

# Crosstalk between jasmonic acid, ethylene and Nod factor signaling allows integration of diverse inputs for regulation of nodulation

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## Summary

Plant hormones interact at many different levels to form a network of signaling pathways connected by antagonistic and synergistic interactions. Ethylene and jasmonic acid both act to regulate the plant's responsiveness to a common set of biotic stimuli. In addition ethylene has been shown to negatively regulate the plant's response to the rhizobial bacterial signal, Nod factor. This regulation occurs at an early step in the Nod factor signal transduction pathway, at or above Nod factor-induced calcium spiking. Here we show that jasmonic acid also inhibits the plant's responses to rhizobial bacteria, with direct effects on Nod factor-induced calcium spiking. However, unlike ethylene, jasmonic acid not only inhibits spiking but also suppresses the frequency of calcium oscillations when applied at lower concentrations. This effect of jasmonic acid is amplified in the ethylene-insensitive mutant *skl*, indicating an antagonistic interaction between these two hormones for regulation of Nod factor signaling. The rapidity of the effects of ethylene and jasmonic acid on Nod factor signaling suggests direct crosstalk between these three signal transduction pathways. This work provides a model by which crosstalk between signaling pathways can rapidly integrate environmental, developmental and biotic stimuli to coordinate diverse plant responses.

**Keywords:** calcium spiking, crosstalk, signal transduction, jasmonic acid, ethylene, Nod factor, *Medicago truncatula*, symbiosis.

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## Introduction

Jasmonates (JAs) are lipid-derived signaling molecules in plants that regulate diverse responses to wounding, pathogen attack, reproduction, development, metabolic regulation and abiotic stress (Devoto and Turner, 2003; Howe, 2004). Jasmonic acid (JA) is derived from linolenic acid via the octadecanoid pathway in plastids and peroxisomes. Jasmonic acid and its precursors are structural analogs of prostaglandins, which perform a similar biological function in stimulating host defense responses in mammals (Bergey *et al.*, 1996; Howe, 2004; Liechti and Farmer, 2002). Evidence indicates that cyclic precursors of JA, the cyclopentenones, can also function as potent signals of plant defense responses (Farmer and Ryan, 1992; Stintzi *et al.*, 2001; Weber *et al.*, 1997), as can volatile derivatives of JA, such as methyl jasmonate (meJA) and *cis*-jasmane, that act as airborne signals stimulating plant defenses and repelling insects

(Birkett *et al.*, 2000; Farmer and Ryan, 1990). Jasmonic acid regulates many of the same plant responses as the gaseous hormone ethylene, frequently functioning in parallel. Together, JA and ethylene are required for defense against necrotrophic pathogens (Thomma *et al.*, 2001) and associated gene expression (Lorenzo *et al.*, 2003; Norman-Setterblad *et al.*, 2000; Penninckx *et al.*, 1998; Xu *et al.*, 1994). The transcription factor ERF1 has been proposed to act as a convergence point in synergistic signaling of JA/ethylene (Lorenzo *et al.*, 2003).

In addition to pathogenic interactions with microbes, plants undergo beneficial interactions with some bacteria and fungi. The symbiotic interaction between legumes and rhizobia results in nitrogen-fixing nodules on the roots of the host plant. The initiation of nodule formation is dependent on Nod factor, a lipochito-oligosaccharide signal produced

by rhizobial bacteria. Within minutes, Nod factors induce a series of rapid responses in cells of the legume root including oscillations in cytosolic calcium levels, termed calcium spiking (Ehrhardt *et al.*, 1996; Oldroyd and Downie, 2004). Nod factor-induced calcium spiking is most probably a component of the Nod factor signaling pathway, since mutants defective in Nod factor signaling are also defective in the activation of spiking (Harris *et al.*, 2003; Wais *et al.*, 2000; Walker *et al.*, 2000) and a calcium/calmodulin-dependent protein kinase that functions downstream of spiking is essential for Nod factor signal transduction (Levy *et al.*, 2004; Mitra *et al.*, 2004). In other systems, calcium spiking triggers changes in gene expression or other downstream events, with information encoded in either the frequency or amplitude of the calcium spikes (Allen *et al.*, 2001; Dolmetsch *et al.*, 1998; Li *et al.*, 1998). It is not yet known how information is encoded in Nod factor-induced calcium spiking.

Symbiotic nitrogen fixation is crucial to the success of legumes, and nodule formation has been closely integrated into existing plant regulatory networks, such that nodule initiation is regulated by auxin:cytokinin signaling (Cooper and Long, 1994; Hirsch *et al.*, 1989), abscisic acid (Philips, 1971; Suzuki *et al.*, 2004), brassinosteroids, gibberellins (Ferguson *et al.*, 2005), light signaling (Nishimura *et al.*, 2002a,b) and metabolites such as nitrate (Carroll and Gresshoff, 1983). In particular, ethylene signaling has a strong effect on nodule formation. When ethylene levels are low, plant and bacteria interact to form nodules; when high, nodule formation is inhibited. The ethylene-insensitive mutant, *sickle* (*skl*), of *Medicago truncatula* shows a greater than 10-fold increase in the number of nodules (Penmetsa and Cook, 1997).

Ethylene appears to regulate multiple steps during nodulation: nodule number, nodule positioning, growth of the infection thread, Nod factor-induced gene expression and calcium spiking (Oldroyd *et al.*, 2001; Penmetsa and Cook, 1997). The inhibition of Nod factor-induced calcium spiking reflects the suppression of the Nod factor signaling pathway at a very early stage and most probably explains the regulation of nodule number. However, the positioning of nodules and regulation of the growth of infection threads are probably functions of ethylene action independent of its effect on Nod factor signal transduction, and this is supported by the restrictive rhizobial induction in roots of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, a key enzyme in ethylene biosynthesis (Heidstra *et al.*, 1997).

