The *fas* locus of the phytopathogen *Rhodococcus fascians* affects mitosis of tobacco BY-2 cells

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Abstract The effect of *Rhodococcus fascians*, the causal agent of leafy gall disease, on the mitotic behavior of synchronized tobacco Bright Yellow-2 (BY-2) cells was investigated. Incubation of aphidicolin-synchronized BY-2 cells with *R. fascians* cells specifically resulted in a broader mitotic index peak, an effect that was linked to an intact and expressed *fas* virulence locus. The obtained results pointed towards an effect of *R. fascians* on the prophase of mitosis. The relevance of these results to the virulence of the bacterium is discussed.

Key words: Aphidicolin; Cytokinin; Mitotic index; Propyzamide; Virulence

1. Introduction

The Gram-positive phytopathogen *Rhodococcus fascians* interacts with many plant species, provoking a variety of symptoms related to a massive production of shoot primordia [1,2]. Disease development depends on several virulence loci that reside on a large linear plasmid, called pFiD188 [3,4]. The *fas1* mutant, carrying an insertion into the gene encoding isopentenyl transferase (*ipt*) is completely non-virulent [3], indicating the essential role of the *fas* locus in pathogenesis. Several observations suggest that a *R. fascians* elicited effect on the plant cell cycle. In all eukaryotes, the cell cycle is controlled at specific transition checkpoints. The key catalytic enzymes exerting this control are the cyclin-dependent kinases (CDKs), the activity of which is subjected to a complex regulation (for reviews, see [5,6]). Meristematic tissues are sensitive to the action of *R. fascians* [7–10] and are thought to contain the highest levels of $p34^{cdc2}$, the 34-kDa product of the *cdc2* gene [11]. In *Arabidopsis thaliana*, the highest levels of *cdc2aAt* mRNA (now designated *CDKA;1* [12]) and promoter activity are found in areas with higher frequencies of, or potential for, cell division [13,14]. Abundant shoot formation provoked by *R. fascians* on *A. thaliana* has been correlated with a strong induction of *CDKA;1* expression [2]. Although *CDKA;1* expression can be induced by cytokinins [14], the unaltered expression levels in plants infected with *R. fascians* mutant strain D188-5, which lacks pFiD188, show that this induction is not mediated by cytokinins originating from the activity of chromosomally encoded proteins. The induced *CDKA;1* expression must thus be provoked by a pFiD188-encoded factor [2].

To study aspects of the cell cycle, cell cultures of *Nicotiana tabacum* L. cv. Bright Yellow-2 (BY-2) are very suitable: they grow very fast and homogenously and a high synchrony in cell division can be induced [15]. Several compounds interfering with a specific step in the cell cycle can be used to block the cells at certain points, allowing the investigation of the effect of external signals on cell cycle progression [15]. The aim of this study was to determine the effects of *R. fascians* on the cell cycle progression of synchronized BY-2 cells. Our data indicate that induced bacterial cells specifically affect the cell cycle of tobacco BY-2 cells at the prophase of mitosis. This effect requires the presence of a *fas*-dependent molecule. The importance of this molecule in the interaction between *R. fascians* and its host is discussed.

2. Materials and methods

2.1. Bacterial strains and growth conditions

R. fascians strains were grown on YEB at 28°C [16]. The strains used were D188 (a highly virulent wild-type isolate), D188-5 (an non-virulent derivative strain without any plasmid [17]), and *fas1* (a non-virulent mutant strain derived from D188, carrying an insertion in the *ipt* gene [3]). *R. fascians* cells were induced as described [18]. When appropriate, media were supplemented with chloramphenicol (25 μg/ml).

2.2. Amaranthus betacyanin bioassay

This standard cytokinin bioassay was carried out as described [19]. The amount of betacyanin produced was calculated as follows: $[OD_{542}-OD_{600}]_{sample}-[OD_{542}-OD_{600}]_{dark\ control}$.

