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Self-incompatibility in *Papaver* Pollen: Programmed Cell Death in an Acidic Environment

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Abstract

Self-Incompatibility (SI) is a genetically controlled mechanism that prevents self-fertilisation and thus encourages outbreeding and genetic diversity. During pollination, most SI systems utilise cell-cell recognition to reject incompatible pollen. Mechanistically, one of the best-studied SI systems is that of *Papaver rhoeas* (poppy), which involves the interaction between the two *S*-determinants, a stigma-expressed secreted protein (PrsS) and a pollen-expressed plasma-membrane localised protein (PrpS). This interaction is the critical step in determining acceptance of compatible pollen or rejection of incompatible pollen. Cognate PrpS-PrsS interaction triggers a signalling network causing rapid growth arrest and eventually programmed cell death (PCD) in incompatible pollen. In this review, we provide an overview of recent advances in our understanding of the major components involved in the SI-induced PCD (SI-PCD). In particular, we focus on the importance of SI-induced intracellular acidification and consequences for protein function, and the regulation of soluble inorganic pyrophosphatase (Pr-p26.1) activity by post-translational modification. We also discuss attempts at the identification of protease(s) involved in the SI-PCD process. Finally, we outline future opportunities made possible by the functional transfer of the *P. rhoeas* SI system to Arabidopsis.

Keywords:

Acidification, Arabidopsis, caspase-like activity, *Papaver rhoeas*, pH, pollen, programmed cell death (PCD), proteases, self-incompatibility (SI), signalling

Abbreviations:

$[Ca^{2+}]_{cyt}$	Cytosolic free calcium
$[pH]_{cyt}$	Cytosolic pH
ABPs	Actin-binding proteins
ADF	Actin-depolymerising factor
CAP	Cyclase-associated protein
F-actin	Filamentous actin
GFP	Green fluorescent protein
PBA1	Proteasome β subunit 1
PCD	Programmed cell death

PrpS	*Papaver rhoeas* pollen S
PrsS	*Papaver rhoeas* stigma S
PrVPE1	*Papaver rhoeas* vacuolar processing enzyme1
ROS	Reactive oxygen species
SI	Self-incompatibility or Self-incompatible
SI-PCD	Self-incompatibility induced programmed cell death
sPPases	Soluble inorganic pyrophosphatases
UPS	Ubiquitin-proteasome system
VPEs	Vacuolar processing enzymes

Introduction

Programmed Cell Death (PCD) is essential for a range of developmental and defence-related processes in plants. Processes associated with the plant reproductive life cycle in flowering plants display a particularly rich collection of tightly controlled and executed PCD. This includes cell death-related events on the male side in the tapetum associated with microsporogenesis and male fertility, and in pollen tubes triggered by the self-incompatibility (SI) response in *Papaver rhoeas*. On the female side PCD is triggered in the embryo sac to ensure proper embryo development and in seeds to ensure their proper development and germination (Wu and Cheung, 2000; Domínguez and Cejudo, 2014; Van Hautegem *et al.*, 2015). Despite the importance of PCD events for the reproductive success of plants, many of the underlying components and processes remain to be elucidated.

The interaction between the pollen and the pistil is one of the most important steps in the reproductive process of flowering plants, involving cell-cell recognition and signalling events (Dresselhaus and Franklin-Tong, 2013). Following penetration of the stigma, pollen tubes grow through the style towards the ovule, delivering the sperm cells to the female gametophyte. The communication and coordination between the pollen and the pistil establishes the limits of inbreeding and outbreeding of a species (Swanson *et al.*, 2004). An estimated 40-50% of flowering plant species have developed a genetically controlled SI mechanism that prevents self-fertilisation and thus encourages outbreeding and genetic diversity (Darlington and Mather, 1949; Igic *et al*., 2008). For this reason, SI has made a significant contribution to the evolutionary success of flowering plants. During pollination, SI generally utilises cell-cell recognition to prevent self-fertilisation by rejection of "self" (incompatible) pollen. In many SI systems, this involves inhibition of pollen tube growth. In *P. rhoeas*, which represents one of the best understood SI systems at a mechanistic level, rapid growth arrest of incompatible pollen is followed by PCD.

The stigma of the *Papaver* pistil secretes a small polymorphic protein (PrsS) which acts as a signalling ligand. With a "self" pollination, PrsS interacts specifically with pollen expressing the male *S*-determinant, the plasma membrane-localised PrpS. A bioassay in which the SI response can be triggered in *in vitro* growing *Papaver* pollen tubes by the addition of recombinant PrsS proteins (Franklin-Tong *et al*., 1988; Foote *et al*., 1994) has allowed analysis of events triggered in incompatible pollen grains and tubes. Depending on the

combination of *S*-haplotypes used, either an incompatible/SI response or a compatible situation can be achieved. Analysis can be carried out on either individual pollen tubes (fixed or live), using microscopy (e.g. Thomas and Franklin-Tong, 2006; Poulter *et al*., 2010; Wilkins *et al*., 2011), or on a larger scale, making extracts for biochemical or proteomic analysis (Rudd et al., 1996); see Franklin-Tong (2008) for more detail. This bioassay has been fundamental to achieve our current understanding of the mechanisms involved in *Papaver* SI. A cognate interaction between PrpS and PrsS triggers a signalling network in incompatible pollen, starting with an almost immediate increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) in incompatible pollen (Franklin-Tong *et al*., 1993, 1995, 1997), followed by transient increases in reactive oxygen species (ROS) and nitric oxide (Wilkins *et al*., 2011). These processes exhibit distinct temporal "signatures". After SI induction, the cytoskeleton is rapidly depolymerised, and F-actin reorganises to form stable "punctate foci" that increase in size (Geitmann *et al*., 2000; Snowman *et al*., 2002; Poulter *et al*., 2010). These alterations in actin dynamics are integral to the network leading to PCD (Thomas *et al*., 2006). Cytoplasmic acidification is a more recently identified regulator of developmental PCD (Fendyrch *et al*., 2014) and SI in *Papaver* induces a substantial and rapid decrease of the cytosolic pH ($[pH]_{cyt}$) in incompatible pollen tubes. The acidification is both necessary and sufficient for triggering several key hallmark features of the SI-PCD signalling network, including formation of punctate actin foci and generation of DEVDase/caspase-3-like activity (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). Other early targets of SI-induced signalling in incompatible pollen include the mitogen-activated protein kinase (MAPK) p56 (Rudd *et al*., 2003; Li *et al*., 2007) and soluble inorganic pyrophosphatase, Pr-p26.1 (Rudd *et al*., 1996; de Graaf *et al*., 2006), which are phosphorylated. Thus, a relatively well-integrated signalling network regulating *Papaver* SI is emerging (Wilkins *et al*., 2014; Figure 1).