The rapidity of the effect of ethylene on Nod factor-induced calcium spiking suggests crosstalk between Nod factor and ethylene signal transduction pathways. Although the point of intersection is not known, there are multiple effects of ethylene on calcium spiking. First, high concentrations of ethylene can inhibit calcium spiking after initiation (Oldroyd *et al.*, 2001). Second, ethylene can reduce the

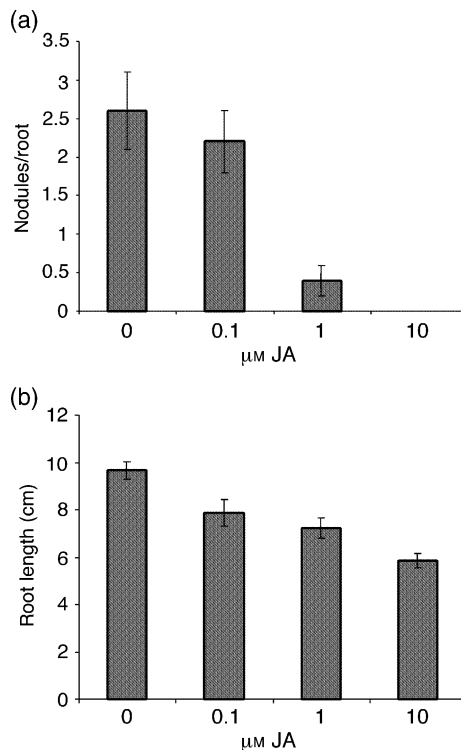
responsiveness of the plant to Nod factor for induction of calcium spiking (Oldroyd *et al.*, 2001). Finally, the ethylene-insensitive mutant, *skl*, exhibits a longer spike period in response to Nod factor (Oldroyd *et al.*, 2001). A longer spike period in an ethylene-insensitive mutant suggests that the role of ethylene itself is actually the opposite, namely, to shorten the spike period, although such an effect has not been observed in ethylene-treated roots, due to the difficulty of delivering a gas in a liquid medium. The multiple effects of ethylene on Nod factor-induced calcium spiking could be due to a single intersection between the Nod factor signaling pathway and that of ethylene. Alternatively, the different effects of ethylene on root hair calcium spiking might represent multiple instances of crosstalk between the two signaling pathways.

In this paper we demonstrate a novel role for JA in regulating the ability of legumes to form nitrogen-fixing nodules. We show that JA, like ethylene, is a negative regulator of nodulation and can suppress the expression of plant genes associated with the earliest steps of nodulation. In addition, we find that JA regulates the same three aspects of Nod factor-induced calcium spiking as ethylene: (i) maintenance of spiking, (ii) responsiveness of spiking to the concentration of Nod factor and (iii) spike period. However, our data show that although JA and ethylene have similar effects on the maintenance of spiking and response to the concentration of Nod factor, they have opposite effects on the spike period. We found that JA lengthens the spike period, while ethylene is predicted to shorten the spike period. The distinct roles of ethylene and JA demonstrate that the initiation of calcium spiking and the regulation of spike frequency represent separate steps in the plant's response to Nod factor and are differentially controlled. In addition, our analysis reveals crosstalk between the ethylene and JA signaling pathways in the regulation of calcium spike frequency and in the regulation of responsiveness to Nod factor. We find that the *skl* mutant reveals greater sensitivity to JA for the effect on calcium spike frequency, but not for nodule formation, indicating the presence of both antagonistic and synergistic interactions between the ethylene and JA pathways. It would appear that the decision to induce calcium spiking and the nature of the calcium spiking response is the result of a subtle interplay between the concentrations of Nod factor, ethylene and JA.

## Results

### *Jasmonic acid inhibits nodulation in Medicago truncatula*

In order to assess the role of JA in regulating nodulation we grew *M. truncatula* plants on Petri plates containing varying concentrations of JA. We found that as the concentration of JA increased, nodulation decreased (Figure 1a). At concentrations of JA that were effective on nodulation, we also saw



**Figure 1.** Inhibition of nodulation by JA at concentrations that inhibit root growth. Data are expressed as the means  $\pm$  SE. (a) Nodulation at 24 days (21 d.p.i.). (b) Root length of the same plants at 8 days.

inhibition of root elongation (Figure 1b) that has been extensively characterized by other groups (Feys *et al.*, 1994; Lorenzo *et al.*, 2004; Staswick *et al.*, 1992). This co-suppression of nodulation and root elongation indicates that the JA concentrations tested are within the physiological range for roots. Although root elongation is decreased at 10  $\mu$ M JA, the roots continue to grow and shoots appear green and healthy.

#### *Jasmonic acid does not affect the growth of free-living rhizobia or the induction of bacterial nod gene expression ex planta*

Nodulation requires the active participation of both plant and bacterial partners. One possible explanation for the inhibition of nodulation by JA is its negative effects on rhizobial viability or the suppression of bacterial *nod* genes that are essential during nodulation. Growth of *Sinorhizobium meliloti* was unaffected by the addition of 10  $\mu$ M JA to liquid cultures, a concentration that reliably inhibits nodule formation (Figure S1a). Using a *nodF*-GUS fusion (Wais *et al.*, 2002) we found that the induction of the *nodF* gene by the plant flavonoid signal luteolin is also unaffected by the addition of 10  $\mu$ M JA to the medium (Figure S1b). Together,

these observations suggest that the primary target of JA activity is the response of the legume host to its rhizobial partner.

