AM fungal exudates activate MAP kinases in plant cells in dependence from cytosolic Ca2+ increase

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Abstract

The molecular dialogue occurring prior to direct contact between the fungal and plant partners of arbuscular-mycorrhizal (AM) symbioses begins with the release of fungal elicitors, so far only partially identified chemically, which can activate specific signaling pathways in the host plant. We show here that the activation of MAPK is also induced by exudates of germinating spores of *Gigaspora margarita* in cultured cells of the non-leguminous species tobacco (*Nicotiana tabacum*), as well as in those of the model legume *Lotus japonicus*. MAPK activity peaked about 15 min after the exposure of the host cells to the fungal exudates (FE). FE were also responsible for a rapid and transient increase in free cytosolic Ca^{2+} in *Nicotiana plumbaginifolia* and tobacco cells, and pre-treatment with a Ca^{2+}-channel blocker (La^{3+}) showed that in these cells, MAPK activation was dependent on the cytosolic Ca^{2+} increase. A partial dependence of MAPK activity on the common Sym pathway could be demonstrated for a cell line of *L. japonicus* defective for *LjSym4* and hence unable to establish an AM symbiosis. Our results show that MAPK activation is triggered by an FE-induced cytosolic Ca^{2+} transient, and that a Sym genetic determinant acts to modulate the intensity and duration of this activity.

Highlights

► MAPK activation is induced by AM fungal exudates (FE) in cultured cells of leguminous and non-leguminous plants. ► FE induce also a transient $[Ca^{2+}]_{cyt}$ increase. ► MAPK activation by FE is dependent on the $[Ca^{2+}]_{cyt}$ increase.► the Sym component Castor is needed for full MAPK activation by FE.

Keywords

- Arbuscular-mycorrhizal fungi;
- Signaling;
- Diffusible factor;
- MAPK;
- Calcium;
- Sym pathway

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1. Introduction

Protein phosphorylation plays a central role in propagating signals from the cell surface to the nucleus initiated by biotic or abiotic stress [1], [2] and [3]. The recognition of the presence of a potential pathogen on a leaf surface has been repeatedly shown to be signaled via mitogen-activated protein kinase (MAPK) cascades. In the model plant *Arabidopsis thaliana*, the three proteins MPK3, MPK4, and MPK6 appear to be activated irrespective of the identity of the pathogen [4]; other MAPKs may also be involved in this process, but these have been only rarely identified as yet, perhaps because of their low abundance and/or faint activation [1]. The same 3 kinases and their homologues in other species have been shown to be activated also by a range of abiotic stresses [5], [6], [7], [8], [9] and [10]. As examples, in *Nicotiana* sp., the activity of both SIPK and WIPK (orthologues of AtMPK6 and AtMPK3, respectively) is inducible not only by various pathogens, but also by wounding and various abiotic stresses [11]. MAPKs are not only activated by biotic and abiotic stress, but also following the perception and synthesis of various phytohormones, and the transduction of certain developmental cues [1]. They have been detected as components of the signaling pathways initiated by abscisic acid, auxin and ethylene, and so play a role in the numerous and diverse processes in which these hormones are involved as regulators [2] and [3]. MAPKs also are key regulators of mitosis, stomatal patterning, and embryo and inflorescence development [12], [13], [14], [15] and [16]. The MAPK signaling pathway represents therefore an example of the elaborate controls necessary for the simultaneous regulation by few transducers of many processes, which in the case of MAPK range from proliferation and survival to metabolism and cell differentiation. Frequently, the modulation of kinetics relies on the activity of specific MAPK phosphatases [1], [17], [18] and [19]. Also, the enzymes acting within the MAPK cascade tend to be physically anchored by scaffold proteins, a device which both maximizes their interaction efficiency – and in some cases, even intensity – and ensures reaction specificity [17].

There is little evidence to date implicating MAPKs as being active in the plant–symbiont interaction, even though certain nodulating bacteria have the capacity to interfere with, and even activate their host's MAPK pathways [20] and [21]. In the arbuscular mycorrhiza (AM)/plant interaction, the only relevant data in the literature pertain to an observed increase in transcription of a single MAPK gene during the pre-contact and the appressorium stage between the barrel medic *Medicago truncatula* and *Glomus mosseae* [22]. Some two thirds of plant species appear able to

establish a symbiosis with AM species in the phylum Glomeromycota [22], [23] and [24]. Signaling pathways in AM symbioses have been explored using forward genetics strategies, particularly based on the model legume species *Lotus japonicus*. These have provided evidence for the existence of a symbiosis pathway (Sym) shared between the rhizobial and the AM processes, and for the use of Ca^{2+} as a secondary messenger in this pathway [25] and [26]. The eight Sym components so far identified in *L. japonicus* encode proteins which are directly or indirectly involved in a signal transduction network required for the development of the intracellular structures needed to accommodate the symbiont (fungus, bacterium) within the host cell. *LjSymRK* encodes a leucine-rich repeat receptor kinase [27], acting upstream of the peri-nuclear Ca^{2+} spiking, which releases Ca^{2+} (probably from the nuclear envelope) via as yet unidentified channels [25]. Among the other components are the two probable cation channels CASTOR (identified from the *L. japonicus sym4* mutant) and POLLUX, which are thought to compensate for the rapid charge imbalances induced by the Ca^{2+} spiking [28] and [29]. NENA, the most recently identified Sym component, is a nucleoporin-related protein that localizes in a conserved sub-complex of the nuclear pore scaffold in *L. japonicus* rhizodermal cells, where it is required for infection [30]. Downstream of Ca^{2+} spiking and NENA action (this latter, in a tissue-specific manner), a Ca^{2+}/calmodulin-dependent protein kinase phosphorylates CYCLOPS, a protein of unknown function, and this complex is thought to transduce the Ca^{2+} signal [25], [26] and [31]. Orthologues of six of the eight above-mentioned genes (the exceptions are *Castor* and *Nena*) have been identified in other legume species [32] and [33].

Mutants defective with respect to the shared symbiosis genes show an AM phenotype in which fungal infection is blocked in the outer cell layers, which is consistent with the notion that the Sym gene products are involved in the early stages of symbiotic signal transduction [34]. Members of the common Sym pathway were shown also to be present in non-leguminous plants, such like rice and liverwort [35] and [36]. An increasing body of data indicates that there must, however, be signaling cues which are specific for AM symbioses. Analysis of *L. japonicus sym* mutants has identified two alternative pathways, one of which is independent of the common Sym pathway, and the other which deviates downstream of the Ca^{2+} spiking mechanism [35]. Similarly, in *M. truncatula*, there is both a *Dmi2*-dependent and a *Dmi2*-independent mechanism for the perception of diffusible fungal signals.

