Evolutionary "hide and seek" between bacterial flagellin and the plant immune system

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https://doi.org/10.1016/j.chom.2021.03.010

Bacterial flagellin is a potent host immune activator. Parys et al. (2021) and Colaianni et al. (2021) dissected effects of flagellin epitope variants on host immune detection and bacterial motility. They report in this issue of *Cell Host & Microbe* that *Arabidopsis*-associated bacterial microbiota differentially evolved flg22 variants that allow tunability between motility and defense activation.

Plants are sessile organisms that interact in a fixed position with a plethora of microorganisms on their roots and leaves. These plant-associated microorganisms are termed plant microbiota, while their collective genomes and functional repertoire are referred to as the plant microbiome (Trivedi et al., 2020). Bacterial members of the plant microbiota can cause devastating diseases, but the majority have a commensal lifestyle or can even provide their host with life-supporting benefits by promoting plant growth, enhancing nutrient uptake, or stimulating the plant's immune system (Pascale et al., 2020). Bacterial microbiota are dominated by the phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, and Acidobacteria. Their relative abundances vary greatly, depending on soil type, host species, and plant compartment (Trivedi et al., 2020). Plants can actively select their microbial partners via exudation of primary and secondary metabolites or by activating specific plant defenses (Pascale et al., 2020). From their side, bacterial microbiota can evade plant immune responses, tolerate plant-released antimicrobial compounds, or competitively colonize nutrient-rich plant niches (Yu et al., 2019a). This balancing act at both the plant and the microbial side represents one of the top questions in plant-microbe interactions research: how do plants engage with beneficial, life-supporting bacteria, while warding off pathogenic ones?

In two elegant tour-de-force studies by the Belkhadir and Dangl groups, Parys et al. (2021) and Colaianni et al. (2021) sought an answer to this question by dissecting co-evolutionary principles between bacterial flagellin and the plant immune receptor FLAGELLIN SENSING 2 (FLS2). Flagellin is the building block of the bacterial flagellum, the locomotory organ of almost all flagellated bacteria. As such, flagellin is essential for bacterial motility, a fundamental function needed for host colonization by pathogens and symbionts alike (Raina et al., 2019). At the same time, flagellin represents a ubiquitous bacterial microbe-associated molecular pattern (MAMP) that can be recognized by its cognate plant immune receptor FLS2 (Couto and Zipfel, 2016). FLS2 detects flagellin from both pathogenic and beneficial bacteria by the 22-amino-acid epitope flg22 (Stringlis et al., 2018). Upon FLS2-flg22 pairing, FLS2 forms a complex with co-receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) (Figure 1A). This activates a number of well-defined MAMP-triggered immunity (MTI) responses that form a first line of defense against colonizing microbes, but also comes with fitness costs due to growth defense tradeoffs (Couto and Zipfel, 2016). Hence, the immunogenic and motility function of flagellin potentially mediates opposing effects on microbial fitness, a concept called antagonistic pleiotropy.

To investigate how single amino acid changes of the immunogenic flg22 motif affect bacterial motility and the interaction with the *Arabidopsis thaliana* immune receptor FLS2, Parys et al. (2021) generated a massive library of 412 flg22 variants in the flagellin gene *fliC* of the pathogen *Pseudomonas aeruginosa* (*Pa*). Transformation into an immotile *ΔfliC Pa* mutant demonstrated that almost 80% of the *fliC* variants did not restore *Pa* motility, indicating that the majority of the flg22 mutations impacted the locomotive func-tion of the bacterial flagellum. Mutations in the first 17 amino acids of the flg22 peptide, representing the "address" segment important for the interaction with FLS2 (Figure 1B), had the strongest impact on the motility function. Mutations in the last five amino acids, representing the "message" segment needed for BAK1 docking (Figure 1B), did not affect motility. Interestingly, systematic analysis of FLS2-flg22 variant interactions showed that more than 70% of the mutant flg22 variants retained interaction with FLS2. This suggests that amino acid changes in the flg22 epitope are much less likely to affect immune detection than motility (Figure 1C). Comparative analysis of all the flg22 variants revealed that Asp[14/15] residues are critical for both the interaction with FLS2 and motility. Phytopathogenic *Pseudomonas* associating with *Arabidopsis* contain selected mutations at the position of Asp[15], yielding flg22 variants that prevent FLS2 interaction but do not affect motility Parys et al. (2021). Hence, some bacterial pathogens seem to have selected the best of both worlds.