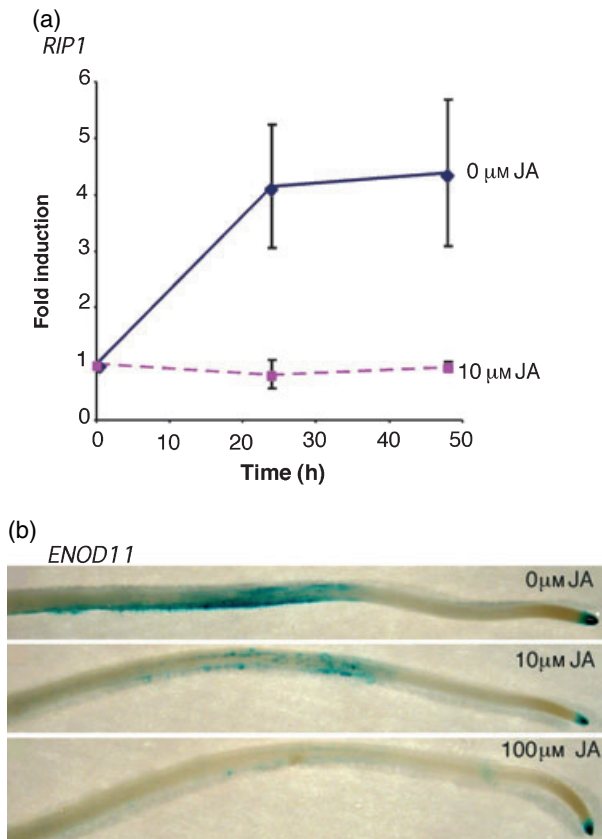
#### *Jasmonic acid inhibits expression of the early rhizobium-responsive genes, RIP1 and ENOD11, in Medicago truncatula*

Since JA-treated roots only rarely form mature nodules (Figure 1a) and do not accumulate immature nodules (data not shown), we hypothesized that the block to nodulation must occur early in the signal transduction pathway. *RIP1* and *ENOD11* represent some of the earliest markers for the plant's response to Nod factor and rhizobia and are therefore candidates for being directly activated by Nod factor signal transduction (Cook *et al.*, 1995; Gamas *et al.*, 1996; Journet *et al.*, 2001). In order to assess the effect of JA on *RIP1* gene expression, we grew *M. truncatula* plants on 0 and 10  $\mu$ M JA and used quantitative RT-PCR (qRT-PCR) to measure *RIP1* expression in root tissue at various time points after inoculation with *S. meliloti*. We found that JA completely inhibited induction of *RIP1* expression in response to *S. meliloti* (Figure 2a), although baseline levels of *RIP1* expression were often higher in uninoculated plants grown on JA than in control plants grown in the absence of JA (data not shown).

To assess the affect of JA on Nod factor-induced *ENOD11* we used *M. truncatula* plants stably transformed with an *ENOD11* promoter-GUS fusion (Journet *et al.*, 2001). Plants grown in the absence of Nod factor showed GUS activity only in the root cap (data not shown) that is the result of non-symbiotic *ENOD11* expression. Treatment with 1 nM Nod factor activates *ENOD11*-GUS in epidermal cells in a restricted region behind the root tip (Journet *et al.*, 2001) (Figure 2b). The addition of 100  $\mu$ M JA coincidentally with 1 nM Nod factor completely eliminated expression of *ENOD11*-GUS in epidermal cells, but did not affect the root cap expression (Figure 2b). Treatment with 10  $\mu$ M JA reduced induction of *ENOD11*-GUS by Nod factor, with fewer cells showing GUS staining (Figure 2b). Together, these results indicate that JA inhibits early Nod factor-induced gene expression.

#### *Jasmonic acid inhibits initiation of Nod factor-induced calcium spiking*

One of the earliest responses of the plant to Nod factor is calcium spiking. To test the role of JA in calcium spiking, we micro-injected the calcium-responsive dye Oregon Green into *M. truncatula* root hairs. Following the induction of calcium spiking by Nod factor, the addition of 100  $\mu$ M JA to the medium caused a rapid suppression of calcium spiking with the first effects visible 10 min post-treatment (Figure 3a). Replacement of the medium with one



