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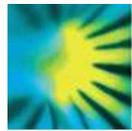
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Nuclear Ca²⁺ signaling in arbuscular mycorrhizal and actinorhizal endosymbioses: on the trail of novel underground signals

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- I. Introduction
- II. Nuclear calcium signaling and the AM symbiosis
- III. Pre-infection signaling during *Frankia*/actinorhizal plant nodulation
- IV. Conclusions & future outlook

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References

1 Summary

2 Root endosymbioses are beneficial associations formed between terrestrial plants and either
3 bacterial or fungal microorganisms. A common feature of these intracellular symbioses is the
4 requirement for mutual recognition between the two partners prior to host-regulated microbial
5 entry. Specific microbial factors activate a highly conserved plant signal transduction
6 pathway, of which a central component is the triggering of sustained Ca^{2+} oscillations in the
7 host epidermis. This then leads to the specialized cellular reprogramming required for the
8 construction of the transcellular apoplastic microbial entry compartments. Here we focus on
9 recent findings concerning this crucial Ca^{2+} -dependent signaling step for endosymbiotic
10 associations involving either arbuscular mycorrhizal fungi or nitrogen-fixing *Frankia*
11 actinomycetes, as well as how this knowledge is contributing to the identification of the
12 respective microbial factors.

13 Key Words:

14 Cameleon calcium reporters; Chitin oligomers; Common symbiotic signaling pathway; LysM
15 receptor-like kinases; Nuclear calcium spiking; Plant-microbe interactions; Root
16 endosymbioses

17 I. Introduction

18 Throughout the evolution of land plants, mutualistic fungal and bacterial associations have
19 provided key metabolites (phosphorus, nitrogen, microelements etc.) to their respective hosts,
20 and thereby played a major role in plant colonization of terrestrial ecosystems. In return, the
21 microbial partners benefited from both a source of photosynthates as well as privileged
22 ecological niches. Striking examples of such beneficial associations are the so-called root
23 endosymbioses, where the microsymbionts are housed within specialized host cell
24 compartments, whether in the inner root cortex for the ancient and widespread arbuscular
25 mycorrhizal (AM) symbiosis, or within *de novo* constructed root organs (nodules) in the case
26 of the more recently evolved rhizobial/legume and *Frankia*/actinorhizal plant nitrogen-fixing
27 symbioses.

28 Studies initially focused on the rhizobial/legume symbiosis using model legumes such as
29 *Medicago truncatula* and *Lotus japonicus* revealed that the successful establishment of this
30 association requires host recognition of specific rhizobial lipo-chitooligosaccharide (LCO)
31 signals known as Nod factors (NFs; Dénarié & Cullimore, 1993). These NF LCOs are
32 perceived *via* legume receptor-like kinases (RLK) belonging to the chitin-binding LysM-RLK
33 family. This then activates a specific host signal transduction pathway in target root hairs, a
34 central feature of which is the triggering of sustained nuclear-associated Ca^{2+} oscillations
35 (known as spiking) which are decoded by a dedicated calcium and calmodulin kinase
36 (CCaMK) (Oldroyd & Downie, 2006). Major cellular reprogramming in the host cells is thus
37 initiated, resulting in the progressive construction of the transcellular compartment (infection
38 thread) within root hairs, through which the rhizobia are conveyed across the root outer
39 tissues (e.g. Fournier *et al.*, 2008). This sophisticated mode of apoplastic root penetration is
40 thought to allow selectivity and regulation of microbial access to inner root tissues.

41 Legume-based research was also instrumental in revealing striking similarities between the
42 molecular and cellular mechanisms of both rhizobial and AM fungal root colonization,
43 including the mode of fungal root entry *via* apoplastic intracellular compartments (Genre *et al.*
44 *et al.*, 2005) as well as the activation of the Ca^{2+} spiking/CCaMK core signaling module (Kosuta
45 *et al.*, 2008; Chabaud *et al.*, 2011) which lies at the heart of the so-called common symbiosis
46 signaling pathway (CSSP; see Fig.1). More recently, a key role for Ca^{2+} signaling has also
47 been demonstrated for the nitrogen-fixing endosymbioses formed between filamentous
48 *Frankia* and their actinorhizal hosts (Chabaud *et al.*, 2015; Granqvist *et al.*, 2015). In this

49 review we will focus on the latest discoveries which throw light on Ca^{2+} signaling during the
50 establishment of both the AM and *Frankia*/actinorhizal root symbioses as well as the resulting
51 approaches which are now being employed to identify the corresponding microbial factors
52 which activate the Ca^{2+} -dependent CSSP.