Here, we set out to define the involvement of MAPKs in the signaling pathways induced by a symbiotic fungus on a legume and non-legume host during compatible interactions, and to define the link, if any, between MAPKs and other signaling components, already known to be involved in the perception of AM fungi and/or the establishment of AMF symbiosis; namely, cytosolic Ca^{2+} increase induced by AM fungal exudates (FE), and the Sym gene *LjSym4*. Since the first step in the establishment of AM symbiosis is a pre-contact molecular dialogue through diffusible molecules [37] and [38], we have taken advantage of a simplified system based on the reaction of tobacco and *L. japonicus* cells to exposure to FE as a means of more easily following the early events happening post symbiont recognition. Tobacco is a good non-legume host for AM [39], while the FE released by the germinating spores of *Gigaspora margarita* are known to contain bioactive molecules [40], [41] and [42], the so-called Myc factor(s) [37]. The chemical nature of at least one of these factors has been recently elucidated; they are lipo-chito-oligosaccharides (Myc-LCOs) very similar to Nod factors [43]. Other bioactive molecules with a chitin-based structure are however expected to be produced by AM fungi [44]. The recent culturing of cells of *L. japonicus* homozygous for the loss-of-function *Ljsym4-2* allele (this mutant is unable to develop an AM symbiosis, due to a lesion in *Castor*) has allowed us to define the link between MAPKs and the common Sym pathway [45] and [46].

2. Results

2.1. Activation in tobacco cells of MBP kinases by *G. margarita* FE

To investigate whether MAPKs are a component of the signaling transduction pathways active in the early phases of AM symbiosis, standard in-gel kinase assays using myelin basic protein (MBP) as a substrate were performed on tobacco cell extracts. This technique allows for the visualization of kinases with MBP-phosphorylating activity (most typically MAPKs), in particular revealing their M_r, although not the identity of the isoform(s) present. In our experimental system, the assay identified a transient increase in activity following exposure of the tobacco cells to *G. margarita* FE, with the signal becoming detectable within 5 min; activity remained high for the next 10 min, and then fell to a background level by 30 min after the FE treatment (Fig. 1). The M_r of the MBP kinase identified was ~50 kDa. Control cells not exposed to FE showed no such kinase activity, while treatment of the cells with cryptogein (positive control) produced a stronger signal (Fig. 1). When challenged with an antibody recognizing the activated form of MAPKs, two bands (M_r ~50 and 46 kDa) were detected (Fig. 2A). The temporal pattern of activation of the former (p50PK) was similar to the one of the MBP kinase detected by the in-gel assay. No equivalent data were available for the temporal activation of p46PK, because its activity was not detectable using the in-gel assay. The denaturation and renaturation steps included in the in-gel assay are known to abolish the activity of some MAPK isoforms [47], so p46PK may only be detectable via western blotting. Note that both p50PK and p46PK were induced by cryptogein (Fig. 2A). To verify that the FE-induced elicitation of MAPK activity was not simply based on variation in the quantity of MAPK protein at various time points, the cell extracts were also challenged by a broadly based anti-MAPK antibody (including non-phosphorylated and inactive forms). This experiment showed that comparable quantities of total MAPKs were present throughout (Fig. 2B).

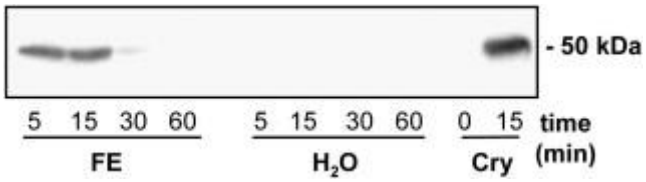

Fig. 1. The activation of host MBP kinases by FE released by germinating spores of *G. margarita*, as analyzed by an in-gel kinase assay. *N. tabacum* cells were treated with FE (5% v/v) for 0–60 min. A negative control was provided by a treatment with an equal volume of water, and a positive one by a 15 min exposure to 50 nM cryptogein. This image is representative of three independent experiments.

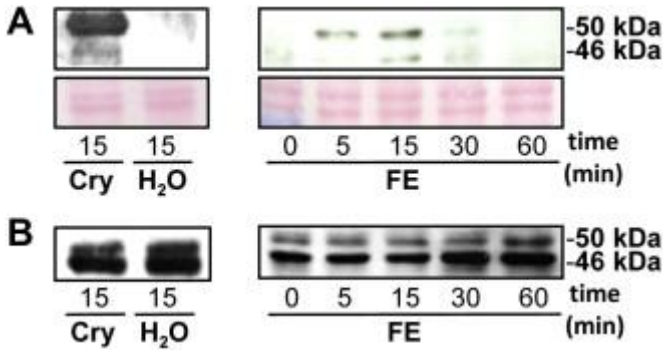

Fig. 2. Immunodetection of activated and total MAPKs in extracts from FE treated *N. tabacum* cells. Cells of *N. tabacum* were harvested over 0–60 min of exposure to 5% v/v FE. Following blotting, membranes were hybridized with either (A) anti MAPK (activated forms), or (B) total MAPK (both active and non active forms) antibodies. The lower panel of (A) shows Ponceau staining, to demonstrate evenness of sampling loading and transfer. Treatment with cryptogein (50 nM, 15 min) represents the positive control. This image is representative of two independent experiments, and shows the results obtained on the same samples analyzed in Fig. 1.

2.2. FE-induced Ca^{2+} elevation in *Nicotiana* cells and Ca^{2+}-dependency of FE-induced MAPK activation

Calcium is known to act as a secondary messenger upstream of MAPKs in several systems, including elicitor-treated cultured plant cells [48]. However, since activation of MAPKs can also occur independently of Ca^{2+} [49], the Ca^{2+} dependence of MAPK activation (if any) needs to be experimentally proven. Navazio et al. (2007) [41] have demonstrated that FE derived from *G. margarita* elicits a cytosolic Ca^{2+} spike in soybean cells. Therefore, to establish whether this response also occurs in non-legume plants, it was first necessary to test the responsiveness of *Nicotiana* cells to FE in terms of their cytosolic Ca^{2+} behavior. Subsequently it was also important to determine whether or not the observed increase in cytosolic Ca^{2+} was due to an influx of ions from outside of the cell.

