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Nuclear Ca^{2+} signaling in arbuscular mycorrhizal and actinorhizal endosymbioses: on the trail of novel underground signals

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Summary

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

Key Words:

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

I. Introduction

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

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

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

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

II. Nuclear calcium signaling and the AM symbiosis

belonging to Glomeromycotina

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

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

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

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

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

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

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

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

In addition to studies on the monocot rice, two additional examples of defective AM phenotypes result from the silencing of LysM-RLKs in non-legume dicots. Firstly, Op den Camp *et al.* (2011) were able to show defects in arbuscule formation in roots of *Parasponia andersonii* following RNAi knockdown of the *PaNFP* gene, although it is not clear whether the initial entry of the AM fungus into the epidermal/cortical tissue is affected. More recently, Buendia *et al.* (2015) have demonstrated by a virus-induced gene silencing approach that knockdown of the *SlLYK10* gene, the tomato orthologue of *MtNFP* and *PaNFP,* leads to a block in AM root entry. In conclusion, these important findings in non-legume AM hosts at last provide convincing evidence that fungal symbiotic signals are indeed chitin-based, but at the same time underline the difficulty of deducing the function and precise ligand structure of LysM-RLK receptors based on their phylogenetic proximity.

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

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

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

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

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

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

IV. Conclusions & future outlook

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

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

tomato provide a powerful argument in favor of the use of such non-legume AM host species for future studies of both the symbiotic fungal signals and the mechanisms of their perception. In the case of rice, mutants such as *oscerk1,* in combination with cameleon-based bioassays for Ca^{2+} signaling/CSSP activation, now offer the means to examine to what extent Myc-CO perception correlates with the AM-defective phenotype, and thus whether short-chain COs 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

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

Figure 2

Nuclear-associated Ca^{2+} spiking in response to bacterial and fungal symbiotic signals

(**a-c**). Nuclear Ca^{2+} responses to AM fungal symbiotic factors were recorded in epidermal atrichoblasts using *Medicago truncatula* (*Mt*) root organ cultures (ROCs) expressing the Nup-YC2.1 cameleon (Chabaud *et al.,* 2011; Genre *et al.,* 2013). Representative spiking observed over a 20 min period are shown for (**a**) cells in direct contact with a fungal hyphopodium prior to infection, or following treatment with either (**b**) a germinated spore exudate (concentrated 10-fold) of *Gigaspora rosea* or (**c**) a 10^{-8} M solution of chitotetraose (CO4). (**d,e**). The identical cameleon reporter was used to compare the nuclear Ca^{2+} spiking observed in (**d**) *M. truncatula* root hairs (intact plant) treated with 10^{-9} M *Sinorhizobium meliloti* (*Sm*) Nod factor with (**e**) *Casuarina glauca* (*Cg*) root hair spiking in response to a crude *Frankia* Cci3 supernatant (SN, diluted 100-fold) (Chabaud *et al.,* 2015). Note that the spiking patterns in response to AM fungal signaling in atrichoblasts are less regular in both frequency and individual spike profile as compared to either *Rhizobium* or *Frankia*-elicited spiking in host root hairs.

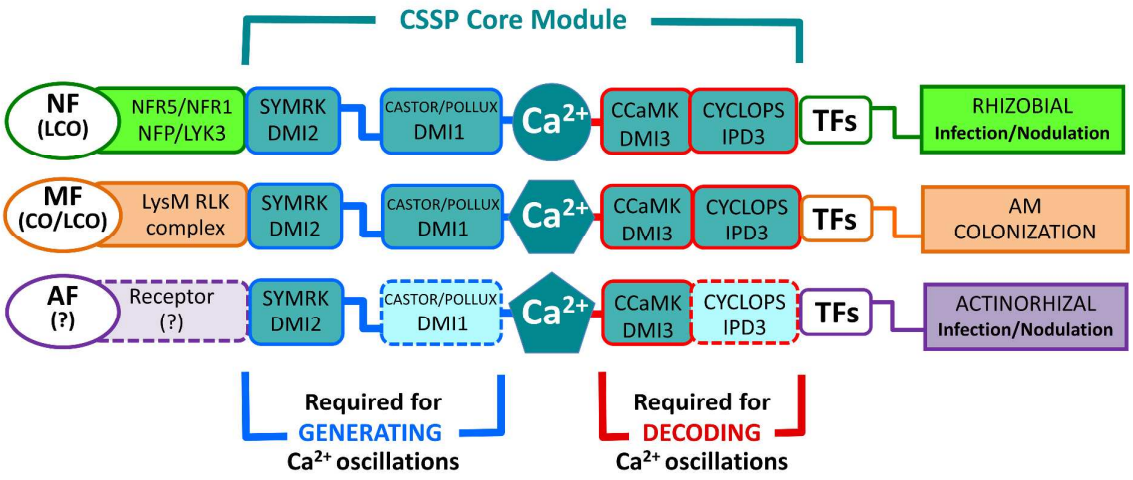

Figure 1

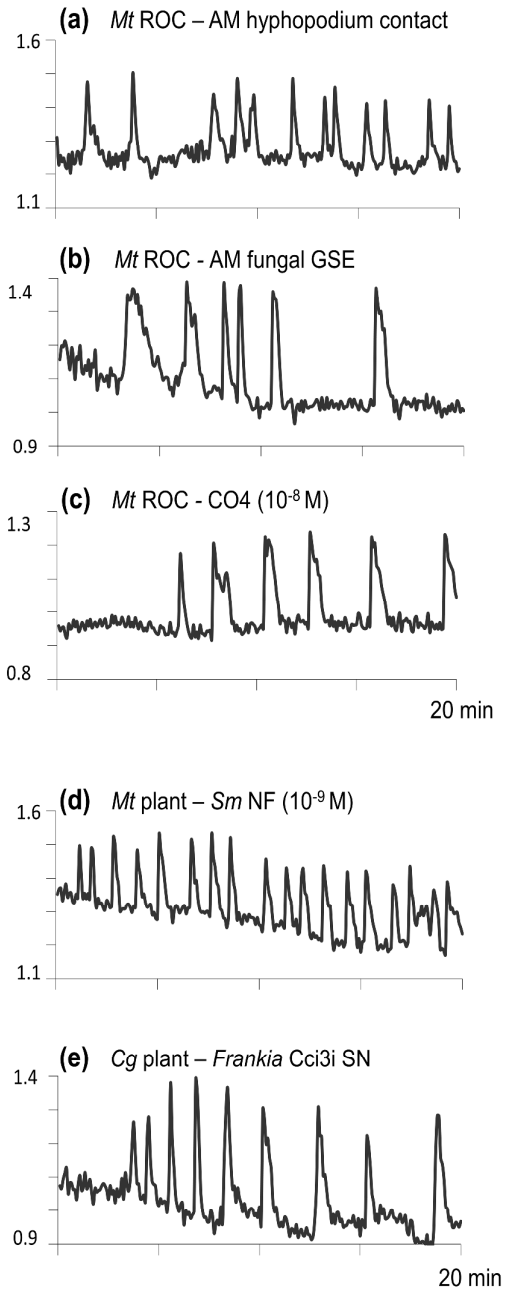
(a) Mt ROC – AM hyphopodium contact
1.6
1.1
(b) Mt ROC - AM fungal GSE
1.4
0.9
(c) Mt ROC - CO4 (10-8 M)
1.3
0.8
20 min
(d) Mt plant – Sm NF (10-9 M)
1.6
1.1
(e) Cg plant – Frankia Cci3i SN
1.4
0.9
20 min

Figure 2