53 **II. Nuclear calcium signaling and the AM symbiosis** 54 **belonging to Glomeromycotina**

54 AM fungi, collectively known as **Glomeromycota**, are widespread obligate biotrophs which
55 are able to colonize roots of the majority (approx. 80%) of plant species, forming elaborate
56 ramified symbiotic structures known as arbuscules within inner cortical cells. Fossil evidence
57 has revealed that analogous structures were present in the tissues of early land plants (over
58 400 million years ago), suggesting that the AM symbiosis played a central role in facilitating
59 plant access to nutrients in a harsh terrestrial environment (Bonfante & Genre, 2008).
60 Furthermore, recent phylogenomic studies have shown that the complete CSSP module is
61 present throughout extant plant clades, thus emphasizing the importance of microbe-host
62 Ca^{2+} -dependent signaling even during these earliest mutualistic associations (Delaux *et al.*,
63 2015).

64 An important advance in the study of oscillatory nuclear Ca^{2+} signaling during the initial
65 stages of endosymbiotic associations was the development of *in vivo* cameleon-based calcium
66 reporters coupled with confocal microscopy imaging (Miwa *et al.*, 2006). In particular, the
67 development of nuclear-localized cameleons such as Nup-YC2.1 (Sieberer *et al.*, 2009)
68 greatly facilitated the detection of Ca^{2+} spiking in atrichoblasts, the non root hair epidermal
69 cells which are the primary targets of AM colonization. Experiments using both legume (*M.*
70 *truncatula*) and non-legume (*Daucus carota*) root organ cultures (ROCs) expressing Nup-
71 YC2.1 were instrumental in first demonstrating nuclear Ca^{2+} spiking in atrichoblasts
72 associated with AM fungal contact and hyphopodium formation (Chabaud *et al.*, 2011; Fig.
73 2a). Significantly, spiking frequency was highest in those cells where the nucleus had
74 migrated to the site of fungal attachment, a key event which precedes the construction of
75 transcellular apoplastic compartment (Genre *et al.*, 2005).

76 The presence of symbiotic fungal signals in germinating spore exudates of several AM
77 species was also examined using Ca^{2+} spiking responses as a bio-assay in both *Medicago* and
78 carrot ROCs expressing Nup-YC2.1. These studies led to the identification of short-chain
79 chitin oligomers (chitotetraose and chitopentaose) as candidate AM signals (Genre *et al.*,
80 2013). Not only can these so-called Myc-COs activate the host CSSP at low concentrations

81 (10^{-8} M) but their levels are greatly enhanced in the spore exudates if the synthetic
82 strigolactone GR24 is present during spore germination. Since plant strigolactones stimulate
83 AM hyphal development prior to initial root contact, these findings provide evidence for
84 reciprocal molecular signaling between host and fungal symbiont during the pre-infection
85 stage. Finally, the nuclear Ca^{2+} spiking elicited in *M. truncatula* atrichoblasts by AM fungal
86 contact, fungal exudates or short-chain Myc-COs (Fig. 2a-c) is generally less regular in both
87 periodicity and spike profile compared to NF-activated spiking in root hairs (Russo *et al.*,
88 2013; Fig. 2d). The reason for this difference in spiking signature is currently unclear, and
89 contrasts with the similar Ca^{2+} spiking profiles associated with both rhizobial and AM
90 infection of cortical cells (Sieberer *et al.*, 2012).