A peak of 0.8 μM free Ca^{2+} was observed over the first 2 min following the FE treatment of the *N. tabacum* cells (Fig. 3A). The *Nicotiana plumbaginifolia* cells reacted even more intensely, reaching a peak ~1 μM free Ca^{2+} (Fig. 3B), often followed by a secondary, smaller, more variable amplitude peak shortly thereafter (data not shown). The signal dissipated within few minutes. Exposure to cryptogein produced a major, persistent but later-appearing peak in both cell lines, of similar height to the ones generated by the FE treatment and in agreement with previously published results [50]. When challenged with 2 mM La^{3+} (an established Ca^{2+}-channel blocker), the Ca^{2+} signal was abolished in both the *N. tabacum* and *N. plumbaginifolia* cells (Fig. 3A, B). The interdependence of the Ca^{2+} peak and MAPK activity was then tested by monitoring the performance of *N. tabacum* cells treated with FE in the presence of La^{3+}. This experiment showed that FE-induced kinase activation 15 min after treatment was inversely correlated to the concentration of La^{3+} within the range 0–2 mM (Fig. 4). MBP kinase activity became gradually attenuated in the presence of 0.5 and 1 mM La^{3+} and was abolished at 2 mM La^{3+}. The same concentrations of La^{3+} were used for cells treated with 50 nM cryptogein and with water (positive and negative controls), with results in accordance with previous reports [48].

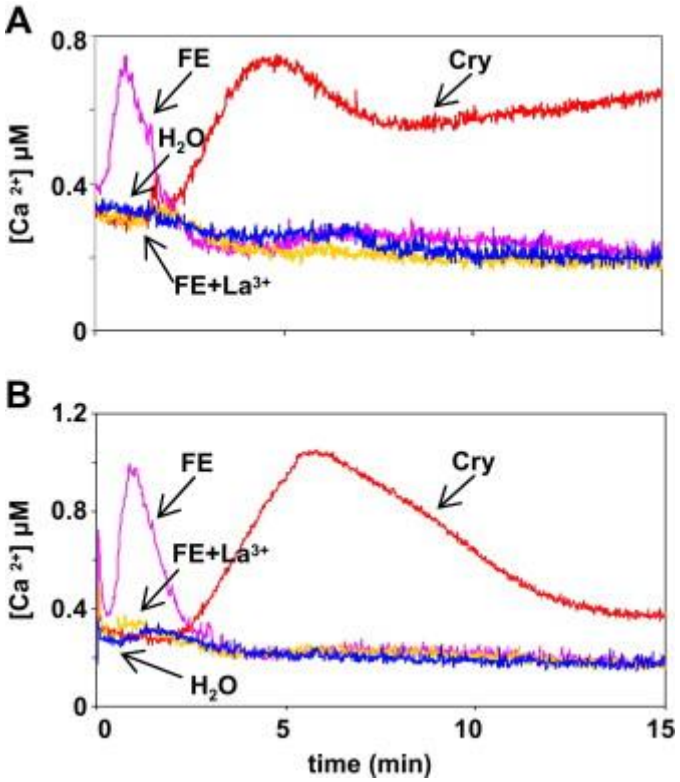

Fig. 3. A spike in cytosolic Ca^{2+} is induced by FE in *N. tabacum* and *N. plumbaginifolia* cells. The effect of FE on cytosolic Ca^{2+} concentration measured for transgenic (A) *N. tabacum* cells, and (B) *N. plumbaginifolia* cells, expressing apoaequorin. FE concentrations were (A) 10% v/v, (B) 5% v/v. Negative control cells were treated with water, positive ones with cryptogein (50 nM). Pretreatment with 2 mM La^{3+} inhibited FE-induced Ca^{2+} influx in both cell types. These graphs are representative of five separate experiments, which gave very similar results.

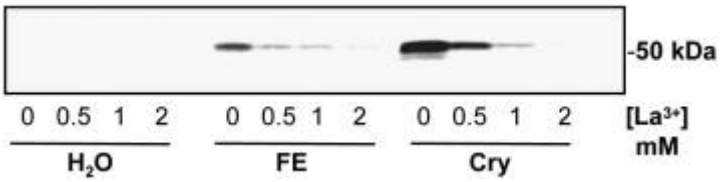

Fig. 4. Dose-dependent inhibition by La^{3+} of the activation of MBP kinases by FE in tobacco cells. MBP kinases were activated by exposing *N. tabacum* cells previously treated with a given concentration of La^{3+} (0–2 mM) to FE (5% v/v, 15 min). The same concentrations of La^{3+} were also used for control cells treated with 50 nM cryptogein (positive control, 15 min) or water (negative control, 15 min). The picture shows one representative set of results of two independent experiments.

2.3. MBP kinase/MAPK activity in *L. japonicus* cells

To investigate the importance of the shared Sym pathway for the induction of MBP kinase activity, the focus was then switched to *L. japonicus*. This choice reflected the situation that it is only in the legumes that Sym genes have been well characterized [26], and that among the various legume models, cell cultures have been established in this particular species [45]. MBP kinase activity in a wild type (WT) *L. japonicus* cell line reacted to exposure to FE in a similar manner as did the tobacco cells (Fig. 5), although the activation appeared to be more long-lived, remaining detectable 60 min after the treatment. The different species can easily account for such difference. The following comparison between the WT cells and those of the *LjSym4* mutant showed that the activity of two kinases, one with an M_r of ~50 kDa and the other 46 kDa, was elicited in both cell lines (Fig. 5). The latter product was either less active and/or less abundant than the former in both cell lines, but their time courses of activity were similar. Both the level of induction and its duration were markedly greater in the WT cells, in which activation peaked within 15 min of exposure to FE (similar to the behavior of the tobacco cells) and remained stable for up to 60 min. In the mutant, the peak time was similar, but thereafter the signal decayed rapidly (Fig. 5). Cells treated with the same volumes of water showed no background activity, while in the positive controls treated with chitin a strong and similarly intense MBP kinase activity was induced both in the WT and mutant cell line, confirming the standard reactivity of the latter to general elicitors (Fig. 5). Further experiments confirmed that the MBP kinase activities induced by FE in both the WT and mutant cells were most likely due to MAPKs. Two products with M_r ~50 and 46 kDa were detected by antibodies targeting phosphorylated, active MAPK isoforms (Fig. 6A). The abundance of the active MAPK as detected by the western blot reflected the kinetics of MBP kinase observed in the in-gel assays, both for the WT and mutant cell line. The positive control based on crab shell chitin induced MAPK phosphorylation equally strongly both in the WT and mutant cell lines. When challenged by a broad anti-MAPK antibody, it was clear that for the same amount of total soluble proteins loaded on the gel, the total amount of MAPKs present in the WT cells was less than that in the mutant line cells (Fig. 6B), demonstrating that the weaker and less persistent MAPK activity shown by the mutant could not have been due to the presence of a lesser quantity of total MAPKs.

