Flagellin perception: a paradigm for innate immunity

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There are surprising similarities between how animals and plants perceive pathogens. In animals, innate immunity is based on the recognition of pathogen-associated molecular patterns. This is mediated by the Toll-like receptor (TLR) family, which rapidly induce the innate immunity response, a first line of defence against infectious disease. Plants have highly sensitive perception systems for general elicitors and they respond to these stimuli with a defence response. One of these general elicitors is flagellin, the main component of the bacterial flagellum. Genetic analysis in *Arabidopsis* has shown that *FLS2*, which encodes a receptor-like kinase, is essential for flagellin perception. FLS2 shares homology with the TLR family, and TLR5 is responsible for flagellin perception in mammals.

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It is common knowledge that plants have the capacity to recognize potentially pathogenic microorganisms and to mount an efficient defence response [1–3]. Much recent research has focused on resistance genes that, in classic formal terms of the gene-for-gene relationship, render a given plant cultivar (carrying a specific resistance gene) resistant to a given race of a specific pathogen (carrying a specific avirulence gene) [4]. This highly specific recognition is reminiscent of the adaptive immune system in animals, which generates antigen-specific antibodies and T-cell receptors by somatic-cell DNA rearrangements. Like the adaptive immune system in animals, the gene-for-gene recognition system usually leads to immunity, based on a vigorous defence response called the hypersensitive response (HR) [5]. However, the plant gene-for-gene recognition system is genetically fixed and does not have the recombinatorial flexibility of animal adaptive immunity [6].

In addition, plants also have the capacity to perceive potentially pathogenic microorganisms in a more general way, through the so-called general elicitors [7,8]. Typically, these general elicitors are molecules that are highly characteristic of a whole class of microorganisms (whether pathogenic or not), such as chitin [9] and ergosterol [10], the hallmarks of the true fungi. The responses to these general elicitors include the production of reactive oxygen species and ethylene, and the induction of pathogenesis-related proteins, but generally do not correspond to an all-out defence of the HR type. This mode of perception of 'non-self' [7] is similar to what has become known as 'innate immunity' in the animal field [11,12]. According to an emerging model, innate immunity in animals is related to the recognition of pathogen-associated molecular patterns (PAMPs) such as fungal mannans and bacterial lipopolysaccharides [13,14]. In mammals, the recognition of such PAMPs mediates inflammatory or pro-inflammatory responses, including the production of reactive oxygen and antimicrobial proteins [15]. This might be important in instructing the development of the adaptive immune response [16] but it is not in itself sufficient to prevent invasion by pathogens [15,16].

Flagellin: a flag for the presence of bacteria

Bacterial motility is based on the flagellum [17], an extracellular propeller constructed from 11 protofilaments, each consisting of several thousand flagellin units [18]. Flagellin from various bacteria have well-conserved N- and C-termini but hypervariable central portions. Although most of the secreted flagellin is usually assembled in the flagellum, flagellin can also accumulate in the bacterial environment as a result of leaks and spillover during the construction of flagellae [19]. Flagellar motility allows bacteria to respond to favourable or unfavourable stimuli in their environment, and is also strongly related to the infectivity of some pathogenic bacteria [20,21]. It would appear to be an ideal PAMP for any cell or organism keen to monitor the presence of bacteria in its surroundings. Indeed, flagellin is recognized by animal cells, inducing defence responses in *Drosophila* [22] and mammals [23–25].

Studies with cell cultures of tomato and *Arabidopsis* revealed that the flagellin of the plant pathogen *Pseudomonas syringae* is a potent elicitor in plants [26]. Based on the idea that plants might recognize the most typical element of the flagellin molecule, a peptide corresponding to the most highly conserved region in the N-terminal domain of flagellin was tested [26]. This synthetic peptide, flg22, was found to be even more active as an elicitor than purified flagellin is, with a threshold of activity in the picomolar range [26]. The flg22 peptide also acted as an elicitor in whole *Arabidopsis* plants, inducing an oxidative burst, callose deposition and ethylene production, and leading to the induction of defence-related genes such as *PR1*, *PR5* [27], *PAL1* and *GST1* [28], but never induced an HR type of necrosis. However, flg22 was found to cause a strong inhibition of growth in *Arabidopsis* seedlings [27].