91 In parallel to these studies, the use of NF bioassays (root hair deformation and early nodulin
92 gene expression) had revealed other potential AM fungal signals in the form of either
93 sulphated or non-sulphated LCOs, present in both AM spore and colonized root exudates
94 (Maillet *et al.*, 2011). These Myc-LCOs structurally resemble rhizobial LCOs and elicit
95 similar Ca^{2+} spiking responses to NFs in root hairs of *M. truncatula* seedlings (Sun *et al.*,
96 2015). Whilst *M. truncatula* mutants defective in the LysM receptor-like kinase NFP (Nod
97 Factor Perception) fail to nodulate and are totally unresponsive to NFs (Ben Amor *et al.*,
98 2003), these same mutants exhibit normal AM colonization. Nevertheless, Ca^{2+} spiking is
99 blocked in *nfp* mutants in response to exogenous Myc-LCOs (Sun *et al.*, 2015). Furthermore,
100 transcriptomic approaches have shown that root gene expression in young seedlings in
101 response to Myc-LCOs is also essentially dependent on NFP (Czaja *et al.*, 2012; Hohnjec *et al.*,
102 2015). These similarities between the perception and biological activities of NF and Myc-
103 LCOs make it difficult to evaluate the extent to which both sulphated and non-sulphated Myc-
104 LCO root responses may result from inappropriate activation of the NF-signalling pathway in
105 legumes.

106 In contrast to Myc-LCOs, the Ca^{2+} spiking activity elicited by Myc-COs is unaffected in an
107 *nfp* mutant background (Genre *et al.*, 2013). In addition to the use of whole plants, *M.*
108 *truncatula* ROCs were also used in these studies since ROCs are readily colonized by AM
109 fungi, but cannot be nodulated and are unresponsive to either rhizobia or NFs. When applied
110 to ROCs Myc-COs are significantly more active in triggering spiking compared to Myc-LCOs
111 (Genre *et al.*, 2013). Together, these results suggest differences in the symbiotic roles played
112 by AM fungal COs and LCOs, and indeed it has been demonstrated that whereas both NF-
113 and Myc-LCOs can stimulate lateral root development (Maillet *et al.*, 2011), this is not the

114 case for chitotetraose (Olah *et al.*, 2005). Further detailed discussions of these and related
115 findings can be found in the recent reviews of Bucher *et al.*, (2014); Nadal & Paszkowski
116 (2013) and Schmitz & Harrison (2014).

117 Unfortunately, the absence of AM fungal genetic approaches makes it difficult to ascribe
118 unequivocal signaling roles for either Myc-COs or LCOs. In addition, *nod* gene orthologs
119 were unfortunately not identified in the recently sequenced genome of the AM fungus
120 *Rhizophagus irregularis* (Tisserant *et al.*, 2013). On the other hand, recent studies on non-
121 legume AM hosts are now throwing fresh light on early AM fungal signal perception and in
122 particular the role of certain LysM-RLK receptors. Two research teams have independently
123 shown that CERK1, the rice receptor kinase associated with chitin-triggered immunity is also
124 required for establishing the AM symbiosis. Both knock-out mutant and RNAi experiments
125 have demonstrated that *OsCERK1* is essential for initial AM fungal infection in rice, with a
126 block at the level of epidermal entry (Miyata *et al.*, 2014; Zhang *et al.*, 2015). In contrast,
127 inactivation of OsCEBiP, the second LysM RLK required for perceiving long chain chitin
128 elicitors and activating host immunity, does not result in a defect in AM colonization (Miyata
129 *et al.*, 2014). Interestingly, the closest legume homologs to rice CERK1 are *M. truncatula*
130 LYK3 and *Lotus japonicus* NFR1, both of which are implicated in rhizobial LCO perception.
131 Recent experiments performed using limiting AM inoculation conditions for the two legume
132 hosts have revealed reduced colonization levels for both *Mtlyk3* and *Ljnfr1* mutants (Zhang *et*
133 *al.*, 2015), thus raising the question of the potential role of these CERK1 orthologs in AM
134 fungal perception in legumes.

