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Early F-actin disorganization may be signaling vacuole disruption in incompatible pollen tubes of *Nicotiana alata*

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Self-incompatibility (SI) systems appeared early in plant evolution as an effective mechanism to promote outcrossing and avoid inbreeding depression. These systems prevent self-fertilization by the recognition and rejection of self-pollen and pollen from closely related individuals. The most widespread SI system is based on the action of a pistil ribonuclease, the S-RNase, which recognizes and rejects incompatible pollen. S-RNases are endocyted by pollen tubes and stored into vacuoles. By a mechanism that is still unknown, these vacuoles are selectively disrupted in incompatible pollen, releasing S-RNases into the cytoplasm and allowing degradation of pollen RNA. Recently, we have studied the timing of in vivo alterations of pollen F-actin cytoskeleton after incompatible pollinations. Besides being essential for pollen growth, F-actin cytoskeleton is a very dynamic cellular component. Changes in F-actin organization are known to be capable of transducing signaling events in many cellular processes. Early after pollination, F-actin showed a progressive disorganization in incompatible pollen tubes. However by the time the F-actin was almost completely disrupted, the large majority of vacuolar compartments were still intact. These results indicate that in incompatible pollen tubes F-actin disorganization precedes vacuolar disruption. They also suggest that F-actin may act as an early transducer of signals triggering the rejection of incompatible pollen.

Keywords: F-actin, Nicotiana alata, self-incompatibility, signaling, S-RNase, pollen, vacuole

Abbreviations: SI, Self-incompatibility; S-RNase, S ribonuclease; SLF, S locus F-box; PCD, programmed cell death; ROS, reactive oxygen species; vPPase, vacuolar pyrophosphatase

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Self-incompatibility (SI) is an ancestral trait in flowering plants that prevents self-fertilization, avoiding in this way the detrimental consequences of inbreeding depression.[1] This mechanism allows plants to maintain genetic variability, and thus be better equipped to cope with environmental challenges. Different SI systems appear to have arisen independently on several occasions during the course of evolution. Many of them are controlled by a single locus, the S-locus, that encodes both female and male determinants of the SI system.[2] One of the most widespread SI types is the S-RNase-based system, which is named after S-RNase, a stylar ribonuclease responsible for pollen recognition.[3,4] Besides, it is generally accepted that S-RNase acts as a cytotoxic agent indispensable for pollen rejection by degradation of pollen RNA.[5] S-RNases gain access to both compatible and incompatible pollen tubes[6] and accommodate into vacuoles.[7] As pollen tubes make their way through the style, these vacuoles are selectively disrupted in incompatible pollinations, whereas in the compatible ones they remain stable.[7]

Thus, compartmentalization appears as an effective mechanism for controlling S-RNase toxicity. The localization of S-RNases in large compartments associated with the absence of cytotoxicity was also observed when they were ectopically expressed in pollen tubes.[8] Although in compatible pollen tubes no signal of S-RNase was seen out of vacuoles, the possibility that a minor pool of S-RNases may reach the cytoplasm cannot be ruled out. The male determinant in

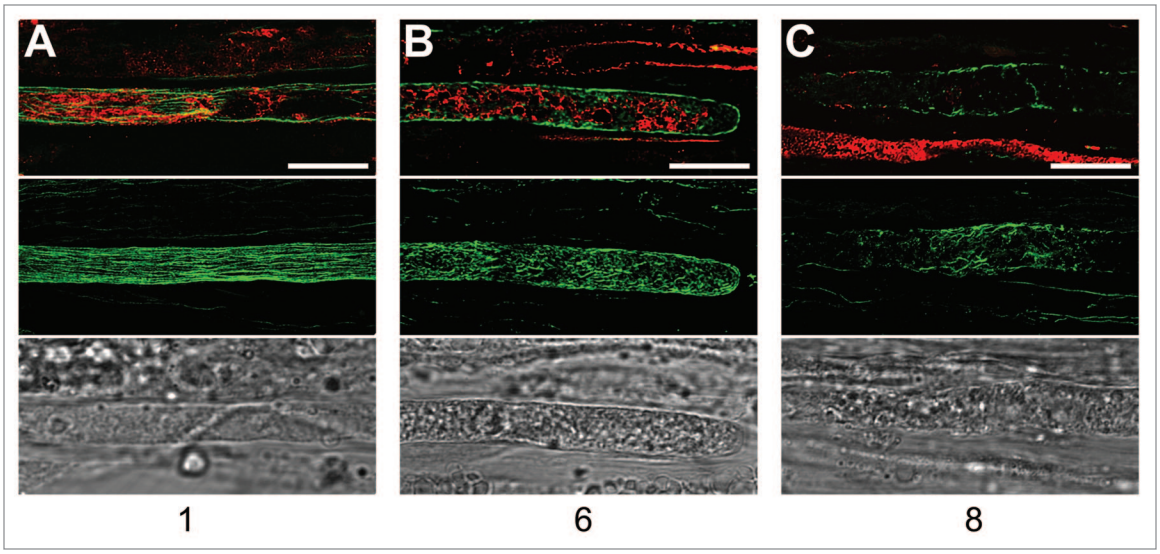

Figure 1. Sequential disorganization of F-actin cytoskeleton and vacuolar endomembrane system in incompatible pollen tubes of *Nicotiana*. Colocalization of F-actin (green) and the tonoplast marker vPPase (red) in incompatibly pollinated pistils. The images show the three patterns observed during pollen rejection: (A) Organized F-actin and vacuolar endomembrane system; (B) Disorganized F-actin and organized vacuolar endomembrane system; (C) Disorganized F-actin and vacuolar endomembrane system. Merged optical sections (top panel), full projections of phalloidin-stained pollen tubes (central panel) and bright fields (bottom panel), are shown. The days after pollination are indicated below the images. Bar 10 μm.

