrosylation of proteins by western blot

Protein extracts were prepared as described above and separated by SDS PAGE. Proteins containing *S*-nitrosylated cysteine were selectively labelled using iodoTMTzero™. S-nitrosylated proteins were visualised by western blotting using anti-TMT antibody using a Pierce™ S-nitrosylation Western Blot Kit according to the manufacturer's instructions. 50 mM DTT (dithiothreitol) was added to controls during protein extraction.

Trypsin digestion

Sample pollen proteins (60 µg) were run into SDS-PAGE and gel plugs containing the proteins were digested using Trypsin Gold (Promega) according to manufacturer's instructions. 10 mM DTT in 100 mM ammonium bicarbonate (pH 8) was added to the protein and incubated for 30 min at 56°C. Samples were cooled to room temperature and alkylated with 50 mM iodoacetamide in the dark for 30 min. Tryptic peptides were analysed for oxidative modifications by mass spectrometry.

Sample desalting for mass spectrometry

Samples were desalted using ZipTip$_{C18}$ (Merck Millipore, Germany). Tips were pre-wet in 100% acetonitrile and rinsed in 2x10 µL 0.1% trifluoroacetic acid. Samples were loaded according to manufacturer's instructions. ZipTip were washed with 0.1% trifluoroacetic acid (3x10 µL) to remove excess salts. Peptides were eluted with 10 µL of 50% acetonitrile/0.1% trifluoroacetic acid. Samples were dried down to remove the acetonitrile, and re-suspended in 0.1% formic acid solution. Chemicals were from Sigma (Gillingham, Dorset, UK), Fisher Scientific (Loughborough, Leicestershire, UK) and J.T. Baker (Philipsburg, NJ).

LC-MS/MS peptide analysis

UltiMate® 3000 nano HPLC series (Dionex, Sunnyvale, CA USA) was used for peptide concentration and separation. Samples were trapped on µPrecolumn Cartridge, Acclaim PepMap 100 C18, 5 µm, 100Å 300µm i.d. x 5mm (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, NY) into the LTQ Velos with Orbitrap™ ETD mass spectrometer (ThermoFisher Scientific, Germany). The data-dependent scanning acquisition was controlled by Xcalibur™ 2.1 software (Thermo Fisher Scientific Inc. USA). The mass spectrometer alternated between a full FT-MS scan (m/z 380 – 1600) and subsequent collision-induced dissociation (CID) MS/MS scans of the 20 most abundant ions. Survey scans were acquired in the Orbitrap™ with a resolution of 30 000 at m/z 400 and automatic gain control (AGC) $1x10^6$. Precursor ions were isolated and subjected to CID in the linear ion trap with AGC $1x10^5$. 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. The width of the precursor isolation window was 2 m/z and only multiply-charged precursor ions were selected for MS/MS.

Identification of modified peptides and criteria for identifying modified proteins

Oxidation modifications of tryptic peptides from CID MS/MS mass spectra were analysed against the NCBInr Green Plant database using the SEQUEST algorithm (Thermo Scientific). As a complete and annotated genome sequence for *Papaver rhoeas* is not currently available, identifications were limited to peptides identical to those found in this database or the few sequences of *P. rhoeas* submitted to EMBL (European Molecular Biology laboratory). Two missed cleavages were allowed and were accepted as real hit proteins with at least two high confidence peptides. The precursor mass tolerance 5 ppm, MS/MS mass tolerance 0.8 Da and FDR 1% were used. The criteria for 'real hit proteins' were accepted as those containing at least two high confidence peptides. Peptides were analysed to identify irreversible and reversible oxidative modifications to amino acids.