135 In addition to studies on the monocot rice, two additional examples of defective AM
136 phenotypes result from the silencing of LysM-RLKs in non-legume dicots. Firstly, Op den
137 Camp *et al.* (2011) were able to show defects in arbuscule formation in roots of *Parasponia*
138 *andersonii* following RNAi knockdown of the *PaNFP* gene, although it is not clear whether
139 the initial entry of the AM fungus into the epidermal/cortical tissue is affected. More recently,
140 Buendia *et al.* (2015) have demonstrated by a virus-induced gene silencing approach that
141 knockdown of the *SILYK10* gene, the tomato orthologue of *MtNFP* and *PaNFP*, leads to a
142 block in AM root entry. In conclusion, these important findings in non-legume AM hosts at
143 last provide convincing evidence that fungal symbiotic signals are indeed chitin-based, but at
144 the same time underline the difficulty of deducing the function and precise ligand structure of
145 LysM-RLK receptors based on their phylogenetic proximity.

146 The fact that non-legume AM hosts such as rice, tomato and carrot do not form additional N-
147 fixing endosymbiotic associations also means that, as for legume ROCs, there should be no
148 interference between AM fungal and rhizobial signaling when studying the activation of the
149 CSSP and associated Ca^{2+} spiking. By introducing a nuclear-localizedameleon into rice, Sun
150 *et al.* (2015) have discovered that Myc-COs (but not Myc-LCOs) are able to elicit Ca^{2+}
151 spiking in rice atrichoblasts, and furthermore at similar concentrations (10^{-8} M) to those used
152 previously for *Medicago* or carrot. Whether the rice *oscerk1* mutant is defective for Myc-CO
153 perception remains to be determined.

154 **III. Pre-infection signaling during *Frankia*/actinorhizal plant nodulation**

155 Gram-positive *Frankia* are filamentous actinomycetes which are able to establish
156 endosymbiotic N-fixing associations with a diverse group of angiosperms (8 plant families
157 and 25 genera) belonging to the Rosid I clade (Santi *et al.*, 2013). These actinorhizal hosts are
158 essentially woody shrubs and trees growing in varied habitats and are natural pioneer species
159 due to their capacity for forming mutually beneficial associations with both *Frankia* and
160 mycorrhizal fungi. Despite major differences between legume and actinorhizal nodule
161 ontogeny and structure, the mechanism of *Frankia* root hair infection is nevertheless highly
162 reminiscent of rhizobial/legume infection. For example, in both *Casuarina* and *Alnus* species,
163 *Frankia* enter the host root *via* infection thread structures formed within root hairs (Wall,
164 2000). Furthermore, homologs of many components of the CSSP signaling module are
165 present in both actinorhizal hosts (Hocher *et al.*, 2011), and the essential roles of at least two
166 of these (SYMRK and CCaMK) in *Frankia* nodulation has now been clearly demonstrated for
167 *Casuarina glauca* using RNAi approaches (Gherbi *et al.*, 2008; Svistoonoff *et al.*, 2013). On
168 the other hand, little is currently known about the *Frankia* signals that activate this conserved
169 endosymbiotic pathway. Indeed, the absence of the suite of canonical *nod* genes required for
170 NF-like LCO biosynthesis in the sequenced genomes of *Frankia* strains which nodulate
171 *Casuarina* and *Alnus* (Normand *et al.*, 2007), as well as the failure of *Frankia* DNA to
172 complement rhizobial *nod* gene mutants argues that *Frankia*/host recognition involves
173 different types of molecular signals (C  r  monie *et al.*, 1998).

