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Early comunication between plants and their symbiont nitrogen fixing bacteria - a minireview

Andra-Sorina Tătar

Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeş-Bolyai University, Cluj-Napoca, Romania. Corresponding author: A.-S. Tătar, tatar.andra@yahoo.com

Abstract. The two participants at the symbiosis, the plant (legume or non-legume) and the bacteria that form nodules on it have to recompose their connection with each generation. The molecular dialogue is extremely complex and is far from being understood, but recently progresses are made, due to the sequencing of the genomes of the symbionts and also the advances in the investigating techniques. First of all, the plant exudes signals in the rhizosphere, mainly the secondary metabolites flavonoids, which are low molecular weight polyphenols that possess a 6C-3C-6C skeleton. The signals are perceived by specific bacteria in different manners, inducing different responses in the microorganism, such as chemotaxis, population enlargement and synthesis of the Nod factor, a lipo-chitooligosaccharide, that will further communicate with the host plant, leading to organogenesis and ultimately to fixing of atmospheric nitrogen into biologically available forms.
Key Words: chemotaxis, flavonoids, nitrogen-fixing bacteria

Rezumat. Cei doi participanţi la simbioză, planta (leguminoasă sau non-leguminoasă) şi bacteria care produce nodozităţi trebuie să re-formeze relaţia cu fiecare generaţie. Dialogul molecular extrem de complex este departe de a fi înţeles, dar recent se fac progrese, datorită secvenţierii genomurilor simbionţilor şi progreselor privind tehnicile de investigare. În primul rând, planta exudează semnale în rizosferă, în principal metaboliţi secundari de tipul flavonoidelor, care sunt polifenoli cu masă moleculară mică cu un schelet 6C-3C-6C. Semnalele sunt percepute de bacteriile specifice, inducând răspunsuri în acestea, în principal sinteza unor lipo-chitooligozaharide numite factori Nod, care vor comunica în continuare cu planta gazdă, ducând la organogeneză, şi în final, la fixarea azotului atmosferic în forme biologic asimilabile.
Cuvinte cheie: chemotaxie, flavonoide, bacterii fixatoare de azot.

Introduction. This mini-review focuses specifically on the communication happening before the actual physical contact between the symbionts, precisely on the substances released by the plant and the reaction elicited in the bacteria. For a detailed analysis of the events between the synthesis of the Nod factor and the activation of the colonisation in plant, see Oldroyd (2013). The conduct downstream of colonisation: the formation of the simbiosomes and development of bacteroids, the complex control of nutrients flow has been reviewed elsewhere (Oldroyd et al 2011).

Most legumes are able to form nodules harbouring nitrogen-fixing bacteria, like *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Azorhizobium*, collectively known as rhizobia, while symbioses with nitrogen-fixing actinomycetes, in particular *Frankia* species, are characteristic for a variety of woody non-legumes belonging to the Betulaceae, Casuarinaceae, Myricaceae, Elaegnaceae, Rhamnaceae, Rosaceae, Coriariaceae, and Datisticaceae families (Abdel-Lateif et al 2012). Also, cyanobacteria, most commonly from the genus *Nostoc*, can produce nitrogen-fixing nodules on plants such as *Gunnera manicata, Cycas revoluta, Blasia pussila* (Nilsson et al 2006).

Flavonoids. Flavonoids are products of the phenylpropanoid pathway and the acetate-malonate pathway, via phenylalanine and malonyl-CoA. Chalcone synthase forms the chalcone precursor of all other flavonoid structures, which can be decorated by

malonysation, hydroxylation, acylation, methylation, prenylation, polymerisation or glycosylation leading to a plethora of end-products (Hassan & Mathesius 2012), the last rendering the flavonoids hydrosolubility (Weston & Mathesius 2013). Main subclasses of flavonoids are: chalcones, flavones, flavanones, flavonols, flavan-3-ols, isoflavones, isoflavans, proanthocyanidins, pterocarpans, coumestans (Cooper 2004). Isoflavones are characteristic to the Legume family. They play a variety of roles, some of which are: UV protection, pathogen protection, pollination and dissemination, as pigments or co-pigments in flowers and fruits, modulation of auxin distribution and, of course, signalling molecules to symbiotic microbes, as chemo-attractants, gene inducers and growth regulators (Philips & Tsai 1992).

