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Cytokinins induce sporulation in *Dictyostelium*

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The social amoeba *Dictyostelium discoideum* diverged from the line leading to animals shortly after the separation of plants and animals but it retained characteristics of both kingdoms. A $\mathrm{GABA_B}$-like receptor and a peptide, SDF-2, with homologs found only in animals, control sporulation, while cytokinins, which act as hormones in plants, keep spores dormant. When SDF-2 binds its receptor DhkA, it reduces the activity of the cAMP phosphodiesterase RegA such that cAMP levels can increase. It has been proposed that the cytokinin discadenine also produces in an increase in cAMP but acts through a different histidine kinase, DhkB. We have found that discadenine and its precursor, isopentenyl adenine, not only maintain spore dormancy but also initiate rapid encapsulation independently of the SDF-2 signal transduction pathway. DhkB and the adenylyl cyclase of late development, AcrA, are members of two component signal transduction families and both are required to transduce the cytokinin signal. As expected, strains lacking the isopentenyl-transferase enzyme chiefly responsible for cytokinin synthesis are defective in sporulation. It appears that SDF-2 and cytokinins are secreted during late development to trigger signal transduction pathways that lead to an increase in the activity of the camp-dependent protein kinase, PKA, which triggers rapid encapsulation as well as ensuring spore dormancy.

KEY WORDS: Discadenine, Isopentenyl adenine, Zeatin, Histidine kinase, SDF-2, Sporulation

INTRODUCTION

Development of *Dictyostelium discoideum* leads to a fruiting body where the mass of spores is held up on a tapering stalk that can be several millimeters high. At the beginning of culmination, migrating slugs become upright and prestalk cells at the tip construct a cellulosic stalk tube. Cells enter the tube and vacuolize to give it added strength. Once the stalk has extended down through the underlying prespore cells to the substratum, further expansion of cells within the stalk extends it upwards. Prestalk cells continue to climb the stalk and enter at the top before vacuolizing. Prespore cells follow behind until the stalk is almost complete, at which point they rapidly encapsulate into dormant spores. The whole process takes about 24 hours (Raper, 1940; Loomis, 1975). As each spore is surrounded by a cellulose reinforced protein coat, prespore cells cannot move once they are encapsulated. Premature encapsulation results in spores that cannot reach the top of the stalk. Therefore, the timing of sporulation must be carefully controlled.

Sporulation of dispersed cells of a strain (KP) with partially constitutive PKA activity has been shown to be density dependent when they are developed on the bottoms of multi-test wells (Anjard et al., 1997). At densities higher than 10^4 cells/cm^2, a phosphopeptide of about 1.2 kDa accumulates in the buffer. When this peptide, SDF-1, is purified and added back to KP cells developing at lower density, it induces sporulation 90 minutes later in a process that requires protein synthesis (Anjard et al., 1997). SDF-1 accumulates in fruiting bodies of wild-type cells where it is found together with several other factors that can also induce encapsulation of KP cells developed at low density. GABA, produced by the enzyme glutamate decarboxylase (GadA) induces sporulation at 1 nM concentration (Anjard and Loomis, 2006). When GABA binds its receptor GrlE, a G-protein-coupled receptor of the $\mathrm{GABA_B}$ metabotropic receptor-like family, it triggers the rapid release of the precursor of a second peptide factor, SDF-2, that can also induce encapsulation in test cells. SDF-2 is a 34 amino acid peptide cleaved from the secreted precursor AcbA (acyl-CoA binding protein) (Anjard and Loomis, 2005). SDF-2 binds the receptor histidine kinase DhkA and inhibits phosphorelay to the internal cAMP phosphodiesterase RegA, resulting in a decrease in its activity. The internal concentration of cAMP can then increase and activate PKA, which leads to rapid encapsulation of prespore cells (Anjard and Loomis, