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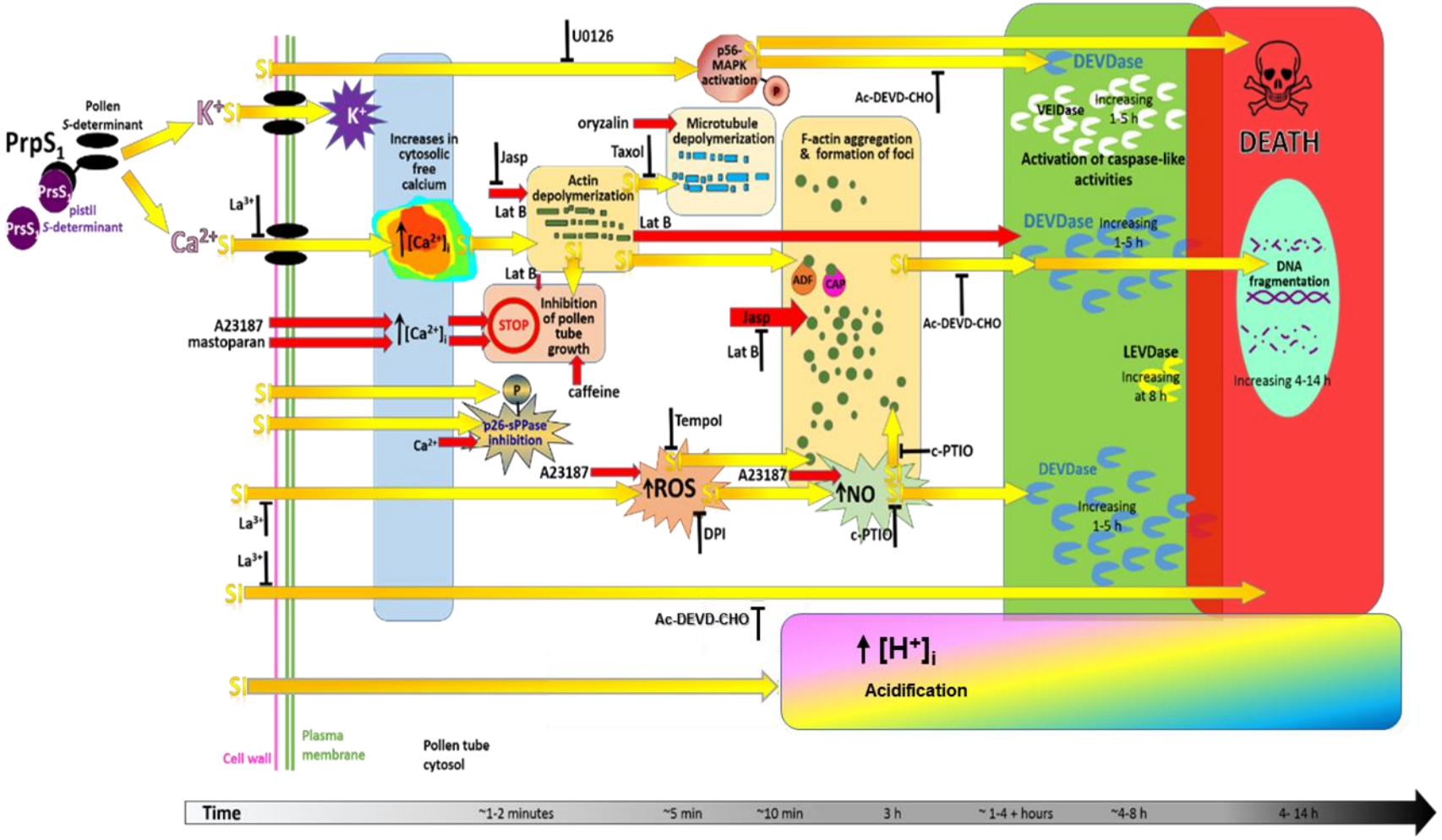
Supplemental Fig. 2. Cartoon showing a model of the integrated self-incompatibility (SI) programmed cell death (PCD) signalling network in *Papaver rhoeas* pollen.



Legend: Supplemental Fig. 2. Cartoon showing a model of the relative timing and integration of the components identified in the self-incompatibility (SI) programmed cell death (PCD) signaling network in *Papaver rhoeas* pollen.

Interaction of a pistil *S*-determinant PrsS with its cognate pollen *S*-determinant, PrpS, in a *S* haplotype-specific manner (e.g. PrsS1 with PrpS1) rapidly triggers the SI signalling network, resulting in the rapid inhibition of pollen tube tip growth and culminating with DNA fragmentation and death of incompatible pollen. Different colored boxes indicate key events triggered by SI; grey arrow indicates approximate timings; yellow arrows show SI-induced events; red arrows indicate agonist drugs used to stimulate events; black symbols show drugs used to inhibit events; pink vertical line represents the cell wall; double green line represents the plasma membrane.