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In rice cell cultures, flagellin from an incompatible strain of *Pseudomonas avenae* induced a resistance response, whereas the flagellin from a related compatible strain did not [29]. Although the details are not yet known, this intriguing finding indicates that plants might detect specific variable features of flagellin in a gene-for-gene manner.

Flagellin-insensitive ecotypes and mutants

To identify genetic determinants involved in flagellin recognition and signalling in *Arabidopsis*, two independent approaches were taken, both taking advantage of the growth inhibition induced by flagellin. The first approach was based on natural genetic variation in *Arabidopsis*. It was found that the Ws-0 ecotype was insensitive to flg22 treatment, and crossing experiments with the La-*er* ecotype showed that sensitivity to flg22 was conferred by a single dominant gene of La-*er*, localized on chromosome V and termed *FLS1* [27]. In the second approach, ethylmethanesulfonate-mutagenized La-*er* *Arabidopsis* seedlings were screened for mutants not affected in growth after flagellin treatment [30]. Several mutants were obtained and three were analysed more closely. These all mapped to the same region of chromosome V as *FLS1* (a region known to contain many disease resistance genes [31,32]), and were characterized as alleles of a single locus termed *FLS2*.

FLS2 is a leucine-rich repeat-containing receptor-like kinase

A map-based cloning strategy was used to identify the *FLS2* gene. This turned out to be a single-copy gene encoding a receptor-like kinase (RLK) [33] composed of an extracellular leucine-rich-repeat (LRR) domain, a single membrane-spanning domain and an intracellular serine/threonine protein kinase domain [30]. The two mutants showed point mutations in the coding sequence, one (*fls2-24*) in the LRR domain and the other (*fls2-17*) in the protein kinase domain. The LRR domain is a common motif of extracellular proteins, including the Toll-receptor family [13]. LRR domains are thought to mediate interactions with ligands, particularly with other proteins or peptides, as exemplified by the crystal structure of the porcine ribonuclease-inhibitor–ribonuclease complex [34]. Thus, it is tempting to speculate that the LRR domain of FLS2 is part of a receptor for flg22 and flagellin [30]. Intriguingly, a resistance gene of rice, *Xa21*, specifying the gene-for-gene resistance of rice against *Xanthomonas oryzae*, encodes a protein with a similar structure and sequence [35].

Reverse-transcription polymerase chain reaction experiments showed *FLS2* to be expressed in roots, stems, leaves and flowers, and ectopic expression of *FLS2* in *Arabidopsis* plants indicated a direct correlation between *FLS2* expression levels and responsiveness to flg22 [30]. Transgenic *Arabidopsis* plants producing β-glucuronidase under the control of the *FLS2* promoter displayed high GUS activity in the vascular tissue but no apparent GUS production in cauline leaves, stamens, seeds or petals. In addition, wounding activated the *FLS2* promoter (L. Gómez-Gómez and T. Boller, unpublished). Because wounds provide entry sites for potential pathogens, enhanced expression of *FLS2* at the wound site might have functional relevance in increasing the awareness of the wound tissue to bacteria.

The genome of *Arabidopsis* encodes at least 610 members of the RLK family but the functions of most are unknown. Intriguingly, the serine/threonine protein kinase domain of RLKs forms a monophyletic group with the animal Pelle family (the protein kinases involved in innate immunity in animals) [33]. This has led to speculation that many of the plant RLKs might be involved in innate immunity [6]. However, plant RLKs might also play a role in development [36]. This is exemplified by *CLAVATA1*, one of the closest homologues of *FLS2*. The protein encoded by this gene, CLV1 [37], interacts with a second receptor-like protein, CLV2, which lacks a protein kinase domain [38]. As a heterodimer putatively located in the plasma membrane, this receptor appears to bind the peptide CLV3 [39], and this interaction is important in the determination of meristem size. It will be interesting to examine whether developmentally important RLKs such as CLV1 also play a role in innate immunity, in analogy to the Toll model for *Drosophila*.