**Figure 2.** JA inhibits induction of plant nodulation genes by *S. meliloti* or Nod factor.

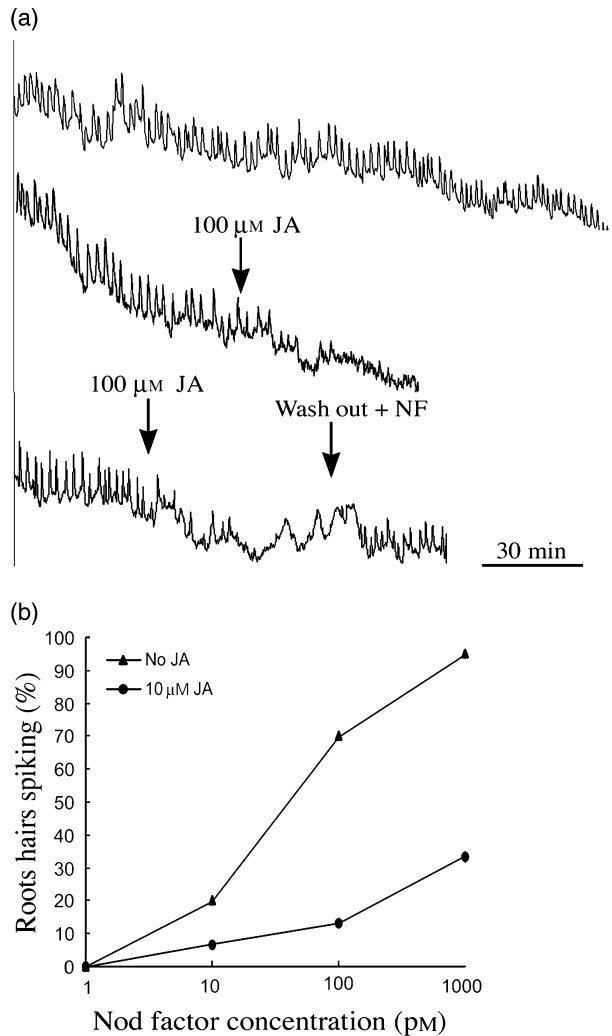
(a) Effect of JA on induction of the *RIP1* gene by *S. meliloti* in wild-type (A17) plants. *RIP1* expression was assessed using qRT-PCR with actin as an endogenous control. Results are expressed as fold induction over expression at the 0 h time point for each condition. These data are the average of three independent experiments  $\pm$  SE. Time = hours post-inoculation.

(b) Plants stably expressing an *ENOD11*-GUS fusion (Journet *et al.*, 2001) were treated with 1 nM Nod factor, simultaneously with different concentrations of JA, and stained for GUS activity after 12 h.

containing only Nod factor reinstated calcium spiking, indicating reversible suppression (Figure 3a). The rapidity of the JA inhibition is similar to what has previously been shown for ethylene (Oldroyd *et al.*, 2001).

#### *Jasmonic acid decreases the responsiveness of root hair cells to Nod factor*

From our previous work with ethylene it is apparent that ethylene levels alter the responsiveness of root hair cells for activation of calcium spiking by Nod factor. High ethylene levels reduce the responsiveness of root hair cells to Nod factor, while low levels increase the responsiveness (Oldroyd *et al.*, 2001). To assess whether a similar situation also exists for JA, we grew *M. truncatula* plants transformed with the calcium reporter cameleon (H. Miwa,



**Figure 3.** Jasmonic acid inhibits Nod factor-induced calcium spiking.

(a) Individual cells of wild-type plants treated with Nod factor and secondarily treated with 100 μM JA where indicated. Cells continue spiking in the absence of JA treatment, upper trace. The traces start 30 min after addition of Nod factor and represent raw fluorescence, measured as an arbitrary unit. The actual fluorescence intensity is dependent on the levels of Oregon Green injected and hence the units of measurement are immaterial.

(b) Plants grown with or without 10 μM JA were treated with 1 pM Nod factor and the concentration increased up to 1 nM with a shift in concentration every 30 min. The concentration at which each root hair cell initiated spiking was assessed. Twenty cells on four plants were analyzed without JA and 15 cells on three plants were analyzed for the 10 μM JA treatment.

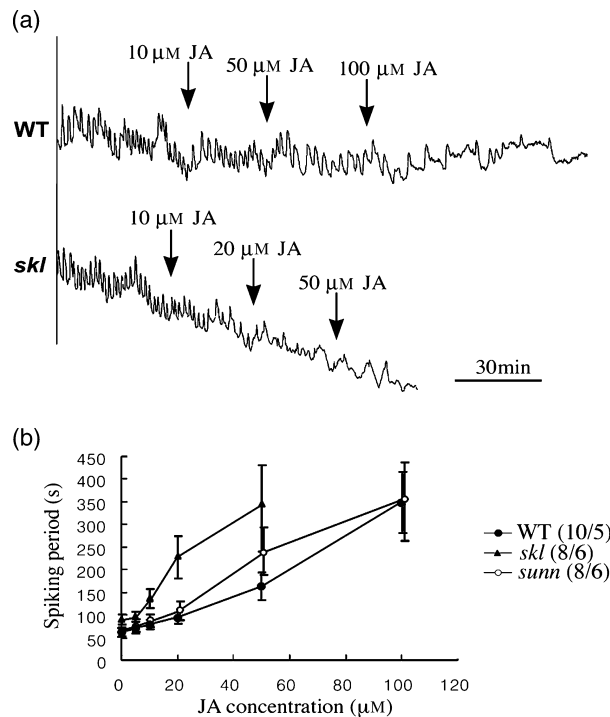
G. Oldroyd and A. Downie, John Innes Centre, Norwich, UK, unpublished data) on 0 and 10 μM JA and assessed the number of root hairs induced for calcium spiking with increasing concentrations of Nod factor while maintaining the appropriate JA concentration. We found that treatment with 10 μM JA causes a shift in the responsiveness of plants to Nod factor, such that higher concentrations of Nod factor are required to activate calcium spiking in an equivalent number of cells (Figure 3b).

### Jasmonic acid modulates the frequency of calcium spiking

The addition of 100  $\mu\text{M}$  JA after induction of calcium spiking causes a mostly complete inhibition of Nod factor-induced calcium spiking; however, low-frequency spiking was occasionally observed (Figure 4a). Lower concentrations of JA did not inhibit spiking, but did affect the period between spikes: 10 and 20  $\mu\text{M}$  JA caused a minor increase in the period between spikes, whereas 50  $\mu\text{M}$  caused on average a doubling of the spike period (Figure 4a,b). This observation suggests that JA can regulate calcium spiking in a number of subtle ways, with high concentrations causing complete inhibition and lower concentrations modulating the nature of the spiking response. The effects of JA and ethylene on spike frequency are opposite: removal of ethylene and addition of JA both suppress the frequency of calcium spiking. This is in contrast to the many similarities we have found in the effects of ethylene and JA on nodulation, nodulin gene expression and induction of calcium spiking.