For the counts we disregarded the carbamidomethyl modifications as these are an artefact of iodoacetamide treatment. [However they are shown in the Supplemental Tables for clarity]. We also counted the number of unique amino acid modifications to oxidatively modified peptides and grouped these according to protein function using the PANTHER classification system; https://www.ncbi.nlm.nih.gov/pubmed/27899595 (Mi et al., 2017). Where a particular ID (GI number) was not in the PANTHER database, we based the protein identify and protein class according to its classification in NCBI, either directly (the same ID) or through the identification of similar proteins by BLAST searches and/or the PANTHER

protein class for a very similar protein. The remaining proteins were labelled “unclassified”, but were placed in a functional class if this was obvious from the protein identified.

p26 analysis and PPase assays

Recombinant His-tagged p26 sPPase proteins (p26.1a and p26.1b) and their triple substitution phospho-mutant versions: phosphomimic with a glutamic acid [E] substitution (p26a/b(3E) and the corresponding phosphonull with an alanine [A] substitution (p26a/b(3A) were for p26a [S13E, T18E and S27E, named p26a(3E)] and p26b [T25E, S41E and S51E, named p26b(3’E)]. They were prepared as described previously (Eaves et al., 2017). The p26 protein was diluted to 10 µM in 50 mM Hepes-KOH, pH 8.0, 50 µM EGTA, 2 mM $MgCl_2$. 250 ng aliquots were assayed for free phosphate production using a discontinuous PPase assay and 2 mM sodium pyrophosphate as substrate (Fiske, 1925); n>3 for each assay. The assay buffer was supplemented with 10 mM H_2O_2 as appropriate. Duplicate assay samples were sent for LC-MS/MS analysis.

Poppy pollen protein extractions for proteasome and caspase assays

Papaver pollen was collected and snap-frozen in liquid nitrogen. Proteins extracts were prepared by grinding pollen using a glass homogenizer in proteasome assay buffer [50 mM Tris-HCl, pH=7.5; 5 mM $MgCl_2$; 250 mM sucrose; 1 mM DTT; 0.05 mg mL^{-1} bovine serum albumin (BSA)]. ATP was freshly added to the buffer to a final concentration of 5 mM before use(Kisselev and Goldberg, 2005). Lysates were sonicated at 10 000 amp for 2×5 s, incubated on ice for 20 min and centrifuged at 13,200 rpm at 4^{o}C for 20 min. The supernatant was collected and protein concentration was determined by the Bradford assay. Protein extracts were aliquoted and stored at -20 ^{o}C for use in the proteasome activity assays. Protein samples for caspase activity assay were extracted using caspase extraction buffer (50 mM Na-Acetate; 10 mM L-Cysteine; 10% (v/v) Glycerol; 0.1% (w/v) CHAPS; pH=6.0)(Bosch and Franklin-Tong, 2007).

Proteasome and caspase activity assays using fluorogenic peptide substrates

Each activity assay (100 µL) contained 10 µg protein lysates and 100 µM of either Z-GGL-amc or Ac-nLPnLD-amc as fluorogenic probes for PBE and PBA1 (both 20S proteasome subunits) activity measurements respectively. Fluorescence was monitored with the excitation at 380 nm and emission at 460 nm every 10 mins over a period of 4 h using a time-resolved fluorescence plate reader (FLUOstar OPTIMA; BMG LABTECH).

Caspase activity was assayed in caspase activity assay buffer (50 mM Na-Acetate; 10 mM L-Cysteine; 10% (v/v) Glycerol; 0.1% (w/v) CHAPS; pH=5.0). Each activity assay (100 µL) contained 10 µg protein lysates and 100 µM fluorogenic probes Ac-DEVD-amc. Caspase activity was monitored in the plate reader as described(Bosch and Franklin-Tong, 2007).

REFERENCES

Aiken CT, Kaake RM, Wang X, Huang L (2011) Oxidative Stress-Mediated Regulation of Proteasome Complexes. Molecular & Cellular Proteomics **10:** R110.006924

Akter S, Huang J, Bodra N, De Smet B, Wahni K, Rombaut D, Pauwels J, Gevaert K, Carroll K, Van Breusegem F, Messens J (2015a) DYn-2 Based Identification of Arabidopsis Sulfenomes. Mol Cell Proteomics **14:** 1183-1200

Akter S, Huang J, Waszczak C, Jacques S, Gevaert K, Van Breusegem F, Messens J (2015b) Cysteines under ROS attack in plants: a proteomics view. Journal of Experimental Botany **66:** 2935-2944

Astier J, Rasul S, Koen E, Manzoor H, Besson-Bard A, Lamotte O, Jeandroz S, Durner J, Lindermayr C, Wendehenne D (2011) S-nitrosylation: An emerging post-translational protein modification in plants. Plant Science **181:** 527-533