FLS2 is part of the flagellin receptor in plants

Using a ^{125}I-labelled derivative of flg22, a high-affinity binding site for the flg22 peptide has been identified and characterized in tomato cells and membranes [40]. Intact flagellin and elicitor-activated flagellin peptides, but not biologically inactive analogues, efficiently competed for binding of radioligand, indicating that the binding site functioned as a flagellin receptor. For a model of receptor activation, a two-step mechanism of the address–message type was proposed in which binding of the N-terminus (address) is the first step and activation of responses with the C-terminus (message) is the second step [40].

With similar techniques, a binding site for flg22 was also found in *Arabidopsis* membranes [41]. Preparations from the Ws-0 plants and preparations from the *FLS2* mutants showed strongly reduced binding of flg22. Thus, responsiveness to flg22 was tightly correlated with the presence of a binding site for flg22. Cross-linking experiments using radiolabelled flg22 and membranes from wild-type plants indicated specific high-affinity binding of flg22 to a membrane protein of ~120 kDa, similar to the expected size of FLS2 (Z. Bauer *et al.*, unpublished). However, at present, there is no direct proof for the physical binding of flg22 to FLS2. Other components might be involved in the function of the flagellin receptor in addition to FLS2.

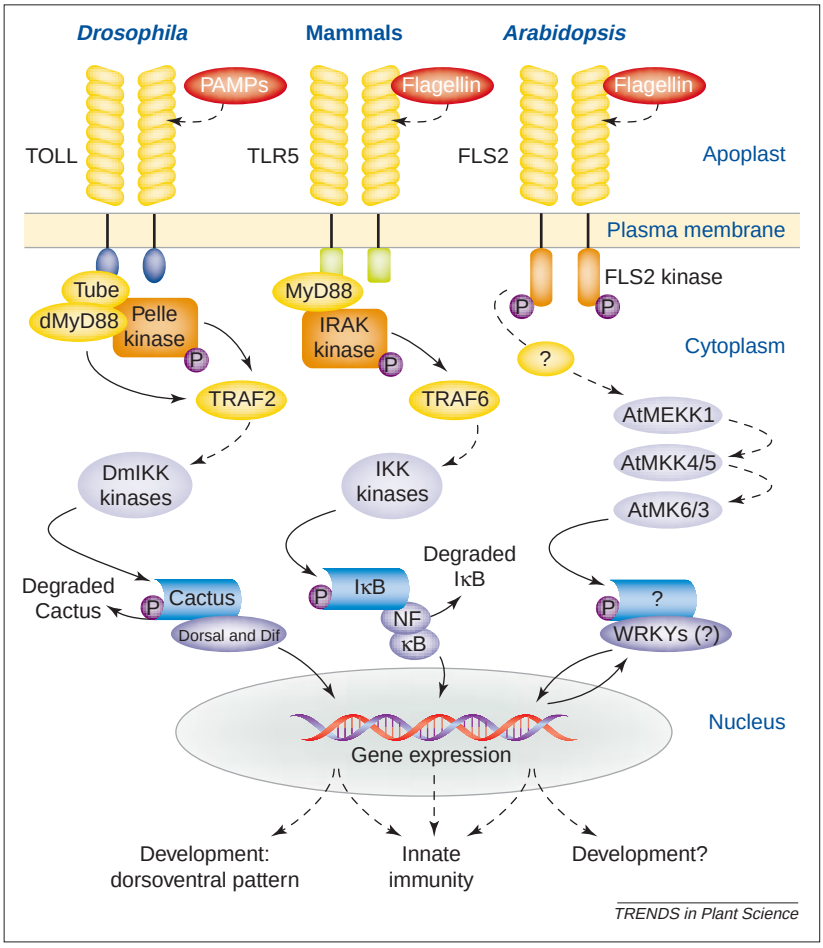

Fig. 1. Main components of the signal transduction pathways in 'innate immunity' in insects (*Drosophila*), mammals and plants (*Arabidopsis*). Toll, TLR5 and FLS2 are used as examples of key parts of the leucine-rich repeats-containing transmembrane receptors involved in the recognition of pathogen-associated molecular patterns (PAMPs) such as flagellin (TLR5 in mammals, FLS2 in *Arabidopsis*) and other microbial stimuli (Toll in *Drosophila*). Pelle and IRAK are animal serine/threonine kinases sharing homology with the kinase domain of the plant receptor kinase FLS2. Activation of Pelle and IRAK is mediated by the adaptor molecules Tube/dMyD88 and MyD88, respectively, leading to autophosphorylation of the kinases. Through poorly characterized steps involving the TRAF proteins, this activates the DmIKK (*Drosophila* homolog of Iκ B kinase) and IKK (cytokine-activated Iκ B kinase) kinases, which in turn phosphorylate Cactus and IκB, respectively. When phosphorylated, these molecules are ubiquitinated and degraded, thereby releasing the transcription factors Dorsal and Dif (Dorsal-related immunity factor) and NFκB, respectively, which then are free to translocate into the nucleus to activate target genes involved in innate immunity or development. In plants, there is no homologous IKK–IκB–NFκB pathway, but FLS2 activation by flagellin leads, through unknown steps, to the activation of a MAPK signalling cascade involving AtMEKK1, AtMKK4/5 and AtMK3/6. A MAPK signalling cascade is also induced by PAMP recognition in mammals (not shown). Through unknown steps that might involve the release of WRKY transcription factors from a negative regulator, this causes the transcriptional activation of defence genes as well as of WRKY factors, which themselves activate defence genes, thereby potentially amplifying the signal. It is not known whether the plant pathway is also involved in development, as in *Drosophila*.