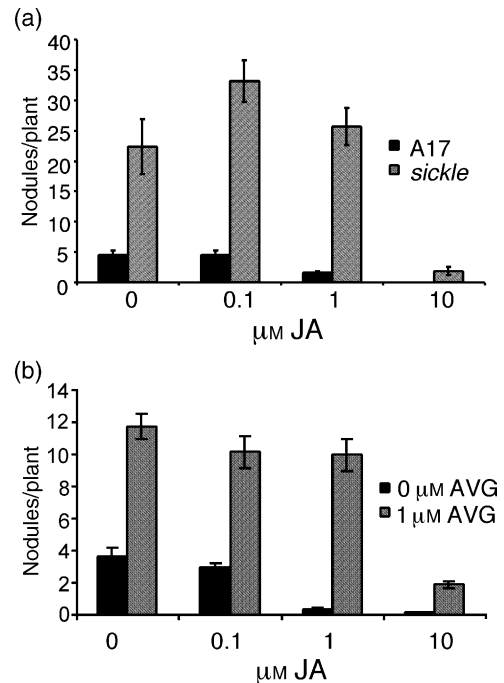
### Ethylene regulates the effect of jasmonic acid

Since ethylene and JA appear to have opposite effects on the frequency of calcium spiking, we next altered the levels



**Figure 4.** Jasmonic acid regulates calcium spiking frequency.

(a) Single cells of wild-type and *skl* plants treated with differing concentrations of JA. The traces start 30 min after treatment with 1 nM Nod factor. (b) The frequency of calcium spiking as measured by assessing the period between spikes from a 15 min section of spiking 30 min after treatment with Nod factor. Numbers in parentheses represent the number of cells/total number of plants analyzed.



**Figure 5.** Inhibition of ethylene signaling or production reduces the sensitivity of nodulation to JA. Data are expressed as the means  $\pm$  SEM.

(a) Nodulation of the ethylene-insensitive mutant, *sickle*, and wild-type plants in the presence of varying concentrations of JA.  $n > 25$  for each genotype  $\times$  condition. The decreased nodulation on 0  $\mu\text{M}$  JA as compared with 0.1  $\mu\text{M}$  JA was not seen in other experiments.

(b) Nodulation of A17 (wild-type) plants grown on medium containing 1  $\mu\text{M}$  AVG and varying concentrations of JA.  $n > 35$  for each condition.

of JA and ethylene simultaneously, in order to examine their interactions in the regulation of Nod factor signaling. The ethylene status of the plant was altered either pharmacologically, using the ethylene biosynthesis inhibitor L- $\alpha$ -(2-aminoethoxyvinyl)-glycine (AVG), or genetically, using the *skl* mutation (Penmetsa and Cook, 1997). We found that reducing the ethylene status of the plant either with 1  $\mu\text{M}$  AVG or with the *skl* mutation reduced the degree to which JA inhibited nodulation, but did not eliminate it (Figure 5a,b). Conversely, as increasing amounts of AVG were added to plants grown on 10  $\mu\text{M}$  JA, we found a reciprocal increase in nodulation (data not shown). These data indicate that the effects of JA and ethylene inhibition on nodule formation are additive and suggest a synergistic interaction between these two hormones for overall regulation of nodulation.

To further assess the interaction of ethylene and JA in the regulation of Nod factor signaling, we next examined the effect of JA on calcium spiking in plants with lowered ethylene status. Both JA and ethylene affect multiple aspects of calcium spiking. We focused on two: regulation of spike frequency and responsiveness to Nod factor, since both are sensitive to low concentrations of JA.

Jasmonic acid and ethylene appear to have opposite effects on the frequency of calcium spiking: both addition of JA as well as removal of ethylene signaling (via the *skl* mutation) result in a decreased frequency of calcium spiking in response to Nod factor (Oldroyd *et al.*, 2001; Figure 4). In order to test the interaction of JA and ethylene signaling in the regulation of spiking frequency, we induced calcium spiking in wild-type and *skl* plants with 1 nM Nod factor, then measured the spike period as increasing concentrations of JA were added. Prior to JA treatment, we observed that *skl* mutants had a slightly increased spike period, as has been previously shown (Figure 4b; Oldroyd *et al.*, 2001). We found that *skl* mutants were hypersensitive to the inhibition of calcium spiking by JA, such that 10 and 20  $\mu\text{M}$  JA show dramatic effects on spike frequency, which was not seen in wild-type plants (Figure 4a,b). As a control we used the *sun* mutant, which also shows a 10-fold increase in nodulation, but is not defective in ethylene signaling (Penmetsa *et al.*, 2003). The *sun* mutant showed a wild-type response to inhibition of spike frequency by JA (Figure 4b). The hypersensitivity of ethylene-insensitive *skl* mutants to the effect of JA on spike frequency suggests that ethylene normally functions to inhibit this action of JA.