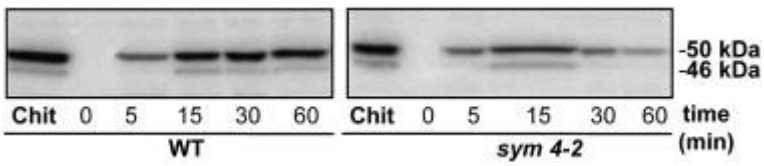

Fig. 5. FE induction of MBP kinase activity in *L. japonicus* cells. Standard in-gel kinase assays were performed on cultured *L. japonicus* cells (WT or *sym4-2* mutant) exposed to FE for 0–60 min. No activation was observed in either cell type when treated with water (negative control), while the positive control (1 µg/µl chitin, 15 min) produced strong activation in both cell types. The image is representative of three independent experiments.

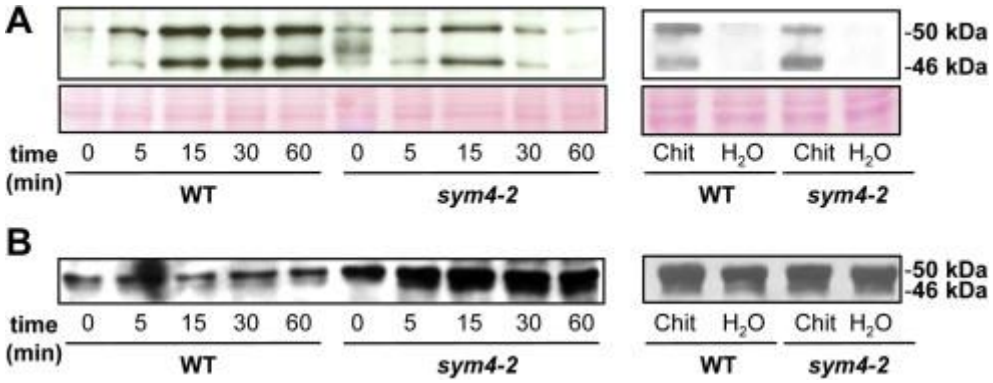

Fig. 6. Immunodetection of activated and total MAPKs in FE treated *L. japonicus* cells. Cells of WT and *sym4-2* mutant *L. japonicus* were exposed to FE (5% v/v) for 0–60 min. Western blot following hybridization with (A) anti MAPK (activated forms), or (B) total MAPK (both active and non active forms). The lower panel of (A) shows Ponceau staining, to demonstrate evenness of sampling loading and transfer. Treatment with chitin (1 µg/µl, 15 min) represents the positive control. This image is representative of three independent experiments, and shows the same samples analyzed in Fig. 5. The minor, low molecular weight product present at time zero in one of the negative controls for *Ljsym4-2* is an artifact due to the presence of the weight ladder in the same lane.

3. Discussion

We have demonstrated that cultured cells of both legume and non-legume plants respond to exudates released during the germination of the spores of an AM fungus by the rapid activation of MAPKs. The simplified system employed has already proven competent to perceive the diffusible signal(s) present in FE [41], and it provides a means of monitoring and analyzing the events which might occur during the pre-symbiotic phase, although it does not fully represent the *in planta* establishment of a successful AM symbiosis. Signaling triggered by symbiont recognition and symbiont accommodation in plant tissues are generally recognized as distinct events, and are probably under independent genetic control and regulation [31]. Indeed, even cells that do not participate in the accommodation of the AM fungus, such as root hairs, are able to perceive diffusible fungal factors [25] and [42].

3.1. MAPK activation following the perception of FE from an AM fungus is an early response in both legume and non-legume plants

The MAPKs represented a clear set of candidate transducers of the FE stimulus, as they are known to represent an important connection between the perception of extracellular stimuli and cellular responses. MAPK cascades are activated by a range of different stimuli, including wounding, various elicitors, a number of abiotic stresses, phytohormones and certain developmental and differentiation cues [1], [2] and [4]. The in-gel kinase assays and subsequent immunological analyses in tobacco demonstrated that the FE were able to induce MAPK activation. We are not aware of any reported MAPK activation in compatible interactions between plants and fungi as part of the establishment of AM symbiosis, and in particular prior to any direct contact between the symbiont and its host. The current picture of the molecular events and the identity of the components involved in AM signaling remains based on the analysis of the Sym pathway. For this reason, it was important to provide a link between the activation of MAPK activity by FE in tobacco cells and the AM symbiosis pathway by analyzing the response of *L. japonicus* cells defective for *LjSym4*, a gene encoding a key ion channel in the Sym pathway, now named CASTOR [26]. The *LjSym4* mutant cannot establish a successful AM symbiosis, and the infection process is

aborted at an early stage. Morphologically, this appears to be because the mutant does not develop sufficient structural support to accommodate the fungus within its epidermis [46]. At the molecular level, mutants defective in *Castor* do not undergo Ca^{2+} spiking following perception of the AM fungus [28]; it is thought that a flux of K^+ through the same channel is necessary to compensate for the charge loss associated with the Ca^{2+} spiking at the surface of the nuclear envelope by an as yet unknown Ca^{2+} channel. This implicates CASTOR in peri-nuclear Ca^{2+} spiking, albeit indirectly [26]. When protein extracts from FE-treated *L. japonicus* cells were subjected to an in-gel kinase assay, two MBP-phosphorylating proteins were detected both in the WT and the mutant cells. These appeared to correspond well with those detected by anti-phospho MAPK antibodies, and are likely to represent at least two of many MAPK isoforms. The activation kinetics of the WT differed markedly from those of the mutant cells, with the latter not achieving the degree of intensity and duration as the former did. These differences were demonstrated not to have derived from a lower quantity of total MAPK in the mutant, so must depend upon post-translational phosphorylation events. In fact, the quantity of MAPK proteins in the mutant increased over time (Fig. 6B), perhaps reflecting a compensation mechanism for the poorer level of its kinase activation. This increased MAPK presence may even have generated an over-estimate in the extent of FE-induced MAPK activity in the mutant, and hence an under-estimate of the difference between the two cell types. Both intensity and duration are important determinants of the final outcome of MAPK activation [17]. Whether MAPK activation is essential for the establishment of AM symbiosis remains to be proven. The versatility of the MAPK system, and the many processes in which they have been implicated, makes any extrapolation of their role to an *in planta* situation highly speculative at this point. MAPKs have frequently been implicated in the defense response, and several authors have highlighted the resemblance between the plant-pathogen and the legume-symbiont interactions, especially during their early phases [51] and [52]. As an example, during the nodulation process, NopL (a *Rhizobium* sp. NGR234 effector protein) is activated by protein kinases, and in turn modulates the activation of plant defense reactions [20] and [53]. Therefore, the activation observed in the present experiments may be both associated with decisions which the host cells need to take in order to accommodate the symbiont, and/or to the triggering of defense in response to the perception of the AM fungus as being non-self. The negative effect of the *sym4-2* allele on MAPK activation by FE, though, supports the notion that the phenomenon is AM-related (see following Section 3.2).