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Abbreviations: BAP, 6-benzyl aminopurine; BY-2, Bright Yellow-2; CDK, cyclin-dependent kinase; 2,4-D, 2,4-dichlorophenoxyacetic acid; DAPI, 2′,6-diamidino-2-phenylindole; DMSO, dimethylsulfoxide; ipt, isopentenyl transferase; %MI, mitotic index; TFA, trifluoroacetic acid

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2.3. HPLC analysis

The presence of 6-benzyl aminopurine (BAP) in the BY-2 culture medium was monitored with a HPLC 600LC system (Waters, Milford, MA, USA) connected to a tunable absorbance detector 486 (Waters) and an chromato-integrator D-2000 (Merck-Hitachi, Darmstadt, Germany). The samples were acidified using trifluoroacetic acid (TFA, final concentration 0.1%) and analyzed on a reverse-phase column (4×250 mm, Vydac 218TP, C18, 300 Å, with guard column; Alltech, Deerfield, IL, USA) with water (A) and acetonitrile (B), both with 0.1% TFA as mobile phases. The gradient was linear from 0 to 50% B in 30 min and 50 to 100% B in 5 min, followed by a 15-min regeneration in phase A. The flow rate was 1 ml/min. BAP eluted around 21.30 min.

2.4. Maintenance, culture synchronization, and infection of tobacco BY-2 cells

A cytokinin-independent cell line derived from *N. tabacum* L. cv BY-2 callus (BY-2) was maintained as described [15] by a weekly 70-fold dilution in modified Linsmaier and Skoog medium supplemented with 0.2 mg/ml of 2,4-dichlorophenoxyacetic acid (2,4-D). Cells were synchronized as described [15]. In brief, 11–15 ml of a stationary, 7-day-old culture was grown in 100 ml fresh medium containing 5 mg/l aphidicolin (Sigma, St. Louis, MO, USA) for 24 h. Then the cells were washed five times with 3% (w/v) sucrose and inoculated in 100 ml fresh medium. Samples of 20 ml were inoculated with 1.5 ml of *R. fascians* cells at an OD_{600} of 2.0 or with the appropriate solution of the compounds to be tested. When desired, propyzamide was used at