TLR5 is part of the flagellin receptor in animals

In *Drosophila*, the Toll pathway was first implicated in dorsoventral patterning in the early embryo but, in 1996, it was also found to be essential in innate immunity for the recognition of Gram-positive bacteria and fungal cell-wall components [11,42]. Toll is a transmembrane protein with an extracellular LRR domain and an intracellular domain with significant sequence similarity to the intracellular domain of the interleukin-1 receptor, so it is referred to as the Toll–IL-1R (TIR) domain. This TIR domain interacts through an adaptor complex with the Pelle protein kinase [11].

In mammals, Toll-like receptors with a similar structure have been shown to be involved in innate immunity [43,44]. The best-studied Toll-like receptors are TLR4 and TLR2, which are involved in the recognition of PAMPS such as lipopolysaccharides, lipoproteins glycolipids and fungal cell-wall components [45,46]. Like Toll itself, some Toll-like receptors are known to homodimerize (in the case of TLR4) or to heterodimerize with other Toll-like receptors (in the case of TLR2) to form functional receptors. Their TIR domain interacts through the adaptor MyD88 with the protein kinase IRAK [46]. Recently, it has been found that the innate immune response of mice to purified *Salmonella* flagellin is mediated by the Toll-like receptor TLR5 [47]. TLR5 can generate pro-inflammatory signals as a homodimer and it uses signalling molecules such as MyD88 and IRAK, as do other members of the Toll-like-receptor family [16]. Intriguingly, flagellin-dependent responses in animals are localized to the conserved N- and C-terminal regions of the protein [23,48].

Downstream elements in flagellin perception

Given the functional and structural parallels between the FLS2 and TLR5 receptors, it is tempting to speculate that the flagellin response pathways in *Arabidopsis* and mammals share common features. Signalling via the Toll pathway in *Drosophila* [11,15] and the Toll-like receptors in mammals [15,46] often involves receptor dimers and proceeds through adaptors (dMyD88 and Tube in *Drosophila*, MyD88 in mammals) to activate the protein kinases Pelle and IRAK, respectively (Fig. 1). This leads, through several steps, to the degradation of Cactus and IκB [inhibitor of nuclear factor κB (NF-κB)], respectively, and to the release of Dif (Dorsal-related immunity factor) and NF-κB, respectively, and their transport to the nucleus [11,15,46]. In addition, at least in mammals, the p38 mitogen-activated-protein kinase (MAPK) pathway is activated [46].

