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Stomata and pathogens
Warfare at the gates

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Bacterial and fungal phytopathogens are capable of triggering stomatal closure through pathogen-associated molecular patterns (PAMPs), which prevents its penetration through these pores. Therefore, stomata can be considered as part of the plant innate immune response. Some pathogens have evolved mechanisms to evade stomatal defense. The bacterial pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*), which infects plants of the Brassicaceae family mainly through hydatodes, has been also reported to infect plants through stomata. A recent report shows that penetration of *Xcc* in Arabidopsis leaves through stomata depends on a secreted small molecule whose synthesis is under control of the *rpf*/diffusible signal factor (DSF) cell-to-cell signaling system, which also controls genes involved in biofilm formation and pathogenesis. The same reports shows that Arabidopsis ROS- and PAMP-activated MAP kinase 3 (MPK3) is essential for stomatal innate response. Other recent and past findings about modulation of stomatal behaviour by pathogens are also discussed. In all, these findings support the idea that PAMP-triggered stomatal closure might be a more effective and widespread barrier against phytopathogens than previously thought, which has in turn led to the evolution in pathogens of several mechanisms to evade stomatal defense.

Stomata are small pores located in the leaf surface that allow plants to exchange gases with the environment. They play an essential role in the intake of CO_2 for photosynthesis, but at the same time they allow water loss by transpiration. Their position at the interface between internal plant tissues and the environment make them convenient gates for endophytic colonization by phytopathogens. For this reason plants have evolved the capacity to adjust stomatal apertures not only in response to hormones like abscisic acid (ABA) and to diverse environmental factors such as light, air humidity, carbon dioxide, but also in response to pathogens. Past studies, conducted with fungal and bacterial pathogens that enter leaves through stomata, have shown that many of these organisms display tropic movements towards them. After infection, these microorganisms may affect stomatal behaviour in diverse ways, a fact which has been attributed to the interplay between fungal and plant compounds secreted during the plant-pathogen interaction (reviewed in ref. 1). The effect of some of these purified compounds on stomatal movements has been reported. For example, the fungal elicitors oligogalacturonic acid and chitosan,[2] as well as the bacterial toxin syringomycin,[3] trigger stomatal closure, while *Pseudomonas syringae* pv. *tomato* (*Pst*) derived coronatine[4] and *Fusicoccum amygdali* derived fusicoccin[5] promote stomatal opening. In spite of these findings, the role of stomata in the defense against pathogens have often been overlooked.[6] However, the recent finding that the ubiquitously present bacterial pathogen-associated molecular patterns (PAMPs) flagellin and lipopolysaccharide (LPS) are capable of triggering stomatal closure provided convincing evidence that stomata effectively function as part of the plant innate immunity.[7] In the same study it was shown that coronatine, whose chemical structure is similar to methyl jasmonate, can revert bacteria-induced stomatal closure, allowing *Pst* to gain access into leaves even after initial stomatal response.

Only relatively high concentrations of bacteria have been reported to trigger stomatal closure (10^7–10^8 c.f.u./ml),[5,7] which might explain why the normal microbial flora living on the phylloplane does not promote stomatal closure. Biofilm formation, which leads to bacterial aggregation, not only improves epiphytic survival of bacteria such as the phytopathogen *Xanthomonas axonopodis* pv. *citri*,[8] but also appears to be a prerequisite for endophytic colonization by some pathogenic and beneficial endophytes.[9] In the bacterial pathogen *Xanthomonas campestris* pv *campestris* (*Xcc*), the *rpf*/DSF cell-to-cell signaling system controls the bacterial density dependent expression of many genes required for pathogenicity and environmental adaptation.[10-12] Some of these genes, like those involved in the synthesis of the extracellular polysaccharide xanthan, are also essential for biofilm formation.[13,14]

Xcc uses hydathode pores as main route on endophytic colonization of Brassicaceae, however, it has also been reported that can penetrate leaves through stomata, at least under certain conditions.[15] For this reason, we investigated if penetration of *Xcc* through stomata can occur passively, when either environmental or physiological conditions favors stomatal opening, or if on the contrary this process is aided by some compound similar to coronatine or fusicoccin.

We found that the *Xcc* is capable of manipulating stomatal closure of Arabidopsis through a secreted small molecule whose

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production is under control of the *rpf/DSF* gene cluster.[16] Both living *Xcc* and an extract from an *Xcc* culture supernatant, can inhibit PAMP- and ABA-induced stomatal closure in Arabidopsis. By contrast, *rpfF* and *rpfC Xcc* mutants, affected in respectively the synthesis and perception of the cell-to-cell communication signal cis-11-methyl-2-dodecenoic acid, cannot interfere with stomatal movements. The secreted factor most likely plays an important role in virulence, as *Xcc* supernatant extracts enhanced the ability of a *Pseudomonas syringae* pv. *tomato* (*Pst*) coronatine deficient mutant to penetrate Arabidopsis leaves.