the S-RNase-based SI is a group of cytoplasmic F-Box proteins, called SLF[9,10] (S Locus F-box). Since recognition between S-RNase and SLF is envisioned as a direct molecular interaction, the presence of small amounts of S-RNase in the cytoplasm, to form an S-RNase-SLF complex, seems to be a reasonable assumption. Since the SLF protein contains a conserved F-box domain and interacts with members of E3 ubiquitin ligase complex, it has been proposed that the ubiquitination of non-self S-RNases and their subsequent degradation by the proteasome 26S may function as a possible detoxification system in compatible pollen tubes.[11] Several recent in vitro studies have provided robust evidence of preferential interaction between SLF proteins with non-self S-RNases (S-RNases from a different haplotype). This interaction was followed by ubiquitination and degradation of non-self S-RNases via proteasome 26S.[12,13] Overall, it becomes clear that these two mechanisms, S-RNase compartmentalization and non-self S-RNase degradation, may be contributing to maintain the integrity of compatible pollen tubes. Now, the focus is shifting toward the pollen rejection mechanism. It seems unlikely that a small amount of S-RNases, gaining access to the cytoplasm to interact with SLF, could be entirely responsible for massive RNA degradation. Therefore, the large pool of S-RNases, initially sequestered in the vacuoles and then released to the pollen cytoplasm, must be playing a decisive role in pollen rejection. Still, the sort of signals involved in the breakdown of incompatible pollen vacuoles continues to be puzzling. The stabilization of HT-B, a non-S-linked pistil component, is thought to be critical for mediating this effect.[7] Earlier specific alterations in incompatible pollen tubes may also contribute to signal tonoplast disorganization. In a recent work, we have demonstrated that in *Nicotiana alata*, vacuolar disruption of incompatible pollen tubes is preceded by the disorganization of F-actin cytoskeleton.[14] The integrity of F-actin cables is absolutely necessary for normal pollen tube growth. We found that early after compatible and incompatible pollinations, F-actin cytoskeleton is well organized in both types of pollen tubes. As expected, this pattern remained unchanged throughout the trajectory of compatible pollen tubes, which reached the ovary in about 2 d. Conversely, the F-actin cables of incompatible pollen tubes evidenced a gradual disorganization, in parallel with a clear reduction in growth rate. The F-actin in these tubes was progressively fragmented -presumably depolymerized- into short segments, reaching 80% of pollen tubes by day 6 after pollination. Importantly, at that time, pollen tube growth was fully arrested but the large majority of vacuolar compartments were still intact (**Fig. 1**). These results indicate that cellular dismantling of incompatible pollen tubes occurs in an organized way, following a specific sequence of events that culminate in vacuolar disruption and the consequent release of large quantities of S-RNase to the cytoplasm.[7,14]

The relevance of these results emerges in the light of current understanding of F-actin role in signal transduction processes. Both in animal and plant cells, F-actin cytoskeleton functions as a major target and transducer of signaling cascades.[15] Then, during the SI reaction in Nicotiana, F-actin disorganization itself is crucial to inhibit pollen growth. That may constitute an early stage in the pollen rejection mechanism. At the same time, the disruption of F-actin would also act as an efficient signal transducer for downstream events, presumably mediated by HT-B and other unknown factors. In the late stage of rejection, the large pool of S-RNase is released from vacuoles, giving way to the massive degradation of pollen tube RNA. This event could warrant the

rejection process, making it irreversible. Although this assumption is speculative, it may explain the reversion of some pollen tubes from incompatible to compatible phenotypes, observed when incompatibly styles were grafted onto compatibly styles.[16]

The role of F-actin as target and effector of signaling cascades has been well documented in the *Papaver rhoeas* SI system, which has been reproduced in vitro, mimicking in vivo pollen rejection.[17] Almost immediately after SI induction, a massive Ca^{2+} influx in pollen cytoplasm triggers an extensive depolimeryzation of F-actin. This in turn, initiates a network of events that leads to programmed cell death (PCD) of incompatible pollen tubes.[18,19] Somewhat similar results have been found in *Pyrus pyrifolia*, where pollen rejection is also an S-RNase-based system. In this case, in vitro SI induction is triggered by the sole presence of an S-RNase of the same pollen haplotype in the pollen culture medium.[20] These authors showed that in incompatible pollen tubes, S-RNase induces actin depolymerization, ROS disruption and DNA degradation, among other changes.[21] Thus, they postulate an alternative model, suggesting that RNA degradation by S-RNase may occur early in the sequence of events, leading to pollen rejection by a PCD mechanism. However, it is difficult to accommodate this model to the one of Nicotiana because S-RNase by itself is not sufficient for pollen rejection. In this species, additional factors not linked to S-locus, such as 120k and HT-B, are required for pollen rejection to take place.[3] Notwithstanding this, there are other relevant differences between the SI system in Rosaceae with respect to the ones of Solanaceae and Plantaginaceae.[22] Future research on this direction will reveal how divergent the S-RNase based SI systems in these families are and if these differences are sufficient to consider them as having two different mechanisms of pollen rejection.

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