The first indication suggesting the involvement of PCD in the SI response came from evidence that DNA fragmentation, generally considered a hallmark of late PCD, was specifically triggered in incompatible, and not compatible, pollen with Ca^{2+} signalling implicated in this process (Jordan *et al*., 2000). Dramatic alterations in the morphology of cellular organelles, including mitochondria, Golgi bodies and ER within 1 h of SI induction and condensation of the vegetative and generative nuclei further implicated the involvement of PCD in the SI response of *P. rhoeas* pollen (Geitmann *et al*., 2004). Conclusive evidence establishing that SI triggers PCD came from a study showing the involvement of a

DEVDase/caspase-3-like activity (Thomas and Franklin-Tong, 2004); see later for details. Hallmark features associated with the execution of PCD (e.g. the detection of caspase-like activities and DNA-fragmentation), were only detected several hours after SI-induction.

In this review, we will provide an overview of several recent advances in our understanding of the signalling components involved in *Papaver* SI-PCD and how these integrate in the signalling cascade leading to PCD. A particular focus is the SI-induced protein phosphorylation, and intracellular acidification and consequences for protein function. We will also discuss attempts to identify the protease(s) responsible for the DEVDase/caspase-3-like activity involved in the PCD process. Lastly, sparked by the successful functional transfer of the *Papaver* SI system to *Arabidopsis thaliana* (de Graaf *et al*., 2012; Lin *et al*., 2015), we will discuss new opportunities that have emerged because of this functional transfer of SI to further elucidate and dissect key mechanisms and components involved in SI-PCD.

Cytosolic pH alterations involved in SI-PCD

Although cytosolic acidification is not considered a marker for PCD, it has been observed in several developmentally regulated PCD systems in plants (Bosch and Franklin-Tong, 2007; Young *et al*., 2010; Fendrych *et al*., 2014; Wilkins *et al*., 2015). Using the ratiometric pH indicator 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein (BCECF), dramatic and rapid acidification during the SI-response was observed in the pollen cytosol of *Papaver* (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). Within 10 min of SI induction, the $[pH]_{cyt}$ was found to decrease from pH ~7 to a pH of approximately 6.4, followed by a continuous pH drop to reach levels as low as pH 5.5 one hour after SI induction (Wilkins *et al*., 2015; Figure 2). By manipulating the pH of the pollen tube cytosol *in vivo* using propionic acid, cytosolic acidification was found to be both sufficient and necessary for SI-PCD (Wilkins *et al*., 2015). Here, we review a series of events relevant to SI-PCD signalling that have been established as targets of this physiological alteration and discuss potential mechanisms involved in generating the SI-induced acidification of the pollen tube cytosol.

Targets of SI-induced cytosolic acidification

The actin cytoskeleton plays important roles in regulating pollen tube growth (Gibbon *et al*., 1999; Vidali *et al*., 2001; Qu *et al*., 2017). In the *Papaver* SI response, the usual actin filament bundles largely disappear and the level of filamentous actin (F-actin) in pollen tubes undergoes a rapid and dramatic reduction (Geitmann *et al*., 2000; Snowman *et al*., 2002). Rapid actin depolymerisation was demonstrated after SI induction (Snowman *et al*., 2002), followed by the formation of highly stable F-actin foci (Geitmann *et al*., 2000; Snowman *et al*., 2002; Poulter *et al*., 2010). By manipulating the $[pH]_{cyt}$ of pollen tubes with propionic acid buffered at pH 5.5, it was established that the formation of F-actin foci can be triggered. Importantly, pre-treatment of pollen tubes with propionic acid buffered at pH 7 followed by treatment with recombinant PrsS to induce the SI response, prevented SI-induced cytosolic acidification and the formation of F-actin foci (Wilkins *et al*., 2015). These observations demonstrated that the actin cytoskeleton is a major target of pollen tube cytosolic acidification during SI (see also Figure 1) and provided evidence that the drop of $[pH]_{cyt}$ is required for the dramatic changes in actin cytoskeleton configuration/organisation.

Two actin binding proteins (ABPs), cyclase-associated protein (CAP) and actin-depolymerising factor (ADF)/cofilin, were found to be colocalised with large F-actin foci that are formed after SI induction (Poulter *et al*., 2010), suggesting that they may play crucial roles in the formation of F-actin foci. Later studies showed that in *Papaver* pollen tubes the colocalisation of CAP and ADF with F-actin is triggered by the acidification of the pollen tube cytosol (Wilkins *et al*., 2015; Figure 1). In this study, pollen tubes treated with propionic acid (pH 5.5) for three hours had ADF and CAP colocalising with F-actin foci, which resembled the phenomenon observed in the SI-induced pollen tubes (Poulter *et al*., 2010). Critically, pollen tubes buffered by pre-treating with propionic acid (pH 7) before SI induction showed a significantly lower level of F-actin colocalisation with either ADF or CAP (Wilkins *et al*., 2015), showing that acidification was required for these alterations. It has been established that the activities of most ADFs in plants are sensitive to cytosolic pH (Carlier *et al*., 1997; Gungabissoon *et al*., 1998; Allwood *et al*., 2002; Chen *et al*., 2002; Lovy-Wheeler *et al*., 2006). Studies have shown that at normal $[pH]_{cyt}$ (approximately seven), ADF possesses the ability to sever F-actin filaments, while at acidic pHs the function of ADF

is altered to bind and stabilise F-actin (Carlier *et al*., 1997; Bamburg *et al*., 1999; Allwood *et al*., 2002; Wilkins *et al*., 2015). This suggests that in SI pollen tubes with acidified $[pH]_{cyt}$ the depolymerising activity of ADF is altered and ADF binds and stabilises actin; this could account for the formation and remarkable stability of F-actin foci decorated by ADF in incompatible pollen. Mammalian CAP1 can sever actin filaments at basic pH, but not at neutral and acid pH (Normoyle and Brieher, 2012). However, no such pH dependency has yet been reported in plants and other actin-regulatory proteins may affect how CAP controls actin dynamics (Ono, 2013). As CAP is associated with stable F-actin foci in SI pollen, this suggests that its severing activity is lost and that this may be due to acidic $[pH]_{cyt}$ conditions. Wilkins *et al.* (2015) suggested that under the acidic condition induced by SI, CAP might act to produce filament ends to facilitate the assembly of actin by ADF. The observation that both artificial cytosolic acidification and SI induction result in the colocalisation of ADF and CAP with F-actin suggests that these two ABPs are targets of SI-induced $[pH]_{cyt}$ acidification, an intriguing hypothesis to explore in future studies.