Berlett BS, Stadtman ER (1997) Protein Oxidation in Aging, Disease, and Oxidative Stress. Journal of Biological Chemistry **272:** 20313-20316

Bosch M, Franklin-Tong VE (2007) Temporal and spatial activation of caspase-like enzymes induced by self-incompatibility in Papaver pollen. Proceedings of the National Academy of Sciences USA **104:** 18327-18332

Cabiscol E, Piulats E, Echave P, Herrero E, Ros J (2000) Oxidative stress promotes specific protein damage in Saccharomyces cerevisiae. Journal of Biological Chemistry

Cecarini V, Gee J, Fioretti E, Amici M, Angeletti M, Eleuteri AM, Keller JN (2007) Protein oxidation and cellular homeostasis: Emphasis on metabolism. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research **1773:** 93-104

Chai L, L Tudor R, S Poulter N, Wilkins K, Eaves D, Franklin C, E Franklin-Tong V (2017) MAP Kinase PrMPK9-1 contributes to the Self-Incompatibility Response, Vol 174

Cooperman BS, Baykov AA, Lahti R (1992) Evolutionary conservation of the active site of soluble inorganic pyrophosphatase. Trends in Biochemical Sciences **17:** 262-266

Couturier J, Chibani K, Jacquot J-P, Rouhier N (2013) Cysteine–based redox regulation and signaling in plants. Frontiers in Plant Science **4:** 105

Dalle-Donne I, Rossi R, Milzani A, Di Simplicio P, Colombo R (2001) The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radical Biology and Medicine **31:** 1624-1632

de Graaf BHJ, Rudd JJ, Wheeler MJ, Perry RM, Bell EM, Osman K, Franklin FCH, Franklin-Tong VE (2006) Self-incompatibility in *Papaver* targets soluble inorganic pyrophosphatases in pollen. Nature **444:** 490-493

Eaves DJ, Haque T, Tudor RL, Barron Y, Zampronio CG, Cotton NPJ, de Graaf BHJ, White SA, Cooper HJ, Franklin FCH, Harper JF, Franklin-Tong VE (2017) Identification of phosphorylation sites altering pollen soluble inorganic pyrophosphatase activity. Plant Physiology **173:** 1606-1616

Farah ME, Amberg DC, Boone C (2007) Conserved Actin Cysteine Residues Are Oxidative Stress