3.2. MAPK activation by *G. margarita* FE is dependent on a cytosolic Ca^{2+} increase

The tobacco cells responded to FE with a rapid, transient increase in cytosolic Ca^{2+}. This response is very much in line with the behavior of soybean cells, as reported by Navazio et al. (2007); and also with the finding that the same FE preparations induce peri-nuclear Ca^{2+} spiking in atrichoblasts of legume and non-legume plants [42]. These results show that the perception of AM factors, in terms of both cytosolic Ca^{2+} increase, peri-nuclear Ca^{2+} spiking and MAPK activation, evolved long before the appearance of the legumes. The evolution of the Leguminosae subfamily Papilionidae has been dated to ~50–60 Mya [54], which is some 400 My after plants had colonized dry land, and AM symbiosis had evolved [55]. Thus although direct experimental evidence is lacking (since no *L. japonicus* cell lines expressing aequorin are as yet available), it is highly probable that *L. japonicus* reacts to microbial symbiont signals, as other legumes do, with a rapid Ca^{2+} influx in the cytoplasm, in addition to a pronounced Ca^{2+} spiking at the nuclear level [42] and [56]. We sought to establish whether there was any relationship between MAPK activation and the observed variation in cytosolic Ca^{2+} content by treating tobacco cells with FE in the presence of La^{3+}, a known inhibitor of Ca^{2+} channels. La^{3+} was already shown to block elicitor-induced reactive oxygen species (ROS) production, plasma membrane depolarization, cytosol acidification, chloride efflux and MAPK activation in our same experimental system [48] and [57]. FE-induced Ca^{2+} influx and MAPK activation were blocked by the presence of 2 mM La^{3+}, showing that MAPK activation by

FE depends on cytosolic Ca^{2+} elevation (at least in tobacco). The same test was inconclusive on Lotus cells, since La^{3+} treatments in the mM range appeared to stimulate basal MBPK activity even in the absence of elicitor treatment, at least under our conditions (data not shown). Given that the identity of the all bioactive component(s) of FE is not as yet known, one cannot exclude the hypothesis that general elicitors (such as chitin fragments) are present in our FE preparations. A chitin backbone was already suggested to be part of the Myc factor(s) molecule [38], and a Nod-factor-like molecule was proven to be produced by AMF fungi [43]; so a simple biochemical assay for the presence of chitin would not solve this issue. However, WT and mutant *L. japonicus* cells respond differently to *G. margarita* FE in terms of MAPK activation, while they do not to chitin (Fig. 5).

In addition to the Ca^{2+}-MAPK relationship in tobacco, our results demonstrate the dependence of FE-induced MAPK activation on components of the Sym pathway, downstream or at the level of Sym4 (synonym of CASTOR in *L. japonicus*). The CASTOR protein is not a Ca^{2+} channel itself, but its activity is required for normal peri-nuclear Ca^{2+} spiking following perception of the AM fungus and for symbiosis. A connection between the peri-nuclear Ca^{2+} spiking in the context of the common Sym pathway and cytosolic Ca^{2+} influx induced by FE cannot be proven at this stage; rather, the two are currently thought to be processes separated functionally and in time [31]. However, since the MAPK activation was less intense in the *Ljsym4-2* mutant (where the functionality of the Sym pathway is affected), it is legitimate to speculate that at least in theory the Ca^{2+} involved in peri-nuclear spiking may contribute *inter alia* to the maintenance of the Ca^{2+} concentration above a critical threshold in the cytoplasm. Such a hypothesis could be tested using a sym-defective *L. japonicus* cell line transformed to express aequorin. More probably, the common Sym pathway modulates MAPK activity via a more indirect mechanism, that may or may not include peri-nuclear Ca^{2+} spiking. In any case, that MAPK activation was triggered by FE treatment in both the WT and mutant *L. japonicus* cell lines indicates that CASTOR activity, indispensable for peri-nuclear Ca^{2+} spiking, is not the initial MAPK-triggering stimulus, but rather produces a signal directly or indirectly required for the expression of sustained and intense MAPK activity.

4. Methods

4.1. Biological material

Cell cultures of *N. tabacum* var. Xanthi and *N. plumbaginifolia* expressing apoaequorin, and of wild type *N. tabacum* var. Xanthi were grown in Chandler's medium [58] under shaking conditions (150 rpm). The wild type line was cultivated in the dark. Cells were maintained in the exponential phase and sub-cultured one day prior to utilization. Cell suspensions of *L. japonicus* Gifu (WT and mutant *Ljsym4-2*) were grown in B52 medium [Gamborg B5 medium with supplementary vitamins (Duchefa), sucrose (2% w/v) and (2-4D) 0.02% w/v] at 25 °C on a rotary shaker (80 rpm) [45]. *G. margarita* BEG34 spores were produced from mycorrhizal infected sand-grown clover (*Trifolium repens*) plants. Spores were collected from the soil, counted by stereomicroscopy, held at 4 °C for two weeks and surface-sterilized, by immersing ~100 spores per 2 ml Eppendorf tube twice for 10 min in 3% w/v chloramine T, 0.03% w/v streptomycine sulphate in distilled water, followed by three washes of 10 min each in 1.5 ml sterile water each wash. The sterilized spores were kept in sterile water (100 spores/ml) at 30 °C in the dark for seven days, and the germination rate monitored by stereomicroscopy. The fungal germination medium was collected, concentrated tenfold by lyophilization and stored at −20 °C until required.