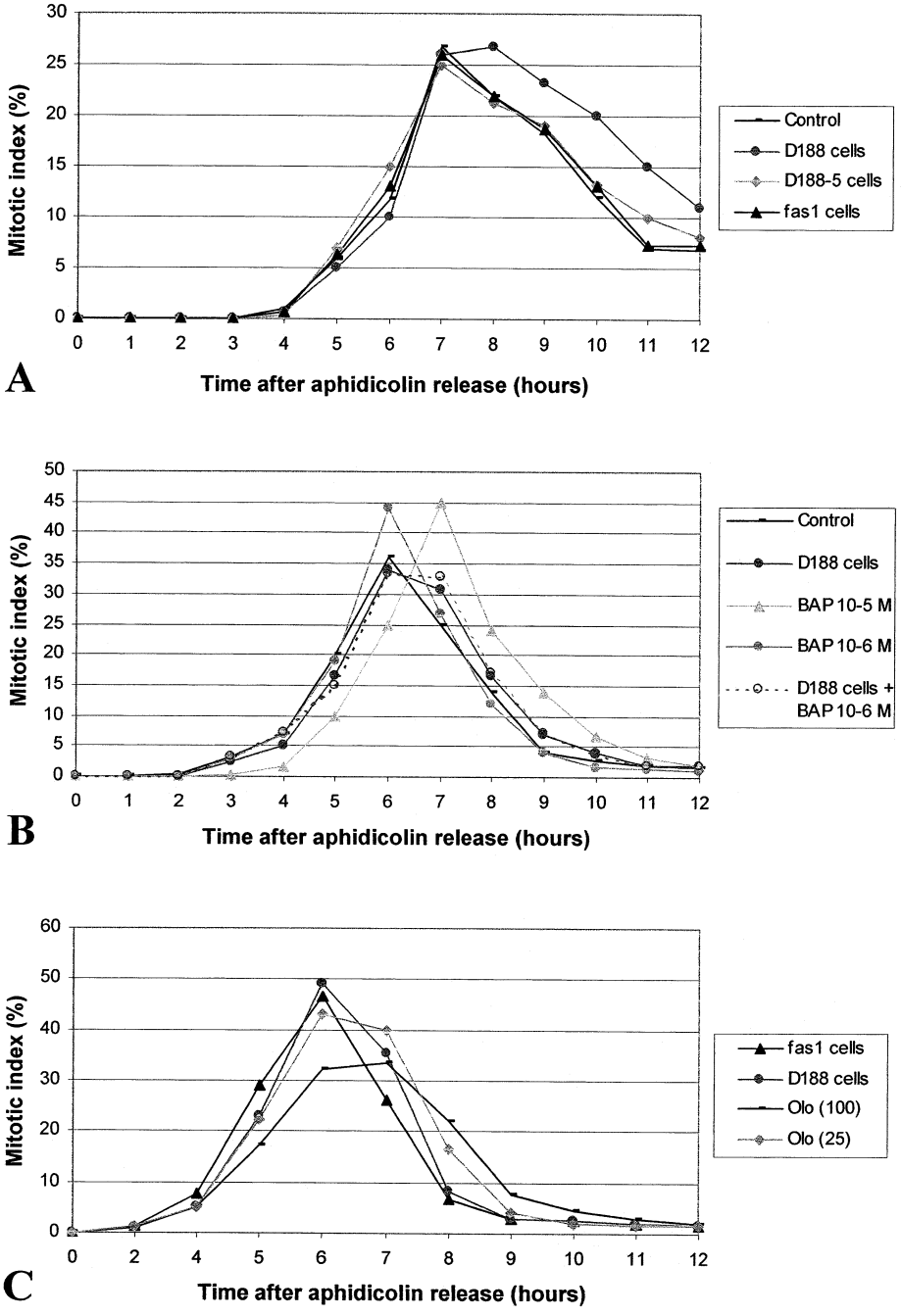

Fig. 1. %MI evolution in aphidicolin-synchronized BY-2 cells. Effect of (A) induced *R. fascians* cells, (B) BAP (10^{-6} M and 10^{-5} M) and (C) olomoucine (100 and 25 μM). The sample of interest is added immediately after release of the aphidicolin block at time point zero.

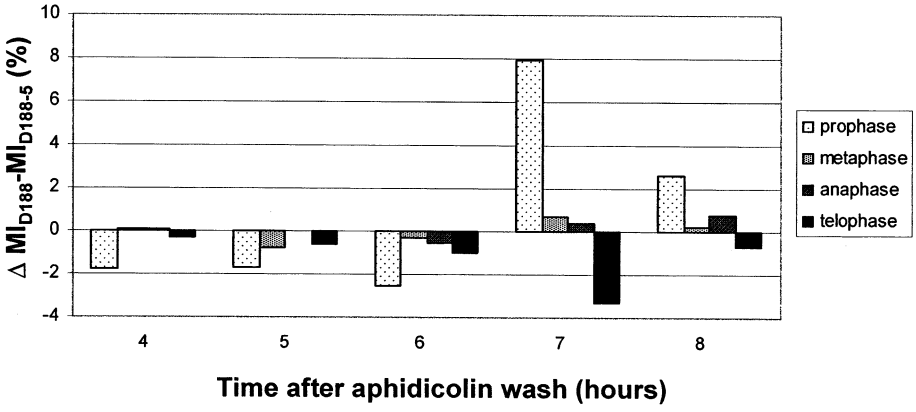

Fig. 2. Effect of *R. fascians* on the contribution of each mitotic phase to the total percentage of %MI.

a concentration of 3 μM, equally followed by extensive washing. For each sample, 750 μl of the cell culture was allowed to sediment on ice. After the supernatant was removed, the cells were fixed with 1 ml ethanol/acetic acid (3/1 (v/v)) and kept at 4°C. To determine the percentage of mitotic cells in a population (mitotic index, %MI), cells were washed with 1 ml of water and stained with 2′,6-diamidino-2-phenylindole (DAPI). Cells displaying mitotic figures were counted using an Axioscope microscope (Zeiss, Jena, Germany) equipped with a filterblock 02 (G365 excitation filter, FT 395 beam splitter, LP 420 barrier filter). For each sample, at least 1000 BY-2 cells were scored.

3. Results

3.1. *R. fascians influences the cell cycle of BY-2 cells*

By using aphidicolin (Section 2), a synchronized BY-2 cell culture was obtained that generally showed a maximal %MI peak between 25 and 35%, 6–7 h after the drug was released (Fig. 1A). Higher levels of synchrony, comparable to those described originally [15], were achieved in some experiments (Fig. 1C). Initial experiments in which synchronized BY-2 cultures were confronted either with non-induced wild-type D188, mutant D188-5, or *fas1* cells of *R. fascians* did not reveal any difference in %MI (data not shown). However, when D188 cells were induced for *fas* gene expression prior to infection [18], an effect on the cell cycle behavior of the tobacco cells was observed. The amount of cells that are in mitosis at the peak of the %MI curve was not affected (maximum difference of 2% between the samples within the experiment, Fig. 1A). 1 h later, with pre-induced D188, consistently 5.6 ± 1.6% more cells were found in mitosis, resulting in a broadening of the %MI peak, an effect that was not observed with pre-induced D188-5, *fas1* or non-treated control cells. Cytological analysis did not reveal any difference in cytology among the treated BY-2 samples (data not shown).