174 The requirement for a functional CSSP (and in particular CCaMK) in order to initiate *Frankia*
175 infection in actinorhizal hosts also implies that the activation of Ca^{2+} spiking is part of pre-
176 infection *Frankia*-host signaling. This important question has been addressed in two recent
177 publications. Using sonicated extracts from the *Frankia alni* strain ACN14a, Granqvist *et al.*

178 (2015) were able to show that Ca^{2+} oscillations could be elicited in the root hair cytoplasm of
179 *Alnus glutinosa* after microinjection with calcium dyes. In contrast, Ca^{2+} spiking was not
180 observed in response to NFs of the broad host-range *Rhizobium* NGR234. In a second
181 publication, the expression of the nuclear fluorescent probe (Nup-YC2.1) in transgenic roots
182 of *C. glauca* confirmed that sustained nuclear Ca^{2+} spiking can be triggered following the
183 addition of diluted cell-free *Frankia* supernatants (Chabaud *et al.*, 2015; Fig. 2e). These same
184 supernatants can also activate transcription of the infection-related *CgNIN* gene in *Casuarina*
185 root hairs (Clavijo *et al.*, 2015) and furthermore there is a good correlation between nuclear
186 Ca^{2+} spiking and *promoterCgNIN-GFP* expression as a function of the species-specific
187 *Frankia* strains tested (Chabaud *et al.*, 2015).

188 Finally, these two pre-infection responses were used as bio-assays for the preliminary
189 characterization of signaling molecules present in the *Frankia* culture supernatants. These
190 experiments revealed that the *Frankia* factors, in contrast to NF LCOs, partition to the
191 aqueous phase after butanol extraction (Chabaud *et al.*, 2015). Furthermore, since chitinase
192 treatment of the *Frankia* supernatant does not abolish either Ca^{2+} spiking or *ProCgNIN*
193 activity, the *Frankia* symbiotic signals are presumably distinct from those of both rhizobia
194 and AM fungi. If so, this also implies that the host receptors which recognize these signals are
195 unlikely to belong to the chitin-binding LysM-RLK family. Thus, a major priority for future
196 research will be to isolate and chemically identify these novel endosymbiotic signaling factors
197 and their corresponding actinorhizal host receptors.

198 **IV. Conclusions & future outlook**

199 Until now, difficulties in manipulating both the actinorhizal woody host plants and the N-
200 fixing actinomycete *Frankia* have retarded research on early pre-infection signaling.
201 However, the development of genetic transformation systems and extensive databases for the
202 *C. glauca* model have at last made it possible to study host responses to secreted *Frankia*
203 factors at the cellular level. Recent results reviewed here now provide final confirmation that
204 nuclear-associated Ca^{2+} oscillatory signaling is indeed a universal hallmark for the activation
205 of the highly conserved CSSP module in endosymbiotic host plants in response to the
206 perception of the appropriate microbial signals. The stage is now set for the purification and
207 chemical characterization of the novel *Frankia* signaling molecules.

208 Concerning the AM symbiosis, recent successes in identifying infection-defective
209 mycorrhizal phenotypes for certain LysM-RLK mutant or knock-down lines for both rice and

210 tomato provide a powerful argument in favor of the use of such non-legume AM host species
211 for future studies of both the symbiotic fungal signals and the mechanisms of their perception.
212 In the case of rice, mutants such as *oscerk1*, in combination with cameleon-based bioassays
213 for Ca²⁺ signaling/CSSP activation, now offer the means to examine to what extent Myc-CO
214 perception correlates with the AM-defective phenotype, and thus whether short-chain COs
215 may be considered as *bona fide* fungal symbiotic signals.

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355 **Figure Legends**356 **Figure 1**357 ***Schema illustrating the role of the Common Symbiosis Signaling Pathway (CSSP)***