Another target of the cytosolic acidification triggered by SI is the activity of two pollen-expressed soluble inorganic pyrophosphatases (sPPases) in *P. rhoeas*, Pr-p26.1a and Pr-p26.1b. The phosphate-metabolising activity of sPPases generates the thermodynamic driving force for many metabolic reactions, including protein, polysaccharide and nucleotide biosynthesis. A screen for SI-induced phosphorylated proteins showed that the two *Papaver* pollen sPPases are rapidly phosphorylated in a Ca^{2+}-dependent manner after SI induction (Rudd *et al*., 1996) with increases in cytosolic Ca^{2+} and phosphorylation leading to a reduction in their activity (de Graaf *et al*., 2006; Figure 1). Notably, sPPase activity assays using recombinant Pr-p26.1a and Pr-p26.1b proteins showed that the sPPase activity is pH dependent with activities being dramatically reduced in acidic conditions when compared to those at the normal physiological pH 7 (Wilkins *et al*., 2015). The phosphorylation sites for Pr-p26.1a and Pr-p26.1b have recently been mapped and, importantly, the sPPase activities of the p26 phosphomimic mutants were more sensitive to a low pH environment (6.8 to 5.5) compared to the wild-type enzymes or the corresponding phosphonull mutants (Eaves *et al*., 2017). Additionally, when combined with the presence of Ca^{2+} and H_2O_2, the activities of the phosphomimic and phosphonull forms of both sPPases were further reduced at pH 7. The same activity tests using low pH levels related to SI (pH 6.8 to 5.5) demonstrated that, in addition to low pH, both Ca^{2+} and H_2O_2 contribute to the inhibition of p26 activity (Eaves *et*

al., 2017). Considering the rapid stimulation of Ca^{2+} and ROS, as well as the later pH drop induced by SI, these observations suggest that SI not only triggers the Ca^{2+}-dependent phosphorylation of Pr-p26 but also contributes to the reduction of sPPase activity by stimulating several intracellular events that cumulatively contribute to changes in intracellular conditions that inhibit sPPase activity.

A key enzyme activity identified as being involved in SI-induced PCD is a DEVDase/caspase-3-like activity that has been characterised by its ability to cleave the Ac-DEVD-AMC substrate over a range of different pH values relevant to SI (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). It was revealed that the SI-induced DEVDase activity is optimal at unusually acidic pH, with a narrow pH optimum between pH 4.5 and pH 5.5 (Bosch and Franklin-Tong, 2007), almost exactly matching the $[pH]_{cyt}$ from one hour onwards after SI-induction (Figure 1 and 2). The results of an *in vivo* test utilising the live-cell caspase-3 probe carboxyfluorescein-DEVD-fluoromethylketone Fluorescent-Labelled Inhibitor of Caspases (FAM-DEVD-FMK FLICA) showed that the addition of propionic acid (pH 5.5) to pollen tubes was sufficient for the induction of DEVDase activity (Wilkins *et al*., 2015), mimicking the SI-induced samples (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). Pre-treatment with propionic acid (pH 7) before SI-induction prevented DEVDase activation (Wilkins *et al*., 2015). Thus, the drop of $[pH]_{cyt}$ is a pivotal event in SI-PCD, acting as a gateway to PCD, by providing the acidic environment that matches the pH optimum for the SI-induced DEVDase activity (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). Although it has been shown that lowering the pH of the germination medium is sufficient to trigger DEVDase activity in pollen tubes (Wilkins *et al*., 2015), further studies are required to determine the exact role of the acidification for the execution phase of this PCD. Crucially, it remains to be established if the SI-induced cytosolic acidification activates the DEVDase by altering cellular conditions to create an optimal pH for DEVDase activity (i.e. directly stimulating DEVDase activation), or whether other cellular components are required to mediate the activation of the caspase-like activities.

Although alterations in $[pH]_{cyt}$ have been reported in many other biological processes in plants (reviewed in Felle, 2001) and animal PCD systems (Park *et al*., 1999; Matsuyama *et al*., 2000; Sergeeva *et al*., 2017), the $[pH]_{cyt}$ changes observed in these studies were transient and mild (with small scale changes of < 1.0 pH unit). This makes the dramatic $[pH]_{cyt}$ shift triggered by SI in *Papaver* pollen tubes a (to date) rare example of PCD-associated cytosolic

acidification in plants (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015). However, a previous study demonstrated the reduction in the fluorescence of a pH-sensitive yellow fluorescent protein (YFP) probe during BAX-induced PCD in onion cells, indicating a large pH drop (Young *et al*., 2010). Utilising a pH-sensitive green fluorescent protein (GFP) variant pHGFP (Moseyko and Feldman, 2001), a sharp drop of $[pH]_{cyt}$ was also observed during PCD controlling root cap development in *A. thaliana* (Fendrych *et al*., 2014), but measurements were not calibrated to establish the absolute change in $[pH]_{cyt}$. Together, these findings of significant $[pH]_{cyt}$ drops related to PCD suggest that this may be a rather more widespread phenomenon than generally thought and is worth further investigation in other plant PCD systems to examine if they also exhibit cytosolic acidification.

Apart from the ABPs, DEVDase, and sPPases mentioned above, the functional properties of most proteins, including their activity, stability, associations, and subcellular translocation are greatly affected by pH (Talley and Alexon, 2010). It is therefore likely that the SI-induced acidification contributes to the termination of incompatible pollen tube growth by altering the properties of many pollen tube proteins responsible for regulating growth. Importantly, by activating a DEVDase activity, the SI-induced cytosolic acidification plays a pivotal role in triggering incompatible pollen tubes to undergo PCD (Bosch and Franklin-Tong, 2007; Wilkins *et al*., 2015).

How is the SI-induced cytosolic acidification achieved?