3.2. *The peak broadening is not a cytokinin effect*

The experiments described above indicate the necessity of an intact, induced *fas* locus for the peak broadening effect. Considering the putative cytokinin-like character of the *fas* product [3,4], the effect of BAP on synchronized BY-2 cells was tested. Addition of BAP to a final concentration of 10^{-6} M (Fig. 1B) or 10^{-7} M (data not shown) resulted in a 10% increase of the %MI peak when compared to the control samples containing D188 or dimethylsulfoxide (DMSO, used to prepare BAP solutions). At the following time point, however, the %MI dropped to levels comparable to those of the DMSO control sample. This result indicates that the *R. fascians*-induced peak broadening differs from cytokinin-mediated changes in BY-2 cell cycle behavior. Higher concentrations of BAP (10^{-5} M) also increased the %MI, although the peak was reached 1 h later (Fig. 1B).

Surprisingly, when a combination of BAP (10^{-6} M) and induced D188 cells was applied to synchronized BY-2 cultures, no cumulative effect (a higher and broader %MI curve) was observed. Instead, the %MI curve was similar to that obtained upon infection with D188, thus a typical broader, but not a higher curve (Fig. 1B). A possible explanation is that *R. fascians* would be able to degrade or inactivate BAP during the experiment. Therefore, the presence and activity of BAP was evaluated after overnight incubation of *R. fascians* in BY-2 culture medium supplemented with 10^{-6} M BAP. HPLC analysis revealed that the amount of BAP did not decrease (Table 1). Furthermore, the activity of BAP measured by using the *Amaranthus caudatus* betacyanin assay [19] also remained unaffected, indicating that another mechanism underlies the dominant effect of *R. fascians* over cytokinins on BY-2 cells.

Table 1
Effect of *R. fascians* on the presence and activity of BAP in BY-2 medium after overnight incubation

Sample	Amount of BAP[a] (peak integrated area × 1000)	Cytokinin activity[b] ((OD$_{542}$ − OD$_{600}$) × 1000)
BAP	181 ± 4	17 ± 4
Medium+BAP	190 ± 5	20 ± 3
Medium+BAP+D188	190 ± 11	23 ± 5
Medium+BAP+D188-5	184 ± 5	22 ± 2

[a]Areas of the BAP peak isolated by HPLC and calculated by the on-line chromato-integrator represent the amount of BAP in each sample; absorbance is measured at 234 nm.
[b]Measured with the *Amaranthus* betacyanin assay; the BAP concentration used is 10^{-6} M.