We next examined the interaction of JA and ethylene signaling in the sensitivity of root hair cells to the concentration of Nod factor. We had previously found that plants treated with AVG or carrying the *skl* mutation exhibited increased responsiveness to Nod factor, such that root hair cells initiated calcium spiking at lower concentrations of Nod factor (Oldroyd *et al.*, 2001). The converse was observed in plants treated with the ethylene precursor, ACC, which led to decreased responsiveness, much as we saw for JA (Oldroyd *et al.*, 2001; Figure 3b). In order to examine the interaction of ethylene and JA signaling on the responsiveness to Nod factor, we grew *M. truncatula* plants in the presence of both AVG and JA. Since AVG increases the responsiveness of root hairs to Nod factor and JA decreases it, we expected that the combined treatment would not differ much from the untreated control. Instead, we found that AVG enhanced the effect of JA on the sensitivity of root hairs to Nod factor: growth of plants on 1  $\mu\text{M}$  AVG with 10  $\mu\text{M}$  JA led to a greater suppression of calcium spiking than 10  $\mu\text{M}$  JA alone (Table 1). In these experiments we scored cells as spiking even if they showed very low frequencies. These data suggest that while ethylene and JA function synergistically for overall regulation of nodulation there is an antagonistic interaction between these hormones during the regulation of Nod factor-induced calcium spiking, such that ethylene acts to inhibit the effect of JA.

## Discussion

Jasmonic acid and its octadecanoid precursors are critical components of signaling pathways that induce the response

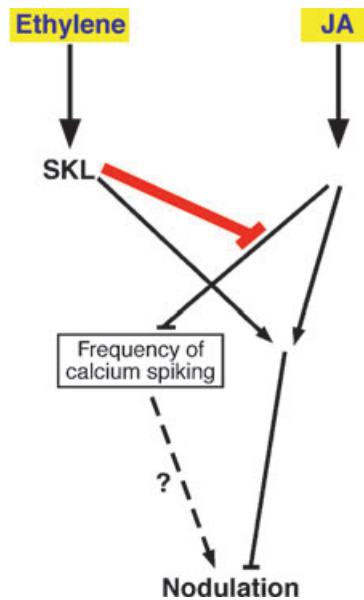
**Table 1** The interaction of JA and ethylene for responsiveness of calcium spiking to Nod factor: the number of cells showing calcium spiking with 1 nM Nod factor is indicated for each treatment relative to the total number of cells analyzed

Treatment	Cells spiking/total cells	% cells spiking
No JA, no AVG	23/29	79
10 $\mu\text{M}$ JA	26/48	56
10 $\mu\text{M}$ JA + 1 $\mu\text{M}$ AVG	16/45	36
100 $\mu\text{M}$ JA	20/58	34

of plants to wounding, herbivory and pathogen attack (Devoto and Turner, 2003; Howe, 2004). Jasmonic acid has recently been shown to modulate a beneficial plant–microbe interaction: the symbiosis between rhizobial bacteria and their legume hosts (Nakagawa and Kawaguchi, 2006). Here we demonstrate that JA inhibits Nod factor signal transduction by modulating the calcium signature of responding root hair cells in the host. Expression of early nodulation genes is also inhibited, and we observe a decrease in nodule formation. Ethylene and JA function additively to inhibit nodulation, but antagonistically in the regulation of Nod factor-induced calcium spiking. This work suggests a mechanism by which crosstalk between signaling pathways can rapidly modulate the plant's response to a specific biotic stimulus.

Ethylene negatively regulates the Nod factor signaling pathway at or above calcium spiking (Oldroyd *et al.*, 2001; Penmetsa and Cook, 1997). The ethylene status of the plant and the concentration of Nod factor together dictate the level of activation of the Nod factor signaling pathway. Ethylene also appears to have a modest effect on calcium spiking frequency, since the ethylene-insensitive mutant *skl* shows a slightly increased period between spikes (Oldroyd *et al.*, 2001). Here we show that JA, like ethylene, negatively regulates the Nod factor signaling pathway at or above calcium spiking. Thus, Nod factor-induced calcium spiking is dependent not only upon ethylene levels but also upon JA status and the concentration of Nod factor. The rapidity of this inhibition suggests direct crosstalk between the ethylene and JA signaling pathways with the Nod factor signaling pathway, and the similarities of ethylene and JA suppression suggest a common mode of action.

We have shown that JA can directly modulate both the frequency of calcium spikes and the induction of early nodulin gene expression. While the frequency of calcium spiking differs between cells it does not appear to be modified by the concentration of Nod factor (Ehrhardt *et al.*, 1996). Therefore JA regulates the mode of Nod factor signaling in a way that signal input alone is unable to do. It is not known what effect a change in spike frequency could have on nodulation. However, in other systems information is encoded in spike frequency, such that altering the frequency of calcium spiking can have profound effects,



**Figure 6.** Model describing the interactions of JA, ethylene and Nod factor signaling in the regulation of calcium spiking frequency and nodule formation in legume roots.

Ethylene, signaling through *SKL*, acts to antagonize JA signaling to limit its effect on the frequency of calcium spiking. However, both JA and ethylene act synergistically to inhibit nodulation. The effect of changes in calcium oscillation frequency on nodulation is not known. Arrows indicate activation (or increase), bars indicate repression (or decrease).

leading to a change in gene expression or physiological processes (Allen *et al.*, 2001; Dolmetsch *et al.*, 1998; Li *et al.*, 1998). The frequency of spiking will be dependent on the opening of a calcium channel, and JA must modulate this through direct interactions between the channel and components of the JA signaling pathway, via crosstalk between other components of the Nod factor and JA signaling pathways or indirectly through modulating cytosolic calcium levels.