Sensors That Can Regulate Cell Death in Yeast. Molecular Biology of the Cell **18:** 1359-1365
Farah ME, Sirotkin V, Haarer B, Kakhniashvili D, Amberg DC (2011) Diverse protective roles of the actin cytoskeleton during oxidative stress. Cytoskeleton **68:** 340-354
Fiske CH, Subbarow, Y. (1925) The colorimetric determination of phophorous. Journal of Biological Chemistry **66**
Foote HCC, Ride JP, Franklintong VE, Walker EA, Lawrence MJ, Franklin FCH (1994) Cloning and Expression of a Distinctive Class of Self- Incompatibility (*S*) Gene from *Papaver rhoeas* L. Proceedings of the National Academy of Sciences of the United States of America **91:** 2265-2269
Franklin-Tong VE, Gourlay CW (2008) A role for actin in regulating apoptosis/programmed cell death: evidence spanning yeast, plants and animals. Biochemical Journal **413:** 389-404
Geitmann A, Snowman BN, Emons AMC, Franklin-Tong VE (2000) Alterations in the actin cytoskeleton of pollen tubes are induced by the self-incompatibility reaction in *Papaver rhoeas*. Plant Cell **12:** 1239-1251
Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. The Plant Cell Online **11:** 2349-2363
Gourlay CW, Carpp LN, Timpson P, Winder SJ, Ayscough KR (2004) A role for the actin cytoskeleton in cell death and aging in yeast. The Journal of Cell Biology **164:** 803-809
Grune T, Reinheckel T, Davies KJ (1996) Degradation of oxidized proteins in K562 human hematopoietic cells by proteasome. J Biol Chem **271:** 15504-15509
Hancock JT, Henson D, Nyirenda M, Desikan R, Harrison J, Lewis M, Hughes J, Neill SJ (2005) Proteomic identification of glyceraldehyde 3-phosphate dehydrogenase as an inhibitory target of hydrogen peroxide in Arabidopsis. Plant Physiology and Biochemistry **43:** 828-835
Henry E, Fung N, Liu J, Drakakaki G, Coaker G (2015) Beyond Glycolysis: GAPDHs Are Multi-functional Enzymes Involved in Regulation of ROS, Autophagy, and Plant Immune Responses. PLOS Genetics
Jacques S, Ghesquière B, De Bock P-J, Demol H, Wahni K, Willems P, Messens J, Van Breusegem F, Gevaert K (2015) Protein methionine sulfoxide dynamics in *Arabidopsis thaliana* under oxidative stress. Molecular & Cellular Proteomics **14:** 1217-1229
Jacques S, Ghesquière B, Van Breusegem F, Gevaert K (2013) Plant proteins under oxidative attack. PROTEOMICS **13:** 932-940
Kisselev AF, Goldberg AL (2005) Monitoring Activity and Inhibition of 26S Proteasomes with Fluorogenic Peptide Substrates. *In* Methods in Enzymology, Vol 398. Academic Press, pp 364-378
Kornberg A (1962) On the Metabolic Significance of Phosphorolytic and Pyrophosphorolytic Reactions Academic Press, New York
Kunz S, Pesquet E, Kleczkowski L (2014) Functional Dissection of Sugar Signals Affecting Gene Expression in Arabidopsis thaliana. . PLoS ONE **9:** e100312
Lamotte O, Bertoldo JB, Besson-Bard A, Rosnoblet C, Aimé S, Hichami S, Terenzi H, Wendehenne D (2015) Protein S-nitrosylation: specificity and identification strategies in plants. Frontiers in Chemistry **2**
Li S, Samaj J, Franklin-Tong VE (2007) A Mitogen-Activated Protein Kinase Signals to Programmed Cell Death Induced by Self-Incompatibility in Papaver Pollen. Plant Physiol. **145:** 236-245
Lindermayr C, Saalbach G, Durner J (2005) Proteomic identification of S-Nitrosylated proteins in *Arabidopsis*. Plant Physiol. **137:** 921-930
Mi H, Huang X, Muruganujan A, Tang H, Mills C, Kang D, Thomas PD (2017) PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements. Nucleic acids research **45:** D183-D189

Møller IM, Jensen PE, Hansson A (2007) Oxidative Modifications to Cellular Components in Plants. Annual Review of Plant Biology **58:** 459-481

Moreau M, Lindermayr C, Durner J, Klessig DF (2010) NO synthesis and signaling in plants–where do we stand? Physiologia Plantarum **138:** 372-383

Pajares M, Jiménez-Moreno N, Dias IHK, Debelec B, Vucetic M, Fladmark KE, Basaga H, Ribaric S, Milisav I, Cuadrado A (2015) Redox control of protein degradation. Redox Biology **6:** 409-420

Poulter NS, Staiger CJ, Rappoport JZ, Franklin-Tong VE (2010) Actin-binding proteins implicated in formation of the punctate actin foci stimulated by the self-incompatibility response in Papaver. Plant Physiology **10.1104:** pp.109.152066

Pradelli LA, Villa E, Zunino B, Marchetti S, Ricci JE (2014) Glucose metabolism is inhibited by caspases upon the induction of apoptosis. Cell Death & Disease **5:** e1406

Reinheckel T, Sitte N, Ullrich O, Kuckelkorn U, Davies KJ, Grune T (1998) Comparative resistance of the 20S and 26S proteasome to oxidative stress. The Biochemical journal **335 (Pt 3):** 637-642

Rinalducci S, Murgiano L, Zolla L (2008) Redox proteomics: basic principles and future perspectives for the detection of protein oxidation in plants. Journal of Experimental Botany **59:** 3781-3801

Rudd JJ, Franklin FCH, Lord JM, FranklinTong VE (1996) Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*. Plant Cell **8:** 713-724