Despite several independent reports showing that cytosolic acidification is a pivotal event for the cell to enter PCD (Fendrych *et al*., 2014; Wilkins *et al*., 2015), to date, there is no clear mechanism that has been demonstrated to be responsible for this pH shift. Since the vacuole is one of the most acidic organelles, with a pH 5.2-6 (Martinière *et al*., 2013; Shen *et al*., 2013), and is involved in one of the PCD classes in plants (van Doorn *et al*., 2011), one obvious mechanism for the acidification would be that the drop in $[pH]_{cyt}$ is caused by vacuolar rupture, as observed in PCD triggering xylem differentiation (Groover and Jones, 1999; Obara *et al*., 2001). Pollen tube vacuoles exhibit a highly dynamic reticulate structure (Hicks *et al*., 2004) and SI-induction triggers a rapid vacuolar reorganisation, with the typical reticulate structure being lost within 15 minutes (Wilkins *et al*., 2015; see Figure 1). However, at this early stage, the vacuolar membrane system (tonoplast) appears to be intact,

with apparent extensive vacuolar permeabilisation only observed after 30 minutes and complete permeabilisation and possibly breakdown detected one hour after SI-induction, well after the initial cytosolic acidification (Wilkins *et al*., 2015). A similar observation was reported by Fendrych *et al.* (2014) during developmental PCD in *Arabidopsis* lateral root cap cells; $[pH]_{cyt}$ dramatically decreased prior to the permeabilisation of the tonoplast for proteins. Wilkins *et al.* (2015) showed that the artificial acidification of the cytosol in *Papaver* pollen tubes triggered something that resembled the reorganisation and collapse of vacuoles after SI induction. Pre-treatment of pollen tubes with propionic acid (pH 7) prior to SI-induction largely reduced vacuolar reorganisation but did not completely prevent it (Wilkins *et al*., 2015). These observations suggest that the cytosolic acidification, at least in the systems of SI-PCD and lateral root cap developmental PCD, are unlikely to be caused by vacuolar collapse or breakdown. Instead, the initial cytosolic acidification induced by SI appears to occur earlier and upstream of vacuolar permeabilisation and this acidification somehow affects the integrity of the tonoplast. At this stage it can however not be ruled out that the initial cytosolic acidification may result from protons entering the cytosol through channels or other gateways from the vacuole before general permeabilisation of the vacuoles becomes visible using fluorescent probes.

Since the apoplastic pH is acidic, with values between pH 5 and 6 (Felle, 2001), a possibility is that H^+ influx is triggered by the very first SI events, as upon cognate interaction of PrsS with PrpS, Ca^{2+} and K^+ influx are the earliest events observed (Franklin-Tong *et al*., 1993; Franklin-Tong *et al*., 1995; Wu *et al*., 2011). The Ca^{2+} and K^+ influx observed at the shank of incompatible pollen tubes (Wu *et al*., 2011) provides evidence for the activation of Ca^{2+}-conducting/non-specific cation channels, which could also play a key role in triggering the drop of $[pH]_{cyt}$ if they also allowed protons through. It should be noted that treatment of pollen tubes with the calcium ionophore A23187, which also increases the permeability of the plasma membrane, mimicked the increase of $[Ca^{2+}]_{cyt}$ exhibited in SI and could also trigger rapid cytosolic acidification (Wilkins *et al*., 2015). In mammalian systems, an increase in $[Ca^{2+}]_{cyt}$ can lead to intracellular acidification through the Ca^{2+}/H^+ exchange activity of a plasma membrane Ca^{2+}/ATPase (Hwang *et al*., 2011). In Arabidopsis roots, the elevation of $[Ca^{2+}]_{cyt}$ induced by mechanical stimulation showed very similar kinetics to changes in pH and ROS triggered by mechanical stimulation (Monshausen *et al*., 2009). Treatment of the root with A23187 resulted in an increase in ROS and decrease in $[pH]_{cyt}$ (Monshausen *et al*.,

2009). These observations suggest a strong link between elevated $[Ca^{2+}]_{cyt}$ levels and decreased $[pH]_{cyt}$ with changes in both Ca^{2+} and H^{+} ions potentially mediated by the same channels or channels acting together.

Regardless of the identity/nature of the channels involved, it seems likely that pollen plasma membrane-localised PrpS proteins are in some way associated with such channels, or themselves form channels or pores. Structural predictions indicate that PrpS proteins might comprise four transmembrane regions with a relatively short (~34 aa) extracellular loop containing several hypervariable residues between different alleles (Wheeler *et al*., 2009). Although PrpS has no significant sequence homology to any protein in existing databases, it exhibits weak structural homology to a transport protein called Flower, a *Drosophila* protein that multimerises to function as a Ca^{2+}-permeable channel involved in presynaptic vesicle endocytosis (Yao *et al*., 2009; Wheeler *et al*., 2010). Whether PrpS forms a channel/pore at the plasma membrane or whether it is involved in the SI-induced acidification through proton transport remains to be determined.

Investigating the identity of proteases involved in SI-PCD

In SI-induced pollen, besides a caspase-3-like/DEVDase, a VEIDase and later a LEVDase activity was detected; all three exhibiting optimal activity at an acidic pH. The DEVDase and VEIDase were activated within 1–2 h after SI, with peak activity around 5 h after SI induction (Bosch and Franklin-Tong, 2007). SI-induced DNA fragmentation was significantly inhibited following pretreatment with Ac-VEID-CHO but not with Ac-LEVD-CHO, suggesting that the VEIDase activity, but not the LEVDase, is functionally involved in SI-mediated PCD (Bosch and Franklin-Tong, 2007). Since no SI-induced cleavage of the GRR substrate (diagnostic for metacaspases) was observed, the evidence from substrate cleavage assays (Bosch and Franklin-Tong, 2007) indicate that metacaspases are not involved in the execution of PCD in *Papaver* pollen. As mentioned earlier, SI-induced DEVDase activity has been demonstrated to play a crucial role in several physiological hallmarks of SI-PCD. Pre-treatment with the DEVDase inhibitor, Ac-DEVD-CHO, prior to SI induction, markedly alleviated pollen tube growth arrest stimulated by SI (Thomas and Franklin-Tong, 2004), rescued SI-induced loss of pollen tube viability (de Graaf *et al*., 2012; Li *et al*., 2007) and nuclear DNA fragmentation (Thomas and Franklin-Tong, 2004) in incompatible pollen.