358 A number of plant genes and secondary messengers are required for the successful
359 functioning of the conserved CSSP core module, first discovered in the model legume species
360 *Lotus japonicus* and *Medicago truncatula*, and now extended to plant hosts of all known root
361 endosymbiotic associations including the rhizobial/legume, AM and actinorhizal symbioses.
362 Although several important features of this signaling module still remain to be elucidated, we
363 present here a consensus linear representation of the best-studied pathway components which
364 have been identified for both model legumes, organized around the activation of the nuclear-
365 associated Ca^{2+} spiking response observed in cells of the root epidermis (root hairs or
366 atrichoblasts) prior to the initiation of apoplastic microbial infection (see text). In the case of
367 the rhizobial and AM symbioses the CSSP is activated following symbiotic signal perception
368 by plasma membrane (PM) localized LysM-RLK receptors, most probably part of a larger
369 complex including the leucine-rich repeat receptor-like kinase known as LjSYMRK/MtDMI2
370 (Antolin-Llovera *et al.*, 2014). SYMRK can also interact with 3-hydroxy-3-methylglutaryl-
371 CoA reductase (HMGR), a key enzyme in the so-called mevalonate pathway, a source of
372 potential secondary messengers including mevalonate itself (Venkateshwaran *et al.*, 2015).
373 Following signal transduction from the PM to the nucleus, nuclear membrane cation channels
374 known as LjCASTOR/LjPOLLUX/MtDMI1, likely in association with the recently
375 discovered cyclic nucleotide gated-calcium channel complex CNGC15 (Charpentier *et al.*,
376 2016) are then required for rapid Ca^{2+} release and the initiation of nucleoplasmic Ca^{2+}
377 spiking. Efficient re-uptake of Ca^{2+} across the nuclear membrane between repeated spiking
378 requires a calcium ATPase pump which has been identified as MCA8 in *M. truncatula*
379 (Capoen *et al.*, 2011). The subsequent decoding of the intranuclear Ca^{2+} oscillatory response
380 involves two key associated components (LjCCaMK/MtDMI3 and LjCYCLOPS/MtIPD3).
381 Binding of Ca^{2+} to the calcium and calmodulin-dependent kinase CCaMK (both directly and
382 indirectly via Ca^{2+} /calmodulin) leads to phosphorylation of the coiled-coil protein CYCLOPS
383 (Singh *et al.*, 2014). Finally, the activation of a downstream signaling cascade *via* a repertoire
384 of GRAS/ERF transcription factors results in the synthesis of the suite of proteins required for
385 the transcriptional re-modeling of the epidermal cell in preparation for apoplastic infection.
386 Note that only recent references have been included here, and that further details about the
387 CSSP and nuclear-associated Ca^{2+} spiking can be found in a number of comprehensive review

388 articles (e.g. Charpentier & Oldroyd, 2013; Gutjahr & Parniske, 2013; Oldroyd, 2013;
389 Venkateshwaran *et al.*, 2013). Note also that in the case of the N-fixing actinorhizal
390 association, the nature of both the *Frankia* signal (AF=Actinorhizal Factor) and the
391 corresponding host receptor remain to be determined, and that direct evidence for an essential
392 role in microbial/host signaling has only been demonstrated so far for *CgSYMRK* and
393 *CgCCaMK* (see text).

394 **Figure 2**

395 *Nuclear-associated Ca²⁺ spiking in response to bacterial and fungal symbiotic signals*

396 (a-c). Nuclear Ca²⁺ responses to AM fungal symbiotic factors were recorded in epidermal
397 atrichoblasts using *Medicago truncatula* (*Mt*) root organ cultures (ROCs) expressing the Nup-
398 YC2.1 cameleon (Chabaud *et al.*, 2011; Genre *et al.*, 2013). Representative spiking observed
399 over a 20 min period are shown for (a) cells in direct contact with a fungal hyphopodium
400 prior to infection, or following treatment with either (b) a germinated spore exudate
401 (concentrated 10-fold) of *Gigaspora rosea* or (c) a 10⁻⁸ M solution of chitotetraose (CO4).
402 (d,e). The identical cameleon reporter was used to compare the nuclear Ca²⁺ spiking observed
403 in (d) *M. truncatula* root hairs (intact plant) treated with 10⁻⁹ M *Sinorhizobium meliloti* (*Sm*)
404 Nod factor with (e) *Casuarina glauca* (*Cg*) root hair spiking in response to a crude *Frankia*
405 Cci3 supernatant (SN, diluted 100-fold) (Chabaud *et al.*, 2015). Note that the spiking patterns
406 in response to AM fungal signaling in atrichoblasts are less regular in both frequency and
407 individual spike profile as compared to either *Rhizobium* or *Frankia*-elicited spiking in host
408 root hairs.