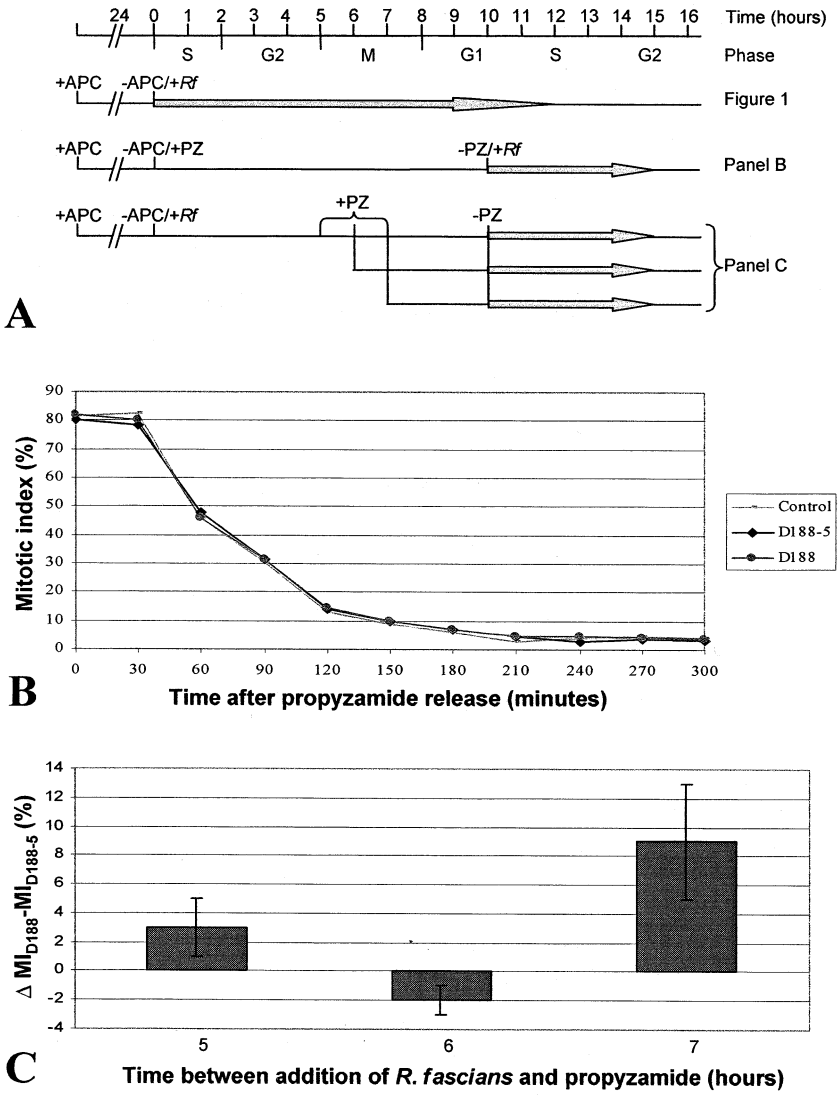

Fig. 3. Effect of *R. fascians* on double-synchronized BY-2 cells. A: Schematic representation of the experiment. APC, aphidicolin; PZ, propyzamide; Rf, *R. fascians* cells (or water for the control). The arrows indicate the period in which samples were collected. B: Effect of D188, D188-5, and water added immediately after release from propyzamide (10-h treatment). C: Effect of D188 and D188-5 added upon aphidicolin release followed by addition of propyzamide. The period between addition of the bacteria and propyzamide is indicated in hours.

3.3. *R. fascians causes an extended prophase upon infection of BY-2 cells*

An effect of *R. fascians* on the duration of one or more of the mitotic phases was considered the basis of the peak broadening. To evaluate this, the number of BY-2 cells in a specific phase of mitosis were counted. Fig. 2 shows a higher percentage of cells in prophase at time point 7 upon D188 treatment when compared to D188-5, suggesting this increase of prophasic cells is the main contribution to the peak broadening effect. To further investigate this possibility, blocking experiments with double-synchronized cells were designed.

Propyzamide inhibits the polymerization of tubulin [20] and causes the cells to arrest in prophase, enabling a cell synchrony up to 90% [15]. In one set of experiments, propyzamide was added to BY-2 cells immediately after release from an aphidicolin block, and was washed away after 10 h. Immediately after release from the propyzamide block, the cultures were treated with D188, D188-5, or water (Fig. 3A). In the double-synchronized cells, D188 had no effect (Fig. 3B).

Previous experiments showed that the peak broadening effect was induced upon D188 infection of synchronized BY-2 cells 6 h after aphidicolin release, i.e. 1 h prior to the %MI peak, indicating that little time is required for D188 to exert its effect (data not shown). The absence of a peak broadening in double-synchronized BY-2 cells thus excludes a prolongation of the mitotic phases following prophase and further suggests that D188 retards the prophase.

In a second set of experiments, bacteria were added to the BY-2 culture upon release of the aphidicolin block, followed by application of propyzamide at various time points (Fig. 3A), allowing a fraction of the cells to pass the prophase before they are blocked. When propyzamide was added 5 or 6 h after aphidicolin release, no major difference in %MI is found between D188- and D188-5-treated samples (Fig. 3C). However, when added after 7 h, the amount of cells blocked in mitosis was higher in the D188-treated sample ($9 \pm 4\%$), implying that BY-2 cells infected with pre-induced D188 cells proceeded through prophase at a slower rate than

D188-5-infected control cells. This also suggests that D188 affects BY-2 cells only in late G2 phase and/or in prophase.