In addition, it was shown that PARP, a classic substrate for caspase-3 activity in animal cells, was cleaved in an *S*-specific manner (Thomas and Franklin-Tong, 2004). Detailed characterisation of the temporal and spatial activation of plant caspase-like enzymes revealed a cytosolic and later nuclear localisation of the SI-induced DEVDase activity (Bosch and Franklin-Tong, 2007). In mammalian systems, caspases are synthesised as precursors (pro-caspases) and often translocated from the cytoplasm into the nucleus after induction of apoptosis (Zhivotovsky *et al*., 1999; Kamada *et al*., 2005). The change in localisation of the SI-induced DEVDase activity suggested that a similar strategy may be employed in the SI-induced PCD in *Papaver* pollen. However, to date, the identity of the SI-induced DEVDase remains a mystery.

Because plants have no caspase homologues, the nature and identity of their caspase-like proteases is of key importance to our understanding of PCD. Although plants contain metacaspases they are unable to cleave synthetic caspase substrates (Vercammen *et al*.,2007) so are not responsible for the caspase-like activities observed during SI-PCD. The identities of several plant proteases that exhibit caspase-like activities involved in PCD have now been revealed. Examples include phytaspases predominantly exhibiting a VEIDase/caspase-6-like activity (Chichkova *et al*., 2010) and vacuolar processing enzymes (VPEs), exhibiting YVADase/caspase-1-like activity (Hatsugai *et al*., 2004). Regarding the identity of DEVDases, two distinct proteases have been confirmed to cleave substrates diagnostic of caspase-3-like activity. Arabidopsis cathepsin B3 has been identified as a DEVDase/caspase-3 involved in UV stress (Ge *et al*., 2016) while the 20S proteasome β subunit 1 (PBA1) has been demonstrated to act as a plant caspase-3-like enzyme in the regulation of pathogen-induced PCD (Hatsugai *et al*., 2009). The 20S proteasome has also shown to be responsible for the caspase-3-like activity during PCD in xylem development (Han *et al*., 2012).

In eukaryotes, proteolysis is mainly carried out by the ubiquitin-proteasome system (UPS) and is crucial to diverse plant physiological events, such as growth and development (Moon *et al*., 2004), responses to abiotic stresses (Stone, 2014), and the SI response in *Solanaceae* and *Brassicaceae* (Entani *et al*., 2014; Indriolo *et al*., 2012, 2014). In mammalian systems, the UPS plays an important role in regulating apoptosis by targeting key cell death proteins, including caspases (Bader and Steller, 2009). Interference with the UPS, such as with the use of proteasome inhibitors, can either trigger apoptosis or protect the cell from apoptosis in mammalian cell lines (Orlowski, 1999), illustrating the complex role of UPS in regulating

apoptosis. Likewise in plants, disruption of proteasome function by silencing has been shown to activate PCD in *Nicotiana benthamiana* (Kim *et al*., 2003) while the proteasome inhibitor MG132 prevented heat shock-induced PCD in *Nicotiana tabacum* Bright-Yellow 2 cells (Vacca *et al*., 2007).

As several studies had identified caspase-3-like proteases involved in plant PCD as proteasomal proteins, we investigated whether the proteasome might be involved in the *Papaver* SI-PCD response. A pharmacological approach was used to test if inhibiting the proteasome might affect the SI response: pollen was pre-treated with MG132, epoxomicin or β-lactone, which are all potent proteasome inhibitors widely used in proteasome-related studies (Kisselev and Goldberg, 2001). However, viability assays using fluorescein diacetate staining showed no alleviation of death of pollen after SI in the presence of MG132 and TUNEL assays revealed that there was no significant alleviation in SI-induced pollen nuclear DNA fragmentation in SI-induced pollen pre-treated with several different proteasome inhibitors compared with non-treated SI pollen (Lin, 2015). Thus, these data suggest that the proteasome is not involved in the SI-PCD signalling pathway. Moreover, *in vitro* activity assays showed that the pH optimum for the *Papaver* pollen proteasome activity was neutral/basic, which contrasts with the acidic pH optimum for the SI-induced DEVDase activity in pollen (Figure 3A). Lastly, the DEVDase inhibitor, Ac-DEVD-CHO, which inhibits the DEVDase/caspase-3-like activity induced by SI, had no inhibitory effect on the PBA1 proteasomal activity (Figure 3B). Together, these data show quite clearly that the identity of the *Papaver* pollen DEVDase is not a proteasomal protein (Lin, 2015). Thus, the identity of this DEVDase remains to be established.

DEVD pull-downs identified a *Papaver* pollen VPE

In an attempt to identify proteins interacting with the DEVDase/caspase-3-like protein, pull-downs of SI-induced pollen protein extracts using a DEVD-biotin probe identified peptides corresponding to a VPE (Bosch *et al*., 2010). VPEs are vacuolar localised cysteine proteases involved in many plant cell death programmes, ranging from developmental PCD to PCD induced by abiotic and biotic stresses (reviewed in Hatsugai *et al*., 2015). VPE silencing suppresses the disintegration of the vacuolar membranes in leaves infected with tobacco mosaic virus, suggesting that vacuolar collapse is VPE-mediated (Hatsugai *et al*., 2004). Although the mechanism of this is unclear, it is thought that this is a key step, initiating the

proteolytic cascade leading to PCD (Fukuda, 2000; Jones, 2001; Hara-Nishimura and Hatsugai, 2011; Hatsugai *et al*., 2015).

Based on this apparent interaction, which suggested that this VPE might be part of the SI-PCD network, a *P. rhoeas* pollen expressed *VPE* (*PrVPE1*) was cloned and characterised. Although recombinant PrVPE1 indeed exhibited some DEVDase activity, its main activity was YVADase with optimum substrate cleavage at acidic pHs. PrVPE1 localised to the vacuolar compartment, like other VPEs. Since a YVADase activity is not required for SI-mediated PCD, it is unlikely that PrVPE1 is directly involved in the SI-PCD response and that the SI-induced vacuolar breakdown is not mediated by the activity of VPEs. However, it is worth mentioning that the characterisation of PrVPE1 revealed several features distinct from other characterised VPEs (Bosch *et al*., 2010). The unprocessed recombinant VPE pre-pro-protein, which is expected to be inactive, displayed YVADase activity while no activity could be detected for the mature (and normally active) form. Moreover, there was no evidence of the pre-pro-protein being processed under acidic conditions, which is unusual. Interestingly, localisation of the YVADase activity using live-cell imaging with a caspase-1 fluorescent probe FAM-YVAD-FMK showed labelling in mitochondria of untreated growing pollen tubes (Figure 4), while >1 h after SI induction this changed to a diffuse cytosolic signal (Bosch *et al*., 2010). This suggests mitochondrial permeabilisation, perhaps releasing YVADase, is triggered by SI. However, since PrVPE1 is not localised to mitochondria, this implies that a different protease is responsible for the observed mitochondrial YVADase activity.