Colaianni et al. (2021) approached the problem from a different angle. They investigated the potential of naturally occurring flg22 epitopes in flagellin of commensal bacteria to trigger FLS2-mediated MTI and consequently inhibit plant growth. The 779 predicted FliC protein sequences of 627 *Arabidopsis*-associated flagellar bacteria were classified into three clades representing 268 unique flg22 sequences. Flg22 sequences from clade 1 (mainly β- and γ-Proteobacteria FliC) and clade 2 (mainly Bacillus and Actinobacteria FliC) resembled that of the immunogenic *Pa* flg22 sequence, while flg22 sequences from clade 3 (mainly
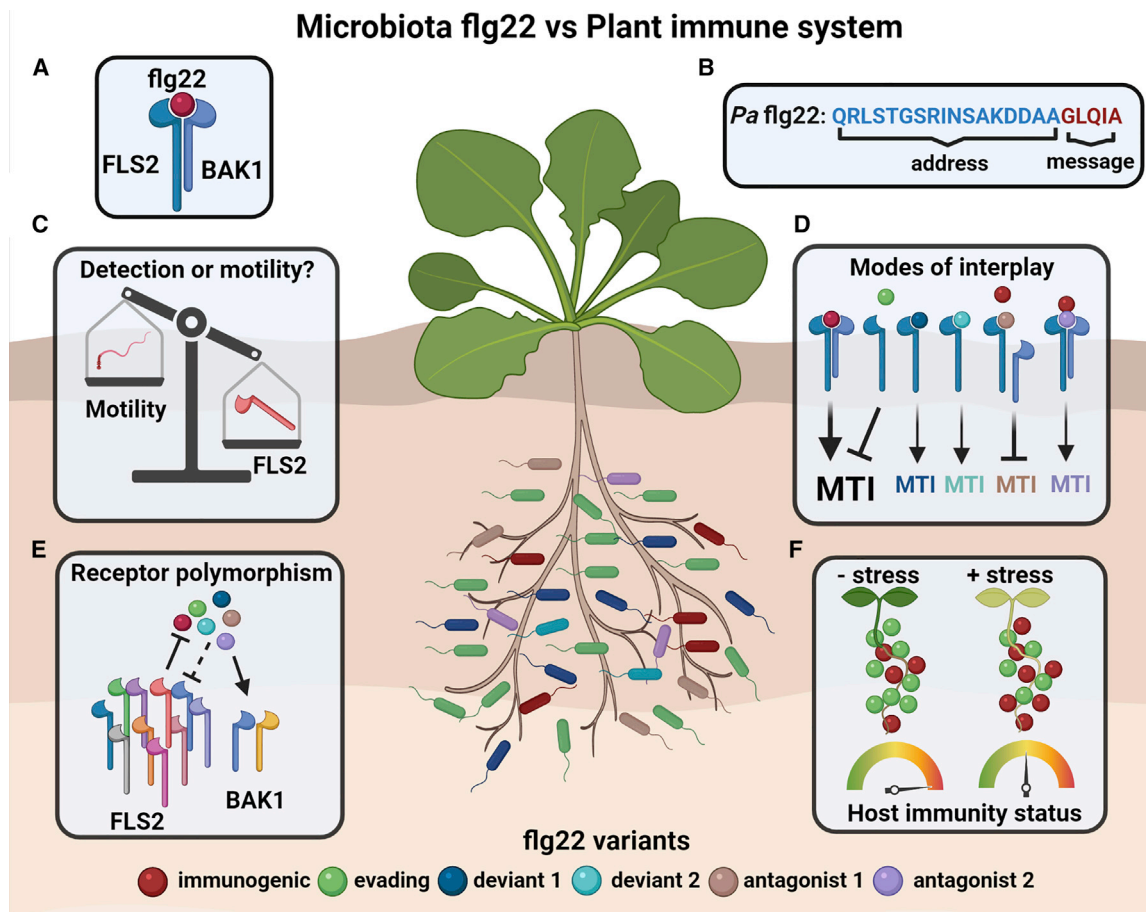
Figure 1. Modes of interaction between bacterial microbiota flg22 variants and the plant immune system
(A) Upon perception of flg22, a complex is formed between the FLS2 receptor and its co-receptor BAK1.
(B) The first 17 amino acids in the flg22 sequence (address segment) interact with FLS2 and the last 5 (message segment) are needed for the formation of the complex with BAK1.
(C) Avoidance of detection by FLS2 is prioritized over optimal motility in commensal bacterial communities, as demonstrated by the balance in immune-evading and motility-affecting natural variants of the flg22 epitope in the genomes of plant-associated bacteria.
(D) Next to immunogenic flg22 variants activating MAMP-triggered immunity (MTI), five different flg22 variants have been evolved in *Arabidopsis*-associated bacteria that either evade activation of FLS2-dependent MTI, activate lower levels of MTI (deviants 1 and 2), or antagonize perception of immunogenic flg22 peptides (antagonists 1 and 2).
(E) There is significant FSL2 sequence variation detected (>100) among *Arabidopsis* accessions, possibly to allow detection of different flg22 variants. Immune-evading flg22 variants tend to target the interaction with BAK1, possibly due to its low polymorphism potential.
(F) Host immune status exerts selection pressure on bacteria based on their flg22 immunogenicity. Healthy, non-stressed plants are mostly colonized by bacteria with non-immunogenic flg22 variants (green circles), while plants experiencing abiotic stress and consequently with a lowered immune status display increased colonization by bacteria with immunogenic flg22 variants (red circles).