4.2. Cell treatments

Cells at the exponential growth phase were collected, washed by filtration in a suspension buffer [175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 10 mM MES (pH5.8)], re-suspended at a rate of 0.1 g fresh weight/ml suspension buffer, and equilibrated by shaking for 2 h on a rotary shaker (150 rpm, 24 °C). FE (5% v/v) was then added, and cell aliquots taken at 5, 15, 30 and 60 min after the treatment commenced. Where indicated, a 10 min pre-treatment with various concentrations of $LaCl_3$ (0, 0.5, 1, 2 mM) was added. As positive controls, cells were treated for 15 min with either 50 nM cryptogein (*Nicotiana* sp.) or 1 mg/ml crab shell chitin (*L. japonicus*), while the negative control consisted of cells incubated in a matching volume of water.

4.3. Protein extraction

Tobacco protein was extracted from 250 mg cultured cells by first grinding in liquid nitrogen, then thawing in the presence of 150 µl 50 mM HEPES (pH7.5) containing 5 mM EDTA, 5 mM EGTA, 10 mM DTT, 1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 5 µg/ml leupeptin, 5 µg/ml antipapain, 1 mM phenylmethylsulfonyl fluoride. Lotus was extracted in the same volume, but with 2× buffer. Following centrifugation (10,000 *g*), aliquots of the supernatant were stored at −80 °C or used immediately. Protein concentrations were quantified using the Bradford method with bovine serum albumin (BSA) as the standard.

4.4. In-gel assay

An in-gel kinase assay was performed as described elsewhere [59], with minor modifications. Twenty-five µg total soluble protein were electrophoresed through 10% SDS-polyacrylamide gels containing 0.25 mg/ml MBP in the resolving gel. After electrophoresis, the SDS was removed by first washing for 1 h in 50 mM Tris–HCl (pH8.0), 20% v/v 2-propanol, and then for a further hour in 50 mM Tris–HCl (pH8.0), 5 mM β-mercaptoethanol. The electrophoresed proteins were denatured by immersing the gel for 1 h in 6 M guanidine-HCl, 50 mM Tris–HCl (pH8.0), 5 mM β-mercaptoethanol, and then allowed to renature over the course of five successive washes over 16 h at 4 °C in 50 mM Tris–HCl (pH8.0), 5 mM β-mercaptoethanol, 0.04% (v/v) Tween-40. Following an equilibration step [30 min at room temperature in 40 mM HEPES (pH7.5), 0.1 mM EGTA, 20 mM $MgCl_2$, 2 mM DTT], the gels were immersed for 1 h in the same buffer supplemented with 25 µM ATP and 10µCi [γ-^{32}P]-ATP (Amersham). The labeling reaction was stopped by extensive washing in 5% w/v trichloroacetic acid containing 1% w/v potassium pyrophosphate. The gels were then dried and exposed to Kodak films. Pre-stained molecular markers (Sigma or Bio-Rad) were included to allow the estimation of M_rs of the MBP kinases.

4.5. Immunoblot analysis

Protein extracts (15 µg total soluble protein) were subjected to electrophoresis through 10% SDS-polyacrylamide gels, and transferred to a nitrocellulose membrane (0.45 µm pore size; HybondC, Amersham) by semi-dry electroblotting using a buffer containing 48 mM Tris–HCl, 39 mM glycine, 0.0187% w/v SDS and 20% v/v methanol. After transfer, the membrane was blocked for 1 h in 10 mM Tris–HCl (pH7.5), 150 mM NaCl supplemented with 1% w/v BSA. Detection of proteins was performed as described by the manufacturer of the ECL western detection kit (Amersham). The polyclonal phospho-specific MAPK antibody used was raised against a synthetic phosphotyrosine peptide corresponding to residues 202–204 of human p44 MAPK (ERK1) or to residues 185–187 of ERK2 (Cell Signalling Technology, working dilution 1:1000). Horseradish peroxidase-anti-rabbit antibody (Bio-Rad) were used as the secondary antibody. For the detection of

total MAPK, the p44/42 MAPK (ERK1/2) antibody (Cell Signalling Technology) was used at a 1:1000 working dilution and detected as above.

4.6. Ca^{2+} measurements

In vivo reconstitution of aequorin was performed by the addition of 1 µM coelenterazine to cells held in a suspension buffer for at least 3 h in the dark (150 rpm, 24 °C). The bioluminescence of 250 µl aliquots of cells was recorded continuously at 1s intervals using a digital luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany). Residual functional aequorin was quantified by the addition of 300 µl 10 mM $CaCl_2$, 2% v/v Nonidet-P40, 20% v/v ethanol, and monitoring the resulting increase in luminescence. The translation of luminescence values into cytosolic Ca^{2+} concentrations was calculated following a published report [60].

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References

J. Colcombet, H. Hirt Arabidopsis MAPKs: a complex signalling network involved in multiple biological processes Biochem. J., 413 (2008), pp. 217–226

N.S. Mishra, R. Tuteja, N. Tuteja Signaling through MAP kinase networks in plants Arch. Biochem. Biophys., 452 (2006), pp. 55–68

T. Zhang, Y. Liu, T. Yang, L. Zhang, S. Xu, L. Xue, L. An Diverse signals converge at MAPK cascades in plant Plant Physiol. Biochem., 44 (2006), pp. 274–283

A. Pitzschke, A. Schikora, H. Hirt MAPK cascade signalling networks in plant defence Curr. Opin. Plant Biol., 12 (2009), pp. 421–426

M. Droillard, M. Boudsocq, H. Barbier-Brygoo, C. Lauriere Different protein kinase families are activated by osmotic stresses in *Arabidopsis thaliana* cell suspensions. Involvement of the MAP kinases AtMPK3 and AtMPK6 FEBS Lett., 527 (2002), pp. 43–50

M.J. Droillard, M. Boudsocq, H. Barbier-Brygoo, C. Lauriere Involvement of MPK4 in osmotic stress response pathways in cell suspensions and plantlets of *Arabidopsis thaliana*: activation by hypoosmolarity and negative role in hyperosmolarity tolerance FEBS Lett., 574 (2004), pp. 42–48

K. Ichimura, T. Mizoguchi, R. Yoshida, T. Yuasa, K. Shinozaki Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6 Plant J., 24 (2000), pp. 655–