Their synthesis begins at the cytoplasmic side of the endoplasmic reticulum (Jorgensen et al 2005); for further storage in the vacuole and glycosylation reactions some complexes seem to also localise to the tonoplast (Aoki et al 2000). Flavonoid accumulation is cell type specific, with the root tip and the root cap cells as primary reservoirs. With specificity to intracellular location, flavonoids have been localised to the cell membrane, the cell wall, the vacuole, the cytoplasm, the nucleus (Hassan & Mathesius 2012).

Flavonoids end up in the rhizosphere passively, from decomposing root cap cells and bordering cells, or actively, as the living root cap cells are programmed to slough (Somers et al 2004). Concentrations of 10^{-6}-10^{-10} M of flavonoids are enough to trigger chemotaxis in bacteria, 1000 fold lower than those necessary for the *nod* induction that leads to the synthesis of the Nod factor (Weston & Mathesius 2013).

Flavonoids are secreted constitutively, but when compatible strains are closely in the rhizosphere and react, producing Nod factors, this leads to other changes in the plant root, also an increase in the concentration of flavonoids exuded, to attract even more symbionts (Recourt et al 1992).

The positive chemotaxis of rhizobia towards unfractionated root exudates is widely observed, but individual compounds can have the same effect. Luteolin, 4,4`-dihydroxy-2`-methoxychalcone, 7,4`-dihydroxyflavone, and 7,4`-dihydroxyflavanone from the alfalfa (*Medicago sativa*) extract induce positive chemotaxis in *Sinorhizobium meliloti*, it's specific symbiont, while naringenin and apigenin do not (Caetano-Anolles et al 1988; Dharmatilake & Bauer 1992). *Rhizobium leguminosarum* bv*. viciae*, the *Vicia* symbiont, is strongly attracted to the naringenin, kaempferol and apigenin (Armitage et al 1988). *Rhizobium leguminosarum* bv*. phaseoli* reacts to apigenin, luteolin, umbelliferone, acetosyringone from *Phaseolus vulgaris,* while naringenin induced a very low response (Aguilar et al 1988). *Bradyrhizobium japonicum* is not chemoattracted to isoflavonoids from soybean (*Glycine max*) root exudate (Kape et al 1991; Barbour et al 1991); however hydroxycinnamic acids did induce a strong chemotactic response. The complexity of these responses, with different bacteria having positive and negative taxis towards flavonoids in different legumes root extracts shows a very intricate control of specific bacteria-host couples, and it is probably the way the rhizosphere is controlled in order to achieve the most productive associations.

The motility and chemotaxis of bacteria have a strong influence on the nodulation, with *Rhizobium* non-motile and non-chemotactic mutants reduced nodulation and *Bradyrhizobium japonicum* hypermotile mutants leading to increased nodulation (Bauer et al 1991).

Other types of chemoattractants. A strain of *R. leguminosarum* bv. *phaseoli* was assayed for chemotaxis for a range of carbohydrates, with xylose, sucrose and raffinose eliciting peak responses at 10^{-4}-10^{-6} M, while glucose, fructose, galactose and maltose produced little or no effect. This suggests that the plant might be using these non-inducing but chemoattractive substances only to guide bacteria in close vicinity to the nodulation site (Aguilar et al 1988). Mannose is another strong attractant of *Rhizobium,* with a peak at 10^{-5}-10^{-8} M (Currier 1980).

Another compound that increases growth and colonisation is vitamin H, the biotin. It is normally present in the rhizosphere and adding nanogram quantities of it increases

Sinorhizobium meliloti growth in the alfalfa rhizosphere and, if avidin is added, the growth and colonisation is strongly reduced (Phillips & Streit 1998).