Rudd JJ, Osman, K., Franklin, F. C. H., Franklin-Tong V. E. (2003) Activation of a putative MAP kinase in pollen is stimulated by the self-incompatibility (SI) response. FEBS Letters **547:** 223-227

Sirover MA (2012) Subcellular dynamics of multifunctional protein regulation: Mechanisms of GAPDH intracellular translocation. Journal of Cellular Biochemistry **113:** 2193-2200

Smirnoff N, Arnaud D (2018) Hydrogen peroxide metabolism and functions in plants. New Phytol **221:** 3

Snowman BN, Kovar, D.R., Shevchenko, G., Franklin-Tong, V.E., and Staiger, C.J. (2002) Signal-mediated depolymerization of actin in pollen during the self-incompatibility response. Plant Cell **14:** 2613-2626

Vidali L, McKenna ST, Hepler PK (2001) Actin polymerization is essential for pollen tube growth. Molecular biology of the cell **12:** 2534-2545

Villa E, Ricci J-E (2016) How does metabolism affect cell death in cancer? The FEBS Journal **283:** 2653-2660

Wang Y, Yun B-W, Kwon E, Hong JK, Yoon J, Loake GJ (2006) S-nitrosylation: an emerging redox-based post-translational modification in plants. Journal of experimental botany **57:** 1777-1784

Waszczak C, Akter S, Eeckhout D, Persiau G, Wahni K, Bodra N, Van Molle I, De Smet B, Vertommen D, Gevaert K, De Jaeger G, Van Montagu M, Messens J, Van Breusegem F (2014) Sulfenome mining in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **111:** 11545-11550

Waszczak C, Akter S, Jacques S, Huang J, Messens J, Van Breusegem F (2015) Oxidative post-translational modifications of cysteine residues in plant signal transduction. Journal of Experimental Botany **66:** 2923-2934

Wheeler MJ, de Graaf BHJ, Hadjiosif N, Perry RM, Poulter NS, Osman K, Vatovec S, Harper A, Franklin FCH, Franklin-Tong VE (2009) Identification of the pollen self-incompatibility determinant in *Papaver rhoeas* Nature **459:** 992-995

Wilkins KA, Bancroft J, Bosch M, Ings J, Smirnoff N, Franklin-Tong VE (2011) ROS and NO mediate

actin reorganization and programmed cell death in the Self-Incompatibility response of Papaver. Plant Physiology

Yang SS, Zhai QH (2017) Cytosolic GAPDH: a key mediator in redox signal transduction in plants. Biologia Plantarum **61:** 417-426

Zaffagnini M, Fermani S, Costa A, Lemaire S, Trost P (2013) Plant cytoplasmic GAPDH: redox post-translational modifications and moonlighting properties. Frontiers in Plant Science **4**

Acknowledgements

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). The Biotechnology and Biological Sciences Research Council (BBSRC) provided funding for research to NS (BB/I020004/1 and BB/N001311/1) and MB & NF-T (BB/P005489/1). This projected was funded by BBSRC grant BB/G003149/1. TH was funded by a Commonwealth PhD studentship. ZL was funded by a PhD studentship from the China Scholarship Council (C.S.C.).

Supplementary data supporting this research is openly available from the University of Birmingham data archive at http://findit.bham.ac.uk/.

Author contributions: VEFT, DJE, TH and ZL designed the research; TH, DJE and ZL, performed research; HJC contributed mass spectrometry expertise, reagents and analytic tools; DJE, TH, VEF-T, MB, NS and CGZ analysed data; NS, VEFT and MB wrote the paper.

Competing interests The authors declare no conflict of interest.

Materials & Correspondence

Correspondence: VEF-T: School of Biosciences, College of Life and Environmental Sciences, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK. Email: v.e.franklin-tong@bham.ac.uk and **NS**: Biosciences, College of Life and Environmental Sciences, University of Exeter, Exeter EX4 4QD, UK. Email: N.Smirnoff@exeter.ac.uk

Materials: MB: Institute of Biological, Environmental & Rural Sciences (IBERS), Aberystwyth University, Gogerddan, Aberystwyth, SY23 3EB, UK