The detection of a caspase-like activity localised to mitochondria in plants is of interest and warrants further investigation, as in mammalian cells, it is known that pro-caspases (inactive precursors of caspases) can localise to the mitochondria, and active caspases have also been found in the mitochondrial fraction (Zhivotovsky *et al*., 1999; Chandra and Tang, 2003). It is well established that mitochondria play key roles in activating apoptosis in mammalian cells, with permeabilisation of the mitochondrial outer membrane and subsequent release of proapoptotic molecules such as cytochrome c into the cytoplasm being involved in the activation of caspases (Wang and Youle, 2009). In *Papaver* pollen tubes, release of cytochrome c from the mitochondria into the cytosol has been detected as early as 10 minutes after SI induction, with levels of cytosolic cytochrome c continuing to increase up to 2 h after SI induction (Thomas and Franklin-Tong, 2004). It has been observed that mitochondria

undergo significant structural changes within 1 h of SI induction, including swelling, loss of cristae and blebbing, similar to PCD/apoptosis-associated changes described for mammalian systems (Geitmann *et al*., 2004). These findings, together with the observation that SI-induces hot spots of ROS production localised to organelles resembling mitochondria (Wilkins *et al*., 2011), suggest a possible critical role for mitochondria in the SI-PCD process that requires further exploration. This is corroborated by recent findings supporting a central role for mitochondria in several plant PCD systems, involving inhibition of the mitochondrial electron transport chain and ROS production (Van Aken and Van Breusegem, 2015; Zhao *et al*., 2018).

Conclusion and outlook

SI in *P. rhoeas* triggers an intricate signalling network leading to PCD of incompatible pollen. Here we have discussed the importance of cytosolic acidification as a physiological regulator of SI-PCD. Up to this date, cytosolic acidification involved in signalling to PCD has been mostly found in animals, with relatively small drop in pH of approximately 0.3–0.4 pH units (Furlong *et al*., 1997; Matsuyama *et al*., 2000; Roy *et al*., 2001; Shin and Loewen, 2011). However, in addition to the *Papaver* SI response, several studies have reported changes in $[pH]_{cyt}$ associated with PCD in plants (Moseyko and Feldman, 2001; Young *et al*., 2010; Fendrych *et al*., 2014). Together, these findings suggest that acidification, perhaps on a larger scale than seen in animal cells, may be a more general phenomenon in plant PCD that requires further characterisation to determine its extent, role and origin.

While *Papaver* SI has provided an excellent model system to investigate the molecular basis of cell-cell recognition and intracellular signalling in plant cells, its limited genetic resources represent a bottleneck to advance the field further. In an attempt to overcome this limitation, it was demonstrated that PrpS expressed in pollen of *A. thaliana* is functional (De Graaf *et al*., 2012). Transgenic *A. thaliana* pollen expressing PrpS undergoes a SI response with key features of *Papaver* SI when challenged with recombinant *Papaver* PrsS proteins, including pollen tube inhibition, actin alterations and PCD involving DEVDase activities (De Graaf *et al*., 2012). More recently, both the *Papaver* SI *S*-determinants have been functionally transferred to *A. thaliana*. Plants expressing PrsS in the stigma and PrpS-GFP in the pollen exhibit robust self-incompatibility and do not set seed (Lin *et al*., 2015). This finding

demonstrates that the two components PrpS and PrsS are sufficient to elicit an SI response in another plant species. The successful transfer, despite the substantial evolutionary distance between *Papaver* and Arabidopsis of some 140 million years, suggests that the network of signalling factors mediating SI-PCD is highly conserved and likely to be ancient, as they could be recruited from a distantly related species (Lin *et al*., 2015). The simplest explanation for why this works in Arabidopsis is that all the components downstream of the "receptor-ligand" interaction of PrpS and PrsS are common and universal (e.g. actin, sPPase etc.) and so when cognate S-determinants interact, all the components are in place to specify the network of events that characterise the SI-PCD response.

The availability of Arabidopsis plants expressing an SI response with all the key features of *Papaver* SI, opens up exciting opportunities to genetically dissect SI-induced signalling networks leading to PCD. Such Arabidopsis plants carrying the *Papaver* SI system allow the exploitation of the full forward and reverse genetics toolbox available for this model plant. For instance, crosses to CRISPR- or T-DNA mutant lines or gene-silencing/overexpression lines and lines with fluorescent markers of interest can further dissect the molecular mechanisms involved in SI-PCD. Forward genetic approaches could be employed to identify new genes involved in SI-PCD.

Likewise, the heterologous Arabidopsis SI system provides opportunities for identifying components involved in the later "execution" phase of the SI-induced PCD process. Although affinity-based approaches using pull-down assays of *Papaver* pollen extracts have identified various proteins that interact with the DEVD tetrapeptide, including the VPE discussed in this review, they have so far not led to the identification of the protease(s) responsible for the SI-induced caspase-3-like/DEVDase activity involved in the execution of PCD. Thus far, annotating peptides following pull-down assays and mass-spectrometry analyses required searches against the "whole green plant" database as the genome of *P. rhoeas* has not been sequenced. The drawback of this approach is that only *Papaver* peptides identical to those present in the "whole green plant" database can be identified. Utilisation of Arabidopsis lines expressing the *Papaver* SI system for pull-down assays with DEVD-based probes would alleviate this drawback, therefore providing an opportunity, together with the availability of

T-DNA lines and/or generation of knockouts/overexpression lines to validate candidates, to identify proteases involved in the execution of PCD in *Papaver* SI.

In summary, the availability of the heterologous Arabidopsis "SI" system should provide a powerful genetic tool for testing new hypotheses about SI-PCD in *Papaver* and to increase our understanding of the cellular mechanisms and genetic components involved in the SI-PCD response.

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Figure legends

Fig. 1. A timeline showing targets of SI-induced cytosolic acidification in *Papaver rhoeas* pollen tubes.