The addition of either JA or ethylene inhibits Nod factor-induced calcium spiking, nodulation gene expression and nodulation itself, and overall these two hormones function synergistically for inhibition of nodulation (Oldroyd *et al.*, 2001; Penmetsa and Cook, 1997). However, JA and ethylene function antagonistically for the regulation of calcium spiking frequency (Figure 6) and for responsiveness to Nod factor. The antagonistic relationship between ethylene and JA in the regulation of responsiveness to Nod factor is particularly surprising, since both signaling molecules independently inhibit the process. These differing interactions between JA and ethylene suggest that these two hormones regulate nodulation at multiple stages in the interaction between the plant and the bacterium. We hypothesize that the antagonistic interaction results from crosstalk between the JA and ethylene signaling pathways that then modulates the Nod factor signaling pathway. In fact,

the decrease in calcium spiking frequency observed in *skl* mutants (Oldroyd *et al.*, 2001) may be due entirely to the fact that repression of JA signaling by ethylene is relieved (Figure 6). Recent work reveals a change in the frequency of oscillations as root hairs mature (H. Miwa, GO and A. Downie, manuscript in preparation) and an appealing hypothesis is that this may reflect a change in the ratio of ethylene and JA at different developmental stages of epidermal cells.

Much of the focus of the study of JA and ethylene interactions has been on synergy; however, antagonistic crosstalk has been increasingly observed. Evidence from multiple species has been accumulating to show that the JA signaling pathway splits into two branches, one of which induces the expression of pathogen-response genes synergistically with ethylene (Lorenzo *et al.*, 2003; Norman-Setterblad *et al.*, 2000; Penninckx *et al.*, 1998; Xu *et al.*, 1994) and a second which is responsible for activating wound-response genes that is negatively regulated by ethylene (Ellis and Turner, 2001; Lorenzo *et al.*, 2004; Rojo *et al.*, 1999; Shoji *et al.*, 2000; Tuominen *et al.*, 2004; Winz and Baldwin, 2001). Interestingly, the transcription factor ERF1, which is proposed to act as a convergence point in JA/ethylene synergistic signaling (Lorenzo *et al.*, 2003), also functions to mediate the antagonistic interaction between ethylene and JA-induced wound-response genes (Lorenzo *et al.*, 2004), suggesting that both synergistic and antagonistic JA/ethylene interactions signal through a common node.

The apparent link between a branch of the JA signaling pathway involved in wound response and *Rhizobium*-induced nodulation is curious. While the common mechanism for rhizobial entry into legumes is through root hairs, in some legume species rhizobia infect via cracks in the epidermis that surround emerging lateral roots (Chandler, 1978; Chandler *et al.*, 1982; Ndoye *et al.*, 1994; Sprent *et al.*, 1989; Tsien *et al.*, 1983). Rhizobia are closely related to *Agrobacterium* (Sawada *et al.*, 2003), which are attracted to wounds and require prior wounding for pathogenesis (reviewed in Escobar and Dandekar, 2003). It is intriguing to speculate that the regulation of Nod factor-induced calcium spiking by antagonistic ethylene–JA interactions in *M. truncatula* may reflect an ancient infection strategy by which rhizobia entered plants primarily via wounds or cracks in the epidermis.

The modulation of Nod factor signaling in root hair cells by JA and ethylene allows the integration of diverse environmental, developmental and biotic factors that are regulated by JA and ethylene (Devoto and Turner, 2003; Howe, 2004; Klee, 2004; Nehring and Ecker, 2004) and hence the rapid coordination of the plant's response to rhizobia with the environmental and developmental status of the plant. The multiple checkpoints in the development of a nodule that are regulated by these two hormones ensure the continuous integration of these developmental processes.

Perhaps the antagonistic and synergistic interactions between JA and ethylene for regulation of Nod factor-induced calcium spiking and nodulation provide alternative checkpoints that integrate different stress or developmental signals transduced by JA and ethylene (Devoto and Turner, 2003; Howe, 2004; Klee, 2004; Nehring and Ecker, 2004). Dissecting the mechanisms of suppression of nodulation by JA and ethylene will provide significant insights into cross-talk between signal transduction pathways.

## Experimental procedures

### Bacterial growth conditions

*Sinorhizobium meliloti* Rm1021 and related strains were grown at 30°C in Luria–Bertani (LB) medium. Growth of Rm1021 was assayed by diluting overnight cultures to an OD<sub>595</sub> of 0.1 in M9 sucrose containing varying concentrations of JA, and assaying the OD<sub>595</sub> every few hours for the next 38 h. To determine bacterial *nod* gene expression, DW386 containing a *nodF* promoter–GUS fusion (Wais *et al.*, 2002) was grown until the OD<sub>595</sub> reached 0.2, at which time the cultures were supplemented with 0 or 10 µM JA with or without 3 µM luteolin (Sigma, St Louis, MO, USA; L 9283). At 0, 3, 6, 9, 21, 24 and 27 h, 1 ml of cells was collected, the OD<sub>595</sub> measured and β-glucuronidase activity was assayed as described (Jefferson *et al.*, 1986).

### Plant growth conditions

*Medicago truncatula* A17 seeds were scarified for 5 min in concentrated sulfuric acid, washed twice in sterile water, surface sterilized for 3 min in undiluted Clorox bleach, then washed five to eight times in sterile water. Seeds were imbibed for 3 h and germinated overnight in the dark at room temperature (20–22°C). Germinated seedlings were transferred aseptically to Petri plates (24085, Nunc, Rochester, NY, USA) containing buffered nodulation medium (BNM) agar at pH 6.5 (Ehrhardt *et al.*, 1992) and placed in a Conviron growth chamber (model MTR30; Winnipeg, Manitoba, Canada) or a growth room at 20–22°C and 16-h days. Where indicated, JA (Sigma, J2500) was added to the medium at the specified concentrations. Plants on plates were flood-inoculated with 10-fold dilution of an overnight culture of *S. meliloti* Rm1021 5 days after plating.