4. Discussion

Cell cycle progression of BY-2 cells depends on the exogenous addition of auxin, but does not require exogenous cytokinin [15]. In aphidicolin-synchronized BY-2 cells, the levels of specific cytokinins sharply increase at the end of the S phase and during mitosis [21], suggesting that these hormones play an important role in cell cycle progression. Similar observations were made with synchronized suspension cultures of the *N. tabacum* L. cv. Xanthi cell strain XD6S, another cytokinin-autonomous cell line, in which the G2-to-M transition is accompanied by a sharp increase of endogenous cytokinin levels [22]. The increases in cytokinin levels most probably result from de novo synthesis and not from hydrolysis of *O*-glucosides [21,23]. Because cytokinins have been shown to influence the plant cell cycle [14,21,23–26], a signal molecule that is produced by the *fas*-locus encoded proteins, one of which is the central enzyme in cytokinin production (IPT; [3]), was assumed to affect cell division. BY-2 cells are cytokinin autonomous and have been selected for their high rate of cell division [15]. By using these rapidly dividing cells, a possible mitogenic effect of (a) *R. fascians*-derived, putative cytokinin-like signal(s) may remain undetected. To trace a stimulating activity of *R. fascians*, or its produced signals, on cell division, the use of cells that essentially do not divide unless they are provided with a mitogen is more appropriate.

However, *R. fascians* was shown to specifically affect the cell cycle of synchronized tobacco BY-2 cells in a way that is distinct from the action of cytokinins. Despite the cytokinin independence of BY-2 cells [15,21,23], addition of BAP (Fig. 1B) or zeatin (data not shown) increases the %MI peak of an aphidicolin-synchronized culture. This indicates that a significant population of BY-2 cells does not divide in the absence of this hormone and can be stimulated to enter M-phase. *R. fascians* alters the mitotic behavior of synchronized BY-2 cells in a different way than exogenously applied cytokinins: the action of (a) *fas*-dependent product(s) consistently results in a broader %MI peak (Fig. 1). The data presented suggest an effect of (a) *fas*-dependent signal(s) on the prophase, i.e. the affected BY-2 cells show an extended prophase (Figs. 2 and 3).

Considering the proposed role for the *fas* locus-encoded proteins to specifically modify an adenine-based cytokinin [4], the action of olomoucine, a C^2-, N^6-, N^9-substituted purine, is of particular interest. Olomoucine has been shown to slow down the prophase-to-metaphase transition in cleaving sea urchin embryos, whereas it does not affect the duration of the metaphase-to-anaphase and anaphase-to-telophase transitions [27]. Such an effect would thus result in a prolonged prophase, which is quite similar to what is found for *R. fascians* D188-infected BY-2 cells. Indeed when the effects of olomoucine were investigated in our experimental system, a peak broadening that was reminiscent of the D188-provoked effect was observed at 25 μM (Fig. 1C) or lower concentrations (10 and 5 μM; data not shown). At 100 μM (Fig. 1C) or 50 μM (data not shown), which are concentrations known to stop cell cycle progression in *Petunia* protoplasts and *A. thaliana* cell suspension cultures [28], significantly less BY-2 cells entered mitosis, possibly because of the toxic effects of the drug. Nevertheless, those cells which enter mitosis do show the characteristic peak broadening. Olomoucine has been found to strongly inhibit only the CDKs CDC2, CDK2, and CDK5 and, to a minor extent, the ERK1-MAP kinase [29] by competing for the ATP binding site. Olomoucine reduces the in vivo tyrosine dephosphorylation of $p34^{cdc2}$, presumably by interruption of the positive feedback loop by which the CDC2 kinase phosphorylates and hyperactivates the CDC25 phosphatase [29].

Cytokinins, on the other hand, have been shown to activate the $p34^{cdc2}$/cyclinB complex by stimulating dephosphorylation of the CDK, allowing the cells to enter mitosis [25]. A burst of cytokinin accumulation in late G2 phase reflects the essential role of this hormone in progression of the plant cell cycle from G2 to M phase [12,21,23]. These data suggest that the mitosis-promoting effect of exogenously applied BAP on BY-2 cells (Fig. 1B) is also mediated by an activation of the CDK/cyclinB complex. Interestingly, simultaneous addition of cytokinins and induced D188 cells did not result in a combination of both effects, but only in a broader %MI peak (Fig. 1B). Because *R. fascians* does not degrade or inactivate the added cytokinin (Table 1), the *fas*-derived molecule(s) might override the response of these cells to cytokinin. A possible implication is that both types of molecules must, at least in part, exert their effect via the same signal transduction pathway or that a feedback mechanism exists between two separate pathways. The exact mode of action of the *fas*-derived product(s) underlying the extension of the prophase remains to be determined.

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