Similarities to the Toll signalling pathway were noted with the cloning of the *Arabidopsis NPR1* gene (also known as *NIM1*), a key element in salicylic acid signalling, which encodes a protein with homology to IκB [49]. However, NPR1/NIM1 acts as a positive regulator of defence responses, in contrast to IκB, which acts as a negative regulator in the Toll pathway [50]. Also, the rapid protein phosphorylation in response to flg22 appears to be independent of the salicylic acid pathway [51]. Although there is currently no evidence in plants for the elicitor-mediated activation of a pathway similar to the NFκB pathway [5], it has been found that a MAPK cascade is activated within minutes after the recognition of general elicitors in a variety of plant species [52]. This is also the case for *Arabidopsis*, in which the flagellin-dependent activation of AtMPK6

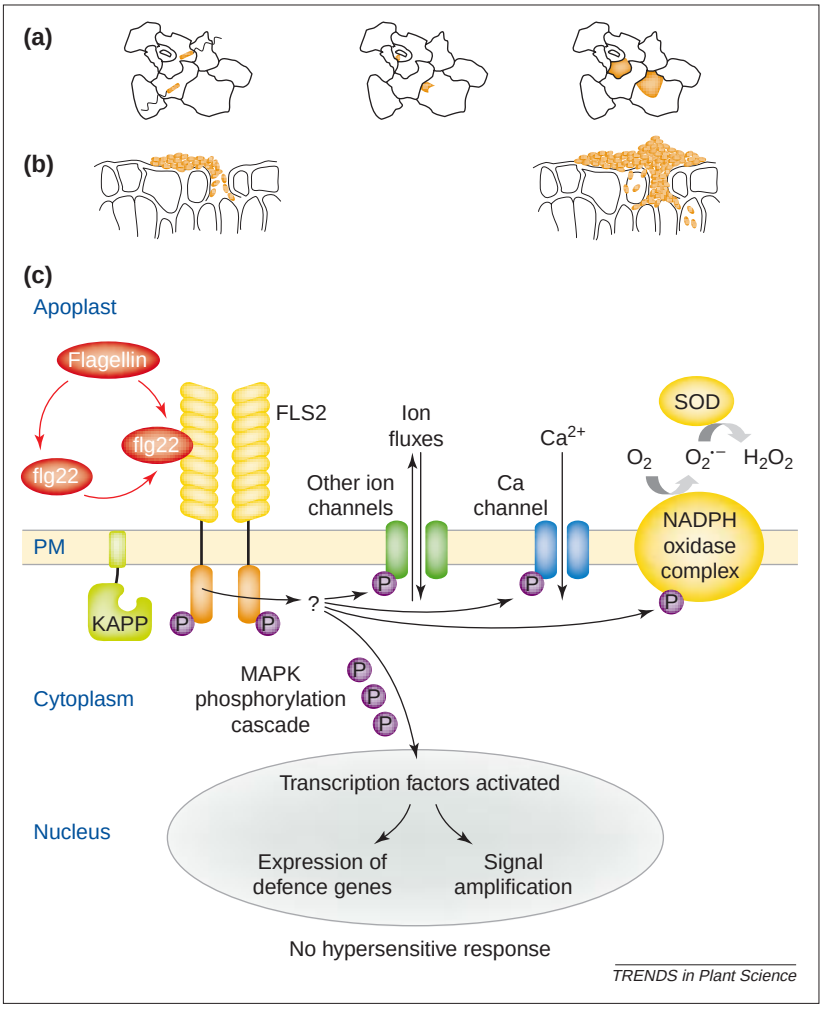

Fig. 2. Model for flagellin signalling in *Arabidopsis*. (a) Bacteria move on the plant surface and enter the plant via natural openings, wounds or hydathodes. A leaf is shown, but roots and stems are equally affected. (b) Bacteria multiply on the surface or in the intercellular space of the host plant. (c) Flagellin (released from bacterial flagellae) or flagellin fragments resembling flg22, are recognized directly or indirectly by the flagellin receptor at the plant plasma membrane (PM), a key part of which is FLS2. This recognition event might lead to autophosphorylation of FLS2, in analogy to the signalling modules in animals. Through unknown signalling pathways, the recognition event also induces the rapid phosphorylation of diverse downstream target proteins, presumably including calcium channels and other ion channels as well as the NADPH oxidase complex, which is involved in the release of hydrogen peroxide in conjunction with superoxide dismutase (SOD). A kinase-associated protein phosphatase (KAPP) is a negative regulator in this pathway. In addition, a MAPK cascade is activated (Fig. 1) that culminates in the activation of unknown transcription factors, causing induced expression of defence genes as well as of genes for WRKY transcription factors that might amplify the signal. In contrast to the defence mediated by resistance genes, this pathway does not lead to a hypersensitive response and cell death.