### Expression analysis by quantitative real-time RT-PCR

Total RNA was extracted from roots with root tips removed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Ribonucleic acid was quantified using a spectrophotometer (Nanodrop-1000, NanoDrop Technologies, Rockland, DE, USA) and checked for quality by gel electrophoresis. The RNA was treated with DNA-free<sup>®</sup> (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The absence of genomic DNA contamination was confirmed by PCR, using *RIP1* primers to exons 2 and 3 that span an intron.

Complementary DNA was prepared from 4 µg total RNA using the SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies, Invitrogen) using oligo-dT primers according to the manufacturer's instructions. Quantitative real time RT-PCR was performed using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SDS 2.2

collection software (Applied Biosystems) and using SYBR Green to monitor double-stranded (ds) DNA synthesis. Reactions were performed in a 96-well plate using the SYBR Green JumpStart<sup>®</sup> Taq ReadyMix (Sigma) and 50 nM of each gene-specific primer. An initial denaturation step of 94°C for 2 min was followed by 40 cycles of 94°C for 15 sec, 60°C for 1 min and 72°C for 1 min. Primers used were: *RIP1.2* for 5'-CAGCAGCTCGTGATTCTGTAG-3', *RIP1* exon 3 rev 5'-GTGGCTCGAGTCTGACCCTTCTGCAATTGAG-3', actin 190F 5'-AAGCTTGCATATGTTGCC-3', actin 190R 5'-CATCCAGTGGGCTGC-CAC-3'. After the completion of PCR amplification, a dissociation curve was run to check for genomic DNA contamination. For this, PCR products were denatured at 95°C, annealed at 60°C and gradually heated to 95°C over the course of 20 min. No DNA contamination was detected in any reactions. Primer–dimer formation was estimated by running a control without template DNA. Results were expressed as a 'threshold cycle' ( $C_T$ ) value. Two replicate reactions were run for each sample, and their  $C_T$  values averaged. These averaged values were used in all subsequent calculations. *RIP1* gene expression was normalized to that of the actin control by subtracting the  $C_T$  value of actin from the  $C_T$  value of *RIP1* to give  $\Delta C_T$ . Fold induction was calculated by normalizing the data for each time series to that of the 0 h sample, the  $\Delta C_T$  value for that sample was then subtracted from the  $\Delta C_T$  of the other samples to give a  $\Delta\Delta C_T$  value. The data were plotted as  $2^{-\Delta\Delta C_T}$ . The data from three independent experiments were averaged and plotted using SE.

### ENOD11–GUS staining

Seedlings of the *ENOD11*–GUS line 416K were grown overnight on BNM and then three plants per treatment were transferred to liquid BNM containing 1 nM Nod factor with or without JA. After 12 h at room temperature and darkness the plants were transferred to 0.1 M potassium phosphate, pH 7.0, 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid and 5 mM EDTA. The experiments were repeated three times, with similar results.

### Calcium spiking experiments

Analysis of calcium spiking was performed as described by Ehrhardt *et al.* (1996), with slight modifications as described by Wais *et al.* (2000). All plants used were grown overnight on BNM in the absence of AVG unless otherwise stated. Micropipettes were pulled from filamented capillaries on a pipette puller (model 773, Campden Instruments, Loughborough, UK). These were loaded with Oregon green dextran (10 000 mW, Invitrogen) and injections performed using iontophoresis with currents generated from a cell amplifier (model Intra 767, World Precision Instruments, Sarasota, FL, USA) and a stimulus generator made to our specifications (World Precision Instruments). Cells were analyzed on an inverted epifluorescence microscope (model TE2000, Nikon, Tokyo, Japan) using a monochromator (model optoscan, Cairn Research Ltd, Faversham, UK) to generate specific wavelengths of light. Images were captured with a charge-coupled device (CCD) camera (model ORCA-ER, Hamamatsu City, Japan) and fluorescent data analyzed using Metaflor (Molecular Devices, Sunnyvale, CA, USA).

Lines expressing the calcium reporter cameleon were generated as described in work by H. Miwa, G. Oldroyd and A. Downie (unpublished). These lines were analyzed on the same inverted epifluorescent microscope (model TE2000, Nikon). During image capture the image was split using the Optosplit (Cairn Research Ltd), and each image was passed through a filter for either Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP)

emissions prior to exposure on the CCD chip. The ratio of CFP:YFP was assayed using Metaflor (Molecular Devices).

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### Supplementary Material

The following supplementary material is available for this article online:

**Figure S1.** Jasmonic acid does not affect the growth of free-living *S. meliloti* or induction of bacterial *nod* gene expression *ex planta*.

The graphs represent a mean of three replicates done in parallel. The experiments were repeated three times and the data presented are each from a single representative experiment.

(a) Growth curve of Rm1021 in LB medium in the presence of varying concentrations of JA as monitored by OD<sub>595</sub>.

(b) Expression of *nodF* as monitored via  $\beta$ -glucuronidase activity of a *nodF*-*uidA* transcriptional fusion (Wais *et al.*, 2002). *nodF* expression was induced by the addition of 3  $\mu$ M luteolin to M9 minimal medium with or without the addition of 10  $\mu$ M JA.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

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