At **0 to 5 min** after SI induction triggered by the interaction of cognate *S*-determinants, pollen tube growth is rapidly inhibited. SI activates nonspecific cation conductance involved in mediating Ca^{2+} and K^{+} influx. At this early stage the vacuolar network appears as a highly dynamic reticulate structure but the $[pH]_{cyt}$ already starts to drop from its normal physiological pH of around 7. F-actin bundles in the shank and an F-actin 'reverse fountain' in the apical area can still be observed. The two p26 proteins are rapidly phosphorylated after SI induction. At about **10 min** after SI induction the $[pH]_{cyt}$ has dropped to around pH 6.4. Much of the F-actin is depolymerised with only filaments adjacent to the plasma membrane remaining. ADF predominantly localises at cortical regions and starts to partly colocalise with F-actin. Vacuolar reorganisation with small aggregations can be observed within 15 min after SI induction. At about **30 min** after SI induction, the $[pH]_{cyt}$ drops to around pH 6 and the typical reticulate structure of vacuoles cannot be observed any more. No F-actin bundles can be observed and small punctate foci start to form. ADF and CAP form small speckles and both colocalise with F-actin foci. DEVDase activity is not detected at this time point. At **1 to 2 hours** after SI induction, the cytosolic pH reaches an equilibrium of pH 5.5. The diameter of F-actin foci increases with their total number decreasing. The level of colocalisation of ADF and CAP with F-actin significantly increases until 1 hour after SI induction. After 1 hour post SI, nearly all of the F-actin foci colocalise with ADF and CAP. Increase in DEVDase activity is rarely observed during this period of time. At about **3 hours** after SI induction, F-actin and both ABPs form larger punctate foci and colocalise. Significantly increased activity of DEVDase is detected at this time point and peaks at 5 hours after SI induction.

Fig. 2. SI-induced pollen tubes undergo rapid cytosolic acidification. Pollen tubes labelled with the pH indicator BECEF-AM were treated with either PrsS to provide an incompatible response (SI) or a compatible response (Compatible), or untreated (UT). Reproduced from Wilkins KA, Bosch M, Haque T, Teng N, Poulter NS, Franklin-Tong VE. 2015. Self-incompatibility-induced programmed cell death in field poppy pollen involves

dramatic acidification of the incompatible pollen tube cytosol. *Plant Physiology* 167, 766-779, by kind permission of the American Society of Plant Biologists (www.plantphysiol.org).

Fig. 3. The proteasome is not involved in the SI-PCD response. (A) *Papaver* pollen proteasome and DEVDase activities have different pH-optima. *Papaver* pollen DEVDase and proteasome activities were profiled in different pHs using fluorogenic substrate-based activity assay. Note that the observed increase in PBA1 activity at pH 5.0 is caused by non-specific cleavage of the fluorogenic substrate Ac-nLPnLD-amc by DEVDase activity present in the pollen protein extract. Black bars: DEVDase activities profiled using Ac-DEVD-amc. White bars: PBA1 activities profiled using Ac-nLPnLD-amc. mean ±SD, n=3. (B) Proteasome activities are not inhibited by a DEVDase inhibitor. The effects of the DEVDase inhibitor, Ac-DEVD-CHO, on *Papaver* pollen PBA1 activity was tested *in vitro*. Pollen protein extracts were incubated with different concentrations (0-50 µM) of the inhibitor for 0.5 h before being subjected to activity measurement. DEVDase activity measurements were included as controls. DEVDase (black bars) and PBA1 (white bars) activities were measured by monitoring the hydrolysis of fluorogenic substrates Ac-DEVD-amc and Ac-nLPnLD-amc, respectively. Ac-DEVD-CHO did not significantly affect PBA1 activity, but dramatically inhibited DEVDase activity. Mean ±SD, n=6.

Fig. 4. A mitochondrial localisation for YVADase activity. (A) FAM-YVAD-FMK provides localisation of YVADase activity in *Papaver* pollen tubes. (B) MT-CMXRos mitochondrial signal. (C) MT-CMXRos and FAM-YVAD-FMK merge shows considerable co-localisation. Detail of (D) FAM-YVAD-FMK, (E) MT-CMXRos and (F) co-localisation. Scale bar, 10 µm. Reproduced from Bosch M, Poulter NS, Perry RM, Wilkins KA, Franklin-Tong VE. 2010. Characterization of a legumain/vacuolar processing enzyme and YVADase activity in *Papaver* pollen. Plant Molecular Biology 74, 381-393, by kind permission of Springer Nature (www.springernature.com).