by dual phosphorylation of a serine and a tyrosine has been directly shown by *in vivo* labelling techniques [53]. Other elicitors activate the MAPK pathway as well [54,55]. A targeted proteomics search has been initiated to identify proteins that are rapidly phosphorylated after flagellin stimulation [51]. Interestingly, the same MAPK pathway is also activated in gene-for-gene interactions, indicating that there are common components in the recognition of general elicitors and specific elicitors (i.e. the products of avirulence genes) in plants [56].

Recent work with *Arabidopsis* protoplasts has shown the importance of the MAPK signalling pathway for the activation of transcription factors by flg22 [28]. In this system, flg22 specifically activates AtMPK3 and AtMPK6. Protoplasts of the *fls2* mutant do not respond unless co-transformed with the wild-type *FLS2* gene. Based on the transient expression of constitutively active or dominant-negative forms of various components of the MAPK signalling pathway, it appears that flagellin signalling initially activates AtMEKK1, which then phosphorylates AtMKK4 and AtMKK5. These kinases in turn phosphorylate and activate AtMPK3 and AtMPK6 [28]. Among the downstream targets of this signalling cascade might be the WRKY transcription factors [57], and the transcriptional activation of such WRKY factors might, in turn, be responsible for the activation of genes involved in defence responses [28].

A negative regulator of signalling by RLKs has been found in the case of the *CLAVATA* pathway: a protein phosphatase associated with CLV1, the so-called kinase-associated protein phosphatase (KAPP) was identified, and it was shown that the overproduction of KAPP resulted in a *CLAVATA* mutant phenotype [58]. Interestingly, *Arabidopsis* plants overproducing KAPP display a *fls2* phenotype when treated with flagellin and are unable to bind flagellin. Furthermore, the protein kinase domain of FLS2 and KAPP appear to interact in a yeast two-hybrid system [59]. These results suggest that KAPP plays a role as negative regulator of FLS2 as well as CLV1, indicating the intriguing possibility of cross-talk between innate immunity and development.

Model for flagellin recognition

Figure 2 depicts a speculative model incorporating our current knowledge of the flagellin response in *Arabidopsis*. In this model, bacteria swim on the wet plant surface, propelled by their flagellae, and enter plant roots, leaves or other organs; in leaves, for example, they might enter through stomata (Fig 2a,b), wounds or hydathodes. The flagellin (or partially degraded flagellin in forms resembling flg22) present in the extracellular medium interacts with the extracellular FLS2 LRR domain (Figs 1,2c). This interaction, which might also involve additional components, leads to activation of the FLS2 kinase domain, possibly through heterodimerization or dimerization of the receptor complex and autophosphorylation, although this remains to be studied. In turn, these activation processes cause rapid phosphorylation of many proteins, through mostly unknown regulatory networks, and lead to the phosphorylation-dependent activation of ion channels and the NADPH oxidase complex. In addition, the FLS2 kinase activity is directly or indirectly responsible for the phosphorylation and activation of AtMEKK1, which in turn phosphorylates AtMAKK4/5 that activates MAPK3/6 [28], which might further cause the activation of WRKY type transcription factors.

These transcription factors activate the expression of defence genes and might also induce transcription of WRKY factors [28], thereby amplifying the defence responses (Fig. 1).

Innate immunity in animals and plants: a primeval recognition system or convergent evolution?