M. Teige, E. Scheikl, T. Eulgem, R. Doczi, K. Ichimura, K. Shinozaki, J.L. Dangl, H. Hirt The MKK2 p.thway mediates cold and salt stress signaling in Arabidopsis Mol. Cell, 15 (2004), pp. 141–152

S. Seo, H. Sano, Y. Ohashi Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase Plant Cell, 11 (1999), pp. 289–298

J.W. Stratmann, C.A. Ryan Myelin basic protein kinase activity in tomato leaves is induced systemically by wounding and increases in response to systemin and oligosaccharide elicitors Proc. Natl. Acad. Sci. U.S.A., 94 (1997), pp. 11085–11089

C. Jonak, L. Okresz, L. Bögre, H. Hirt Complexity, cross talk and integration of plant MAP kinase signalling Curr. Opin. Plant Biol., 5 (2002), pp. 415–

D.C. Bergmann, W. Lukowitz, C.R. Somerville Stomatal development and pattern controlled by a MAPKK kinase Science, 304 (2004), pp. 1494–1497

L. Bögre, O. Calderini, I. Meskiene, P. Binarova Regulation of cell division and the cytoskeleton by mitogen-activated protein kinases in higher plants Results Probl. Cell Differ., 27 (2000), pp. 95–117

O. Calderini, L. Bögre, O. Vicente, P. Binarova, E. Heberle-Bors, C. Wilson A cell cycle regulated MAP kinase with a possible role in cytokinesis in tobacco cells J. Cell Sci., 111 (1998), pp. 3091–3100

S.M. Bush, P.J. Krysan Mutational evidence that the Arabidopsis MAP kinase MPK6 is involved in anther, inflorescence, and embryo development J. Exp. Bot., 58 (2007), pp. 2181–2191

P.J. Krysan, P.J. Jester, J.R. Gottwald, M.R. Sussman An Arabidopsis mitogen-activated protein kinase kinase kinase gene family encodes essential positive regulators of cytokinesis Plant Cell, 14 (2002), pp. 1109–1120

M.D. Brown, D.B. Sacks Protein scaffolds in MAP kinase signalling Cell. Signal, 21 (2009), pp. 462–469

S. Luan Protein phosphatases in plants Annu. Rev. Plant Biol., 54 (2003), pp. 63–92

A. Schweighofer, H. Hirt, I. Meskiene Plant PP2C phosphatases: emerging functions in stress signaling Trends Plant Sci., 9 (2004), pp. 236–243

A.V. Bartsev, W.J. Deakin, N.M. Boukli, C.B. McAlvin, G. Stacey, P. Malnoe, W.J. Broughton, C. Staehelin NopL, an effector protein of *Rhizobium* sp. NGR234, thwarts activation of plant defense reactions Plant Physiol., 134 (2004), pp. 871–879

M. Fernandez-Pascual, M.M. Lucas, M.R. de Felipe, L. Bosca, H. Hirt, M.P. Golvano Involvement of mitogen-activated protein kinases in the symbiosis *Bradyrhizobium- Lupinus* J. Exp. Bot., 57 (2006), pp. 2735–2742

S. Weidmann, L. Sanchez, J. Descombin, O. Chatagnier, S. Gianinazzi, V. Gianinazzi-Pearson Fungal elicitation of signal transduction-related plant genes precedes mycorrhiza establishment and requires the *DMI3* gene in *Medicago truncatula* Mol. Plant–Microbe Interact., 17 (2004), pp. 1385–1393

A.H. Fitter Darkness visible: reflections on underground ecology J. Ecol., 93 (2005), pp. 231–243

S.E. Smith, D.J. Read Mycorrhizal Symbiosis Academic Press, San Diego (2008)

S. Kosuta, S. Hazledine, J. Sun, H. Miwa, R.J. Morris, J.A. Downie, G.E. Oldroyd Differential and chaotic calcium signatures in the symbiosis signaling pathway of legumes Proc. Natl. Acad. Sci. U.S.A., 105 (2008), pp. 9823–9828

M. Parniske Arbuscular mycorrhiza: the mother of plant root endosymbioses Nat. Rev. Microbiol., 6 (2008), pp. 763–775

S. Stracke, C. Kistner, S. Yoshida, L. Mulder, S. Sato, T. Kaneko, S. Tabata, N. Sandal, J. Stougaard, K. Szczyglowski, M. Parniske A plant receptor-like kinase required for both bacterial and fungal symbiosis Nature, 417 (2002), pp. 959–962

M. Charpentier, R. Bredemeier, G. Wanner, N. Takeda, E. Schleiff, M. Parniske *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis Plant Cell, 20 (2008), pp. 3467–3479

H. Imaizumi-Anraku, N. Takeda, M. Charpentier, J. Perry, H. Miwa, Y. Umehara, H. Kouchi, Y. Murakami, L. Mulder, K. Vickers, J. Pike, J.A. Downie, T. Wang, S. Sato, E. Asamizu, S. Tabata, M. Yoshikawa, Y. Murooka, G.J. Wu, M. Kawaguchi, S. Kawasaki, M. Parniske, M. Hayashi Plastid proteins crucial for symbiotic fungal and bacterial entry into plant roots Nature, 433 (2005), pp. 527–

M. Groth, N. Takeda, J. Perry, H. Uchida, S. Draxl, A. Brachmann, S. Sato, S. Tabata, M. Kawaguchi, T.L. Wang, M. Parniske NENA, a *Lotus japonicus* homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhizal fungi and rhizobia but dispensable for cortical endosymbiotic development Plant Cell, 22 (2010), pp. 2509–2526

P. Bonfante, A. Genre Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis Nat. Commun., 1 (2010), pp. 1–11

J.M. Ané, G.B. Kiss, B.K. Riely, R.V. Penmetsa, G.E. Oldroyd, C. Ayax, J. Levy, F. Debelle, J.M. Baek, P. Kalo, C. Rosenberg, B.A. Roe, S.R. Long, J. Denarie, D.R. Cook *Medicago truncatula* DMI1 required for bacterial and fungal symbioses in legumes Science, 303 (2004), pp. 1364–1367

E. Peiter, J. Sun, A.B. Heckmann, M. Venkateshwaran, B.K. Riely, M.S. Otegui, A. Edwards, G. Freshour, M.G. Hahn, D.R. Cook, D. Sanders, G.E. Oldroyd, J.A. Downie, J.M. Ane The *Medicago truncatula* DMI1 p.otein modulates cytosolic calcium signaling Plant Physiol., 145 (2007), pp. 192–