Tables

Table 1. Overlap between oxidatively modified peptides in SI-induced and H_2O_2 treated pollen. Peptides containing the same oxidatively modified amino acids in both SI and H_2O_2 treated pollen are listed in column 3. Peptides identified in the H_2O_2 treated pollen are listed in column 4; those highlighted in grey have additional oxidative modifications. Modified amino acids are indicated by small bold letters, with the type of oxidative modification indicated by superscript numbers as follows: [1]Glu y-semialdehyde (I), [2]AASA (I), [3]Met sulfone (I), [4]Kynurenine (I), [5]Cysteic acid (I), [6]2-oxohistidine (I), [7]Met sulfoxide (R), [8]Carbamidomethyl (R) produced by reaction with iodoacetamide during sample preparation, [9]Deamidation (R), [10]S-nitrosocysteine (R). Most proteins were identified using PANTHER, except a few that were "unclassified" in PANTHER (indicated by *) and identified using a BLAST search.

Protein	protein class	Modified peptide in SI pollen	Modified peptide in H_2O_2 pollen
Actin	cytoskeleton	DLYGNIVLSGGSTMF**p**[1]GIADR	DLYGNIVLSGGSTMF**p**[1]GIADR
Actin		EITALAPSS**m**[3]K	EITALAPSS**m**[3]K
Actin		YPIEHGIVSNWDD**m**[3]EK	YPIEHGIVSNWDD**m**[3]EK
Actin		YPIEHGIVTN**w**[4]DDMEK	YPIEHGIVTN**w**[4]DD**m**[3]EK
Actin		DLYGNIVLSGGTT**m**[3]FPGIADR	DLYGNIVLSGGTT**m**[3]FPGIADR
Alpha-tubulin		**k**[2]LADN**c**[8]TGLQGFLVFNAVGGGTGSGLGSLLLER	**k**[2]LADN**c**[8]TGLQGFLVFNAVGGGTGSGLGSLLLER
Alpha-tubulin		TIQFVDW**c**[4]PTGFK	TIQFVDW**c**[4]PTGF**k**[2]
Beta tubulin		GHYTEGAELIDSVLDVVR**k**[2]	GHYTEGAELIDSVLDVVR**k**[2]
Beta tubulin		NSSYFVE**w**[4]I**p**[1]NNV**k**[2]	NSSYFVE**w**[4]I**p**[1]NNV**k**[2]
Beta tubulin		**m**[3]MLTFSVFPSPK	**m**[3]MLTFSVFPSPK
GAPDH	metabolism	VALQRDDVELVAVNDPFITTDYMTYMF**k**[2]	VALQRDDVELVAVNDPFITTDYMTYMF**k**[2]
GAPDH		DA**p**[1]MFVVGVNEK	DA**p**[1]MFVVGVNEK
sPPase		AIGL**m**[3]**p**[1]MIDQGEKDDK	AIGL**m**[3]**p**[1]MIDQGEKDDK
sPPase		RSVAAH**p**[1]**w**[4]**h**[6]DLEIGPGAPSVVNAVVEIT**k**[2]	RSVAAH**p**[1]**w**[4]**h**[6]DLEIGPGAPSVVNAVVEIT**k**[2]
Enolase		KYGQDATNVGDEGGFAPNIQEN**k**[2]EGLELLK	KYGQDATNVGDEGGFAPNIQEN**k**[2]EGLELLK
Enolase		SFVSDYPIVSIEDPFDQDD**w**[4]E**h**[6]YS**k**[2]	SFVSDYPIVSIEDPFDQDD**w**[4]E**h**[6]YS**k**[2]
HSP70	stress	NQVAMN**p**[1]INTVFDAK	NQVAMN**p**[1]INTVFDAK
Elongation Factor 2*	Signalling/regulatory	GVQYLNEIKDSVVAGFQWAS**k**[2]	GVQYLNEIKDSVVAGFQWAS**k**[2]
Elongation Factor 2*		GVQYLNEIKDSVVAGFQ**w**[4]As**k**[2]	GVQYLNEIKDSVVAGFQ**w**[4]AS**k**[2]
Predicted EF2-like		N**c**[8]DPDGPL**m**[3]LYVSK	N**c**[8]DPDGPL**m**[3]LYVSK
Predicted EF2-like		LYMEAR**p**[1]LEDGLAEAIDDGR	LYMEAR**p**[1]LEDGLAEAIDDGR
Eukaryotic initiation factor 4		VQVGVFSATM**p**[1]PEALEITR	VQVGVFSATM**p**[1]PEALEITR
RAB GTPase		LLLIGDSGVG**k**[2]	LLLIGDSGVG**k**[2]
RAB GTPase		FADDSYLESYISTIGVDF**k**[2]	FADDSYLESYISTIGVDF**k**[2]
RAB GDP dissociation inhibitor		NDYYGGESTSLNLIQLW**k**[2]	NDYYGGESTSLNLIQLW**k**[2]
14-3-3-like protein		QAFDEAISELDTLGEESY**k**[2]DSTLI**m**[3]QLLR	QAFDEAISELDTLGEESY**k**[2]DSTLI**m**[3]QLLR
Methionine synthase	Protein bio-synthesis	**k**[2]LNLPILPTTTIGSFPQTIELR	**k**[2]LNLPILPTTTIGSFPQTIELR
Methionine synthase		GMLTG**p**[1]VTILNWSFVR	GMLTG**p**[1]VTILNWSFVR
Serine hydroxyl methyl-transferase*		GIELIASENFTSFAVIEALGSALTN**k**[2]	GIELIASENFTSFAVIEALGSALTN**k**[2]
Serine hydroxyl methyl-transferase*		IMGLDL**p**[1]SGGHLTHGYYTSGG**k**[2]	IMGLDL**p**[1]SGGHLTHGYYTSGG**k**[2]
SKS (SKU5 similar)	Redox	YALNGVSHTD**p**[1]ETPLK	YALNGVSHTD**p**[1]ETPLKSGKGDGSDA**p**[1]LFTLK**p**[1]GK
2-oxoacid dehydrogenase acyltransferase		RTPVSGPKG**k**[2]PQALQV**k**[2]	RTPVSGPKG**k**[2]PQALQV**k**[2]