In animals, innate immunity has moved to centre stage after decades of neglect [60] and has brought PAMPs into the spotlight. In plants, by contrast, research on general elicitors, the equivalent of PAMPs, received much attention in the 1980s and early 1990s but became a Cinderella in recent years owing to the overwhelming progress and interest in the field of resistance genes and avirulence genes, which result in clearly defined disease resistance based on the HR [2,61]. The cloning of large numbers of resistance and avirulence genes in plants and pathogens, respectively, has overshadowed the plants' need to probe and sense the microorganisms in their environment in a much more general way.

The surfaces of plant organs, both above and below ground, are continuously and permanently exposed to a vast microflora of fungi and bacteria. Many microorganisms landing on these surfaces do not interact with the plant, but some are symbiotic partners and others are potential pathogens. The plants' perception systems for characteristic non-self molecules, exemplified by the recognition system for bacterial flagellin, are highly reminiscent of animals' innate immunity response. There, too, the lipopolysaccharides, glycolipids, mannans and now even flagellin are recognized by the Toll-like receptors. The signalling molecules involved are not just pathogen associated, as the name PAMPs indicates, but are also typical of large groups of microorganisms and are absent from the host. In plants, as in animals, such molecules do not generally induce 'immunity' or 'resistance' against well-adapted pathogens but, in plants, as in animals, they might be important as signals indicating the presence of foreign, non-self organisms.

It should be realized that current reviews often equate animal innate immunity with the HR-based gene-for-gene-type resistance in plants [2,5,61]. In our view, this is conceptually misleading: the perception of PAMPs in animal immunity corresponds more to the perception systems for general elicitors known for decades in the plants, whereas the HR-based gene-for-gene-type resistance resembles the adaptive immune response of animals. In the animal field, connections are beginning to emerge between innate and adaptive immunity [46]. It will be interesting to find analogous connections between the gene-for-gene-type resistance and the response to general elicitors in plants. Such connections are indicated by the striking similarity between some of the responses to general elicitors and specific elicitors involved in gene-for-gene resistance [3,56].

At the moment, we do not know whether the Toll-like receptors for PAMPs in the innate immunity of animals and the perception systems for general elicitors in plants, such as FLS2, belong to an evolutionarily conserved primeval recognition system for non-self or whether they have been shaped by convergent evolution. However, one aspect that is important for the function of these recognition systems has largely been neglected in the animal systems, namely that many of the microorganisms in the environment are potentially mutualistic symbionts rather than pathogens. Recognition of symbionts such as *Rhizobium* bacteria or mycorrhizal fungi by the plant might involve the perception systems for general elicitors as well [7].

Today, we do not fully understand the essence of the mechanism of discrimination between friend and foe, but the perception system of plants for flagellin might provide an interesting example. The flagellin receptor is activated by flg22-type sequences from the flagellins of as widely divergent bacteria as *Pseudomonas*, *Salmonella* and *Vibrio*. However, the flagellins of the plant-associated bacteria *Agrobacterium* and *Rhizobium* have a highly divergent flagellin sequence, and their flagellins or the corresponding flg22-type sequences do not stimulate the flagellin perception system [26]. Does this represent co-evolution of the plant-associated bacteria *Agrobacterium* (a foe) and *Rhizobium* (a friend) to overcome the innate immunity system of plants?

Conclusions

In plants as well as in mammals, bacterial flagellins are recognized by surface receptors containing transmembrane proteins containing extracellular LRR domains (FLS2 and TLR5). In both plants and mammals, stimulation of the receptor by flagellin results in the coordination of activities at the plasma membrane (e.g. the production of reactive oxygen species and ion fluxes), rapid protein phosphorylation, the activation of a MAPK cascade and the activation of defence-related genes. It will be interesting to discover whether these strikingly similar perception systems are the result of convergent evolution or whether they represent an ancient, evolutionarily conserved recognition mechanism for non-self detection that is common to all metazoa.

Note added in proof

In previous work, we stated that the genes *FLS1* and *FLS2* were closely linked but different. However, re-sequencing showed that both Ws-0 and the EMS mutant *fls1-19* contain stop-codon-mutations in the kinase domain of FLS2 (S. Robatzek and T. Boller, unpublished). Thus, we now conclude that the gene formerly called *FLS1* is identical to *FLS2*.

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