One of the earliest events include rapid influx of Ca^{2+} and K^{+}, and almost instantaneous increases in cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$; blue box). $[Ca^{2+}]_{cyt}$ increases are required for many SI events, including phosphorylation and inactivation of soluble inorganic pyrophosphatases (sPPases) Pr-26.1a/b, actin depolymerization, and transient increases in reactive oxygen species (ROS) and nitric oxide (NO). Moreover, mastoparan or the Ca^{2+} ionophore A23187 (red arrows) can mimic SI-induced increases in) $[Ca^{2+}]_{cyt}$. Lanthanum (La^{3+}) has been used extensively to block SI-induced Ca^{2+} events and has demonstrated a requirement for Ca^{2+} as a key mediator of SI signaling to death. This places $[Ca^{2+}]_{cyt}$ signaling as one of the first SI events, upstream of many of the targets.

The end focus of the SI-induced network appears to be DNA fragmentation and cell death (red box), which are inhibited by pretreatment with the tetrapeptide inhibitor Ac-DEVD-CHO, but not Ac-YVAD-CHO. The inferred DEVDase activity has been more directly measured using the tetrapeptide substrate, Ac-DEVD-AMC, which is cleaved by SI-induced pollen extracts. This approach revealed three activities (green box): DEVDase and VEIDase increase between 1–5 h post SI and LEVDase increases later (still increasing at 8 h). DEVDase activity peaks at 5 h and has been shown to be required for DNA fragmentation, which can be prevented by the DEVDase inhibitor Ac-DEVD-CHO. These activities are optimal at pH 5. The caspase-like activities, which do not have any activity at normal cytosolic pH, need the pollen cytosol to acidify. We previously showed this occured in the first few hours of SI; here we have shown it begins at ~10 min and stablizes at ~pH 5.5 at 60 min (rainbow box).

Several components appear to be integrated in signaling to SI-mediated cell death. Central to this is the cytoskeleton (yellow boxes). SI-stimulates actin depolymerization, which causes inhibition of pollen tube growth. Low concentrations of Latrunculin B (Lat B) also inhibits pollen tube tip growth (orange box). SI triggers much higher levels of depolymerization than that required to inhibit pollen tube growth and higher concentrations of LatB that mimic this triggers activation of a DEVDase activity (measured with the Ac-DEVD-AMC substrate) and DNA fragmentation that is prevented by pretreatment with Ac-DEVD-CHO and not Ac-YVAD-CHO. Moreover, low concentrations of actin stabilizing Jasplakinolide (Jasp) can counteract and alleviate SI- or Lat B-induced DNA fragmentation, presumably by lowering the level of actin depolymerization. Subsequently, in SI, F-actin aggregates form highly stable punctate F-actin foci that are resistant to depolymerization and are associated with the actin-binding proteins (ABPs) actin-depolymerizing factor (ADF), and cyclase-associated protein (CAP; yellow box). High concentrations of Jasp can also trigger DNA fragmentation which is alleviated by pretreatment with Ac-DEVD-CHO and not Ac-YVADCHO. The formation of stable F-actin foci may be analogous to the stabilization using Jasp. Caffeine, which inhibits pollen tube growth without affecting the cytoskeleton, does not trigger DNA fragmentation. Together, these data implicate actin dynamics in modulating the DEVDase activity. SI also triggers microtubule depolymerization. This can be mimicked by use of the microtubule depolymerizer, oryzalin. Actin depolymerization using LatB triggers microtubule depolymerization but not vice versa, suggesting that SI-induced F-actin depolymerization signals to microtubule depolymerization. The tubulin-stabilizing drug, taxol, alleviates SI-induced Ac-DEVDAMC cleavage, implicating a requirement for tubulin depolymerization for the DEVDase activation. Disrupting microtubule dynamics alone using oryzalin does not trigger increased cleavage of the DEVDase substrate Ac-DEVD-AMC: this suggests that both actin and microtubule depolymerization are required for DEVDase-mediated SI-induced PCD. Evidence for involvement of a SI-activated p56-MAPK comes from use of the MEK inhibitor U0126 and its negative analogue U0124. Although U0126 inhibits pollen tube growth and p56-MAPK activity, it does not affect pollen viability. These drugs provided evidence that p56-MAPK activation is required for the activation of a DEVDase, DNA fragmentation, and cell death. SI stimulates transient increases in ROS and later NO. Pretreatment of pollen tubes with either the NADPH oxidase inhibitor diphenyliodonium or the NO scavenger cPTIO and then stimulating SI showed that both were required to reduce the SI-induced DEVDase activity. This suggests that ROS and NO act in concert or tandem to signal to activate SI-induced PCD through the activation of a DEVDase activity. Moreover, these drugs alleviated the formation of the characteristic punctate F-actin foci. This implicates ROS and NO signaling for the formation of these F-actin foci.

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