Gaworzewska & Carlile (1982) tested the *Pisum sativum* root exudate composition for chemotaxis for *Rhizobium leguminosarum* and observed that all amino acids have attractive effects on the bacterium at 10^{-5} M, while Lys, Arg, Gly worked even at 10^{-6} M. Four organic acids elicited positive chemotactic responses: citric acid, gluconic acid, malic acid and succinic acid. Only the fraction of the root extract with substances of a molecular weight less than 1000 elicited chemotactic responses and the cations showed stronger effects than the anions. Their work also showed that *Rhizobium leguminosarum* is attracted to both host root exudate and non-host (*Zea mays* - maize, *Sinapis alba* - mustard). However, if the *Rhizobium meliloti* Sym megaplasmid responsible for symbiosis is deleted, the bacteria does not show taxis for the usual chemoattractant, luteolin, but does for other chemical compounds, such as proline, meaning that the two types of chemotaxis are separated, according to their evolutionary purpose (Caetano-Anolles et al 1988).

Flavonoids and bacteria. The flavonoid naringenin accumulates on the inner membrane of *Rhizobium leguminosarum* bv. *viciae* almost instantly after its addition. Its hydrophobicity explains the bilayer accumulation, and the low molecular weight could allow it to pass the outer membrane through aqueous pores almost instantly accumulating at an 80:1 cell-to-medium ratio. Decreasing the temperature to 4°C or adding metabolic inhibitors substantially increased the naringenin levels inside the bacteria, suggesting no implication of specific uptake mechanism or high-affinity sites, but that the proton motive force seems to oppose the accumulation (Recourt 1989).

Once the flavonoids enter the bacterial cells, the NodD protein (a LysR type regulatory protein) activates the operons *nod* boxes, promoters of several *nod* genes, genes involved in the synthesis of the lipo-chitooligosaccharide Nod factor (Broughton et al 2000). Some bacterial species can have more than one NodD proteins, e.g. *Sinorhizobium meliloti* has up to four NodD proteins, NodD1, NodD2, NodD3, SyrM, that interact specifically with different flavonoid inducers (Somers et al 2004). The presence of the flavonoid leads to changes in the DNA topology where the NodD binds, allowing the RNA polymerase to transcribe the *nod* genes (Chen et al 2005). This is not entirely accepted, because in his review, Cooper (2004) stresses that although it was assumed that flavonoids and NodD form complexes there is no evidence of a physical interaction between the two.

Another paradigm that was recently overturned is the one saying that the activation of NodD protein by a flavonoid is the first step of signalling in the rhizobia. It has been observed (Moscatiello et al 2009, 2010), using aequorin^{+} mutants, that a variation in calcium concentration appears in a *Rhizobium leguminosarum* strain and in *Mesorhizobium loti*, meaning that calcium signalling is a universal way for intracellular communication. The transient increase in Ca^{2+} concentration appeared only after treatment with the rhizobial host specific signalling molecules and not with other types of molecules, such as strigolactones. The same Ca^{2+} signature was observed in the *Rhizobium leguminosarum* when the Sym plasmid was removed, so NodD was not present, meaning that the Ca^{2+} reaction happens before the binding of the NodD-flavonoid complex, which would be downstream in the signaling processes.

Conclusions. Flavonoids are plant secondary metabolites with a plethora of functions, some of which are part of the complex communication that leads to the formation of symbioses with nitrogen-fixing bacteria.

There is a strong microorganism-host specificity, with various responses elicited in the bacteria as a reaction to minute components of a root exudate, components that are, of course, in many possible combinations and ratios in every different plant.

Much is still unknown about the immense communication between symbionts, much is just partially understood, and some information might be even speculated and not demonstrated, so a lot more work is to be done in order to fully understand the process.

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Author:
Andra-Sorina Tătar, Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeş-Bolyai University from Cluj-Napoca, Gh. Bilascu Str., No. 44, Cluj-Napoca, Romania,email: tatar.andra@yahoo.com

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