In addition, in our work we provide evidence that Arabidopsis reactive oxygen species (ROS)- and PAMP-activated MPK3 is required for PAMP triggered stomatal closure, as plants expressing a guard cell-specific antisense construct against its coding gene are unable to close stomata in response to bacteria or purified LPS, although they still respond to ABA. Our unpublished observations show that these antisense plants are also unresponsive to the epiphytic fungus *Saccharomyces cereviciae* induced stomatal closure, indicating MPK3 integrates information coming from different receptors involved in pathogen perception.

Since different pathogens and elicitors induce ROS production, it is likely that these compounds act as a signaling link between elicitor perception and MPK3 activation in guard cells. In agreement with this hypothesis, the yeast derived elicitor and chitosan, both capable of triggering plant defense responses, also cause an elevation in guard cell free cytosolic Ca^{2+}.[17] This increase depends on the presence of cytosolic NAD(P)H, the substrate of the NAD(P)H oxidases involved in ROS production. Increases in both ROS and free cytosolic Ca^{2+} are linked to ABA-induced stomatal closure.[18] However, antisense MPK3 plants showed normal promotion of closure in response to ABA but no response to phytopathogens or to H_2O_2. How can this apparent paradox be solved? As ABA is known to trigger many different signaling events within guard cells, we propose that ABA signaling acts redundantly to promote closure in guard cells, while signaling of PAMPs in these cells relies absolutely on H_2O_2, making the presence of MPK3 a necessary requirement for pathogen-induced stomatal closure. Interestingly, MPK3 antisense plants also turned out to be insensitive to the *Xcc* factor in ABA-induced promotion of closure, which suggests that the *Xcc* factor targets some signaling component acting on the same pathway as MPK3.

H_2O_2 has been shown to inhibit guard cell H^+-ATPase activity,[19] it might be possible that the *Xcc* factor acts by indirectly relieving pathogen-induced, H_2O_2-mediated, inactivation of H^+-ATPase activity. In agreement with this proposal, it has been recently found that Arabidopsis RIN4, a negative regulator of plant immunity, is expressed in guard cells and upregulates PM H^+-ATPase activity,[20] *rin4* mutant stomata can not be reopened by virulent *Pst*, indicating that these plants are insensitive to coronatine. The fusicoccin toxin also inhibits H^+-ATPase, although by a different mechanism that involves direct binding to this protein.[21]

The cell-to-cell signaling system *rpf*/DSF regulates in a cell density dependent manner the expression of several genes involved in biofilm formation and endophytic colonization, including the suppressors of plant defenses, xanthan and β-cyclic glucan.[22,23] The factor capable of modulating stomatal responses also suppresses plant innate immunity, and therefore explains one of the multiple mechanisms by which the *rpf*/DSF gene cluster coordinates endophytic colonization of *Xcc.* While biofilm formation helps endophytic colonization, it is unlikely that it is a prerequisite for bacterial penetration through stomata, since this can take place in isolated epidermis aided without biofilm formation, provided that coronatine[7] or the *Xcc* factor[16] are present. Furthermore, even *rpfF* or *rpfC* mutants, unable to synthesize or perceive the *Xcc* cell-to-cell signaling molecule DSF, are capable of migrating through isolated epidermis in the presence of a wt *Xcc* extract.

The chemical nature of the *Xcc* factor has not been elucidated yet. While preliminary characterization indicates that it shares some common properties with coronatine (has a MW of 2,000 kD, and can be extracted from culture supernatants with ethyl acetate), it is unlikely that they are the same molecule, as the enzymes required for coronatine biosynthesis are encoded in a plasmid or chromosome of only some pathovars of *P. syringae.* The fungal toxin fusicoccin is also probably different from the *Xcc* factor as, unlike this, it causes a very strong promotion of stomatal opening.

Recently, it has been reported that the phytopathogenic fungi *Rhynchosporium secalis* and *Plasmopara viticola* can modulate stomatal behaviour[24] and that oxalic acid, a virulence factor produced by many fungi, can promote stomatal opening.[25] In addition, the human pathogen *Salmonella enterica* displays tropism towards photosynthetically active lettuce guard cells and possesses the ability of penetrating through them—suggesting that it may have some mechanism to disable stomatal defense.[26] While *S. enterica* is not a plant pathogen, endophytic colonization may be an important part of it life cycle, before being eaten by a host animal. The examples mentioned above rise the interesting possibility that mechanisms to overcome stomatal innate defense may be more common than previously thought, and that they might have evolved independently in different pathogens. Characterization of more pathogen molecules involved in modulation of stomatal defense and of their targets inside guard cells might provide exciting new tools to study stomatal physiology, as well as helping in the discovery of new strategies to prevent pathogen penetration inside leaves.

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