Table 2. Oxidative modifications identified by LC/LC MS on the recombinant sPPase proteins p26a and p26b after H_2O_2 treatment. Oxidative modifications identified on the recombinant proteins p26.1a/b and their phosphomimic mutants p26a(3E) and p26b(3E) and their phosphonull mutants p26a(3A) and p26b(3A) without and after H_2O_2 treatment. Irreversible (I), or reversible (R).

p26a	**untreated**	**H_2O_2 treated**		
Residue	**p26a**	**p26a**	**p26a(3E)**	**p26a(3A)**
C99	-	-	Cysteic acid (I)	Cysteic acid (I)
M111	-	-	Met sulfone (I)	Met sufoxide (R) Met sulfone (I)
C119	-	-	Cysteic acid (I) Nitrosyl (R)	-
M129	Met sufoxide (R)	Met sufoxide (R)	Met sufoxide (R)	Met sufoxide (R)
M131	-	-	Met sufoxide (R)	Met sufoxide (R)
C145	-	-	-	Sulfinic acid
M202	-	-	Met sufoxide (R) Met sulfone (I)	Met sufoxide (R)
M210	Met sufoxide (R)	-	Met sufoxide (R)	Met sufoxide (R)
M211	Met sufoxide (R)	-	Met sufoxide (R)	Met sufoxide (R)

p26b	**untreated**	**H_2O_2 treated**		
Residue	**p26b**	**p26b**	**p26b(3'E)**	**p26b(3'A)**
M1	Met sufoxide (R)	Met sufoxide (R)	Met sufoxide (R) Met sulfone (I)	Met sufoxide (R)
M150	-	Met sufoxide (R)	Met sufoxide (R)	Met sufoxide (R)
M152	-	-	Met sufoxide (R)	-
M223	Met sufoxide (R) -	Met sufoxide (R) Met sulfone (I)	Met sufoxide (R) Met sulfone (I)	Met sufoxide (R) Met sulfone (I)

Supplemental data

Table S1. Oxidative modifications of pollen proteins after SI induction

Table S2. Oxidative modifications found in proteins from untreated pollen

Table S3. Oxidative modifications of pollen proteins after treatment with H_2O_2

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