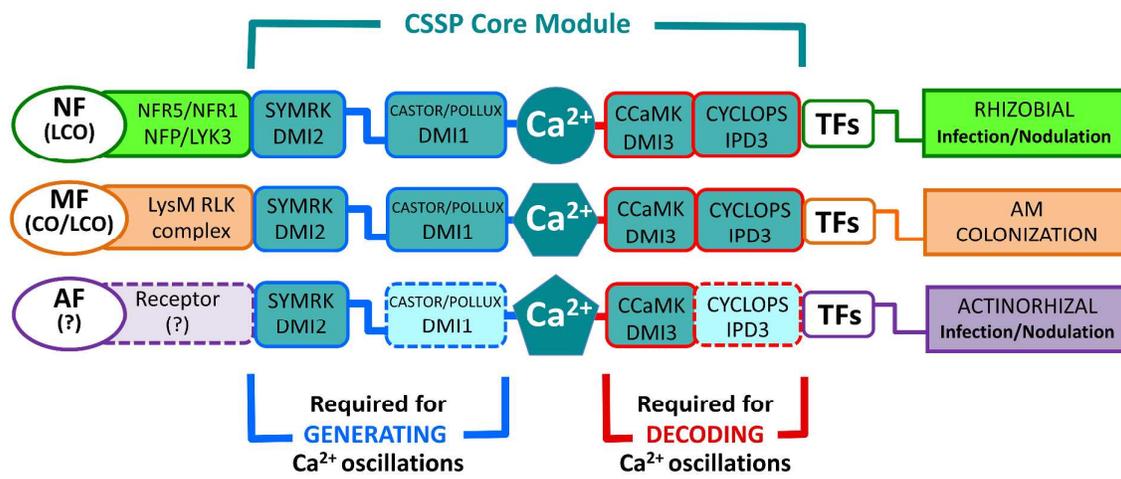


Figure 1

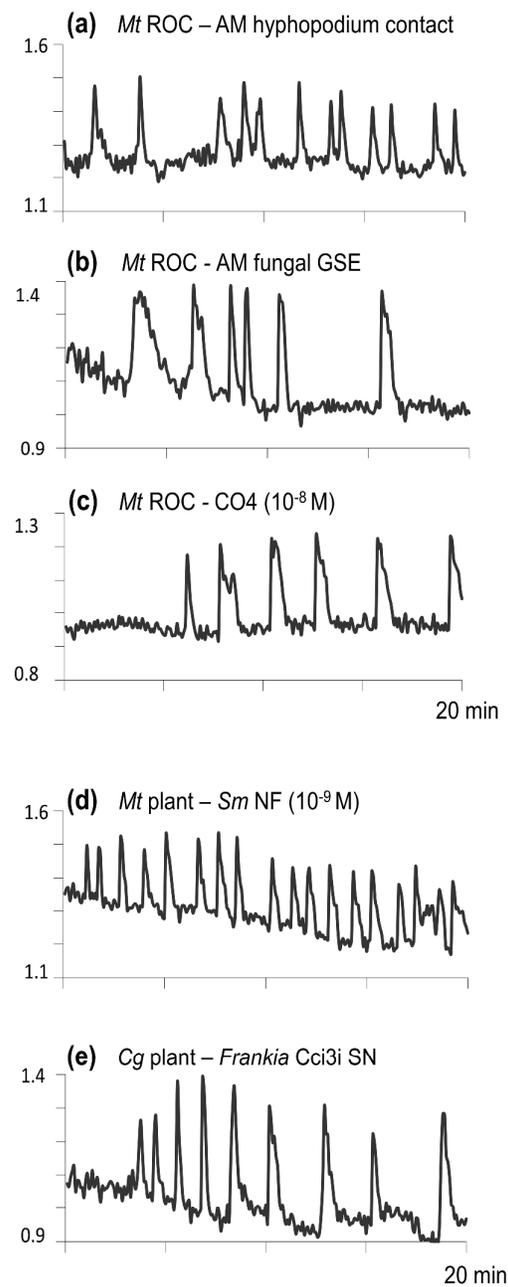


Figure 2