Figure 1

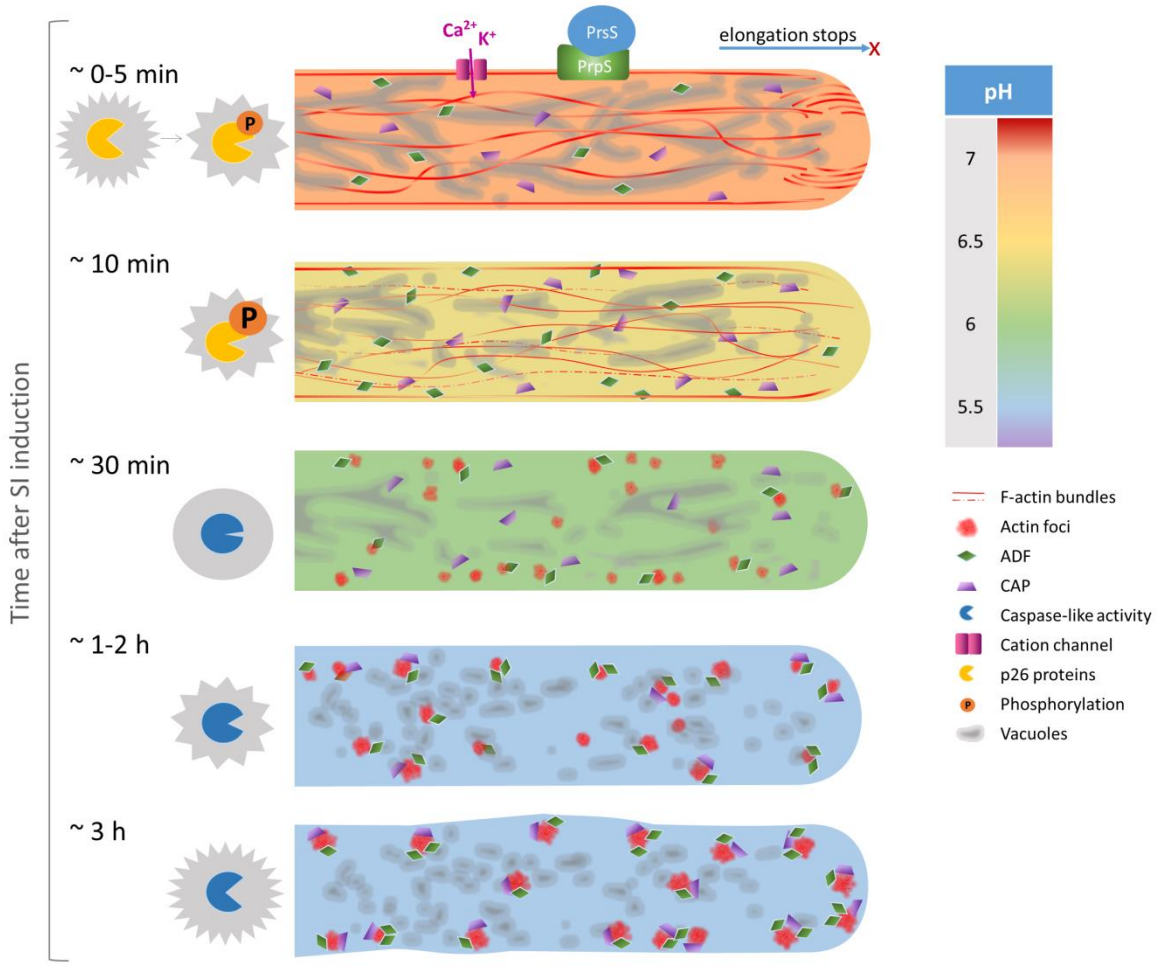

Figure 2

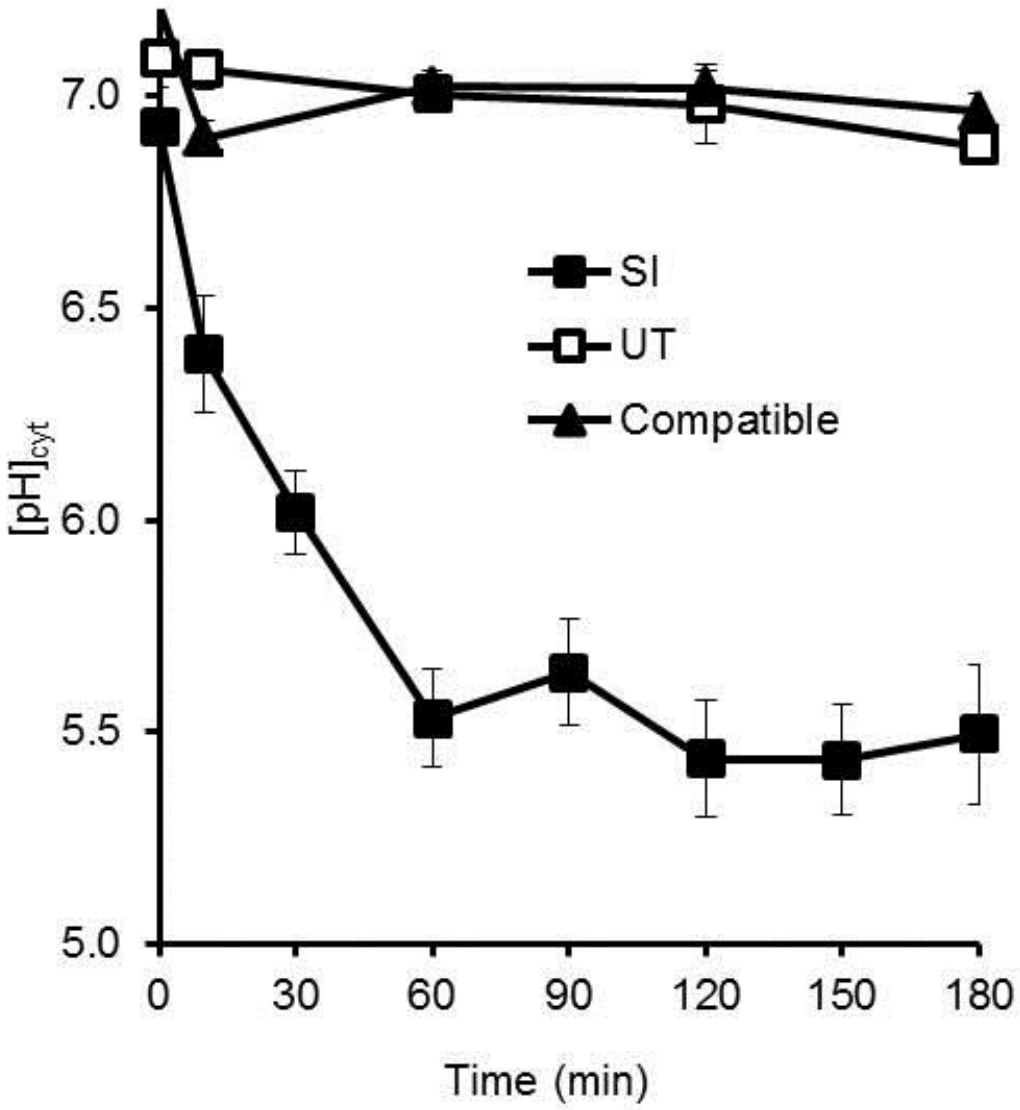

Figure 3

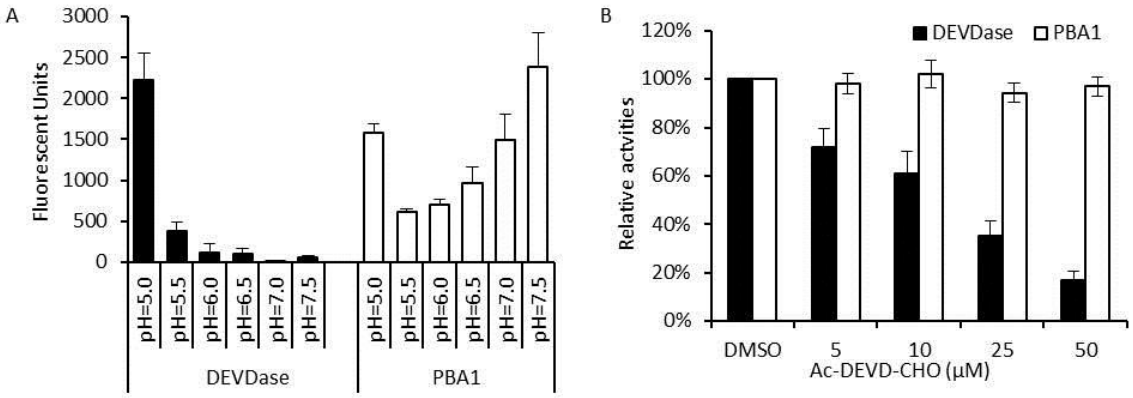

Figure 4