Rhizobiales and Caulobacterales FliC) were much more divergent. Colaianni and coworkers screened 97 representative flg22 peptides from the three FliC clades for FLS2 interaction and downstream MTI-related production of reactive oxygen species (ROS) and inhibition of plant growth. Parys et al., (2021) similarly tested a set of synthetic flg22 variants with varying levels of FLS2 interaction and MTI responses. While most flg22 variants could interact with FLS2, there were variants that did not induce typical MTI-related ROS production or cause growth inhibition. This points to the natural occurrence of flg22 polymorphs in flagellar bacteria that do interact with FLS2 without generating downstream immune responses. Immunogenic ROS-inducing and growth-inhibiting flg22 variants were mostly derived from FliC clade 1, while immune-evading flg22 variants were mostly derived from FliC clade 3. Interestingly, two "deviant" types of flg22 variants were identified that induced ROS production but did not inhibit plant growth. However, they led to differential transcriptional changes of MTI marker genes. These deviant flg22 variants mainly occurred in FliC clade 2. A striking observation was that most of the tested flg22 variants were of the evading or deviant type, suggesting that commensal microbiota contain flg22 variants that evade FLS2 or induce a non-canonical MTI (Figure 1D).

Using the MTI marker gene *CYP71A12,* it was shown that *Arabidopsis* root immune responses triggered by immunogenic flg22 peptides can be actively suppressed by 42% of the tested root-dwelling bacterial microbiota (Yu et al., 2019b). Interestingly, both Parys et al.

and Colaianni et al. found that some of the non-immunogenic flg22 variants can antagonize the immunogenic function of *Pa* flg22 and inhibit *CYP71A12* expression in *Arabidopsis* roots (Figure 1D). Considering the presence of these antagonist flg22 variants among the plant-associated bacterial microbiota, it is reasonable to expect that in a microbial community context, competition between immunogenic and non-immunogenic flg22 variants might affect FLS2 activation and consequently impact the success of host colonization by specific bacteria. Interestingly, epitopes with changes in the message segment of flg22 (Figure 1B) more often displayed reduced immune activation, and when introduced in immotile bacteria more often restored motility. The fact that these mutations were present in 85% of the commensal *Pseudomonas* genomes suggests that they can benefit these flagellar bacteria during colonization and that bacteria prioritize to stay undetected over motility.

While at the bacterial side the flg22 epitope evolved abundantly into diverse variants, at the plant-side FLS2 seems to be particularly prone to evolutionary selection pressure. Genome mining in >1,000 *Arabidopsis* accessions detected >100 amino acid changes in FLS2, but only one in BAK1 (Parys et al., 2021). FLS2 sequence variation may have evolved to allow for the detection of different flg22 variants to safeguard proper immune activation (Figure 1E). On the other hand, polymorphism in the message segment of flg22 can help bacteria avoid BAK1 recruitment and therewith prevent immune activation (Parys et al., 2021). Colaianni et al. further tested FliC abundance and prevalence of different flg22 variants in a previously defined synthetic microbial community (Finkel et al., 2020) after colonization of *Arabidopsis* roots and shoots. *Arabidopsis* roots and shoots were enriched for bacteria with clade 3 FliCs (carrying mostly non-immunogenic flg22 variants), while bacteria with clade 1 FliCs (representing mostly

immunogenic flg22 variants) were depleted. Hence, during colonization of the *Arabidopsis* host, bacteria with immune-evading flg22 variants became more dominant, while immunogenic ones became depleted (Figure 1F). Interestingly, this balance between non-immunogenic over immunogenic flg22-carrying bacteria shifted in favor of the immunogenic variants when plants were exposed to salt stress (Colaianni et al., 2021). This abiotic stress condition lowered the capacity of plants to activate immune responses. Hence, proper functioning of the plant immune system is not only essential to prevent disease, it is also required for flg22-mediated monitoring of commensal and symbiotic bacteria in the plant microbiota.

The significant studies of Parys et al. and Colaianni et al. provide us with deep mechanistic understanding of the interplay between the host immune receptor FLS2 and the numerous variants generated by bacterial microbiota to circumvent their detection while maintaining motility. It is tempting to speculate that similar molecular arms races take place between other immune-receptor-MAMP pairs and in more complex microbial communities consisting not only of bacteria. Collectively, they may contribute to the coordinated activity of different immune-suppressing mechanisms that are employed by plant-associated bacteria (Yu et al., 2019a). The ultimate challenge for the plant-microbe interactions field is to decipher this balancing act of plants and their naturally associated microbiota and harness it to optimize plant health under diverse environmental stress conditions.

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