C. Kistner, T. Winzer, A. Pitzschke, L. Mulder, S. Sato, T. Kaneko, S. Tabata, N. Sandal, J. Stougaard, K.J. Webb, K. Szczyglowski, M. Parniske Seven *Lotus japonicus* genes required for transcriptional reprogramming of the root during fungal and bacterial symbiosis Plant Cell, 17 (2005), pp. 2217–2229

C. Gutjahr, M. Banba, V. Croset, K. An, A. Miyao, G. An, H. Hirochika, H. Imaizumi-Anraku, U. Paszkowski Arbuscular mycorrhiza-specific signaling in rice transcends the common symbiosis signaling pathway Plant Cell, 20 (2008), pp. 2989–3005

B. Wang, L.H. Yeun, J.Y. Xue, Y. Liu, J.M. Ane, Y.L. Qiu Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants New Phytol., 186 (2010), pp. 514–525

H. Kuhn, H. Küster, N. Requena Membrane steroid-binding protein 1 induced by a diffusible fungal signal is critical for mycorrhization in *Medicago truncatula* New Phytol., 185 (2010), pp. 716–733

M. Bucher, S. Wegmuller, D. Drissner Chasing the structures of small molecules in arbuscular mycorrhizal signaling Curr. Opin. Plant Biol., 12 (2009), pp. 500–507

T. Riedel, K. Groten, I.T. Baldwin Symbiosis between *Nicotiana attenuata* and *Glomus intraradices*: ethylene plays a role, jasmonic acid does not Plant Cell Environ., 31 (2008), pp. 1203–1213

C. Gutjahr, M. Novero, M. Guether, O. Montanari, M. Udvardi, P. Bonfante Presymbiotic factors released by the arbuscular mycorrhizal fungus *Gigaspora margarita* induce starch accumulation in *Lotus japonicus* roots New Phytol., 183 (2009), pp. 53–61

L. Navazio, R. Moscatiello, A. Genre, M. Novero, B. Baldan, P. Bonfante, P. Mariani A diffusible signal from arbuscular mycorrhizal fungi elicits a transient cytosolic calcium elevation in host plant cells Plant Physiol., 144 (2007), pp. 673–681

M. Chabaud, A. Genre, B.J. Sieberer, A. Faccio, J. Fournier, M. Novero, D.G. Barker, P. Bonfante Arbuscular mycorrhizal hyphopodia and germinated spore exudates trigger Ca^{2+} spiking in the legume and nonlegume root epidermis New Phytol., 189 (2010), pp. 347–355

F. Maillet, V. Poinsot, O. Andre, V. Puech-Pages, A. Haouy, M. Gueunier, L. Cromer, D. Giraudet, D. Formey, A. Niebel, E.A. Martinez, H. Driguez, G. Becard, J. Denarie Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza Nature, 469 (2011), pp. 58–63

P. Bonfante, N. Requena Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis Curr. Opin. Plant Biol., 14 (2011), pp. 1–7

F. Bastianelli, A. Costa, M. Vescovi, E. D'Apuzzo, M. Zottini, M. Chiurazzi, F. Lo Schiavo Salicylic acid differentially affects suspension cell cultures of *Lotus japonicus* and one of its non-symbiotic mutants Plant Mol. Biol., 72 (2010), pp. 469–483

P. Bonfante, A. Genre, A. Faccio, I. Martini, L. Schauser, J. Stougaard, J. Webb, M. Parniske The *Lotus japonicus LjSym4* gene is required for the successful symbiotic infection of root epidermal cells Mol. Plant–Microbe Interact., 13 (2000), pp. 1109–1120

F. Cardinale, C. Jonak, W. Ligterink, K. Niehaus, T. Boller, H. Hirt Differential activation of four specific MAPK pathways by distinct elicitors J. Biol. Chem., 275 (2000), pp. 36734–36740

A. Lebrun-Garcia, F. Ouaked, A. Chiltz, A. Pugin Activation of MAPK homologues by elicitors in tobacco cells Plant J., 15 (1998), pp. 773–781

M.E. Hoyos, S. Zhang Calcium-independent activation of salicylic acid-induced protein kinase and a 40-kilodalton protein kinase by hyperosmotic stress Plant Physiol., 122 (2000), pp. 1355–1363

O. Lamotte, C. Courtois, G. Dobrowolska, A. Besson, A. Pugin, D. Wendehenne Mechanisms of nitric-oxide-induced increase of free cytosolic Ca^{2+} concentration in *Nicotiana plumbaginifolia* cells Free Radical Biol. Med., 40 (2006), pp. 1369–1376

C. Baron, P.C. Zambryski The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? Annu. Rev. Genet., 29 (1995), pp. 107–129

S.R. Long, B.J. Staskawicz Prokaryotic plant parasites Cell, 73 (1993), pp. 921–935

A.V. Bartsev, N.M. Boukli, W.J. Deakin, C. Staehelin, W.J. Broughton Purification and phosphorylation of the effector protein NopL from *Rhizobium* sp. NGR234 FEBS Lett., 554 (2003), pp. 271–274

Q. Cronk, I. Ojeda, R.T. Pennington Legume comparative genomics: progress in phylogenetics and phylogenomics Curr. Opin. Plant Biol., 9 (2006), pp. 99–103

V. Gianinazzi-Pearson, N. Sejalon-Delmas, A. Genre, S. Jeandroz, P. Bonfante Plants and arbuscular mycorrhizal fungi: cues and communication in the early steps of symbiotic interactions Adv. Bot. Res., 46 (2007), pp. 181–219

G.E. Oldroyd, J.A. Downie Calcium, kinases and nodulation signalling in legumes Nat. Rev. Mol. Cell Biol., 5 (2004), pp. 566–576

A. Pugin, J.M. Frachisse, E. Tavernier, R. Bligny, E. Gout, R. Douce, J. Guern Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway Plant Cell, 9 (1997), pp. 2077–2091

M.T. Chandler, N. Tandeau de Marsac, Y. Kouchkovsky Photosynthetic growth of tobacco cells in liquid suspension Can. J. Bot./Rev. Can. Bot, 50 (1972), pp. 2265–2270

I. Kameshita, H. Fujisawa A sensitive method for detection of calmodulin-dependent protein kinase II activity in SDS polyacrylamide gels Anal. Biochem., 183 (1989), pp. 139–143

D.G. Allen, J.R. Blinks, F.G. Prendergast Aequorin luminescence: relation of light emission to calcium concentration. A calcium-independent component Science, 195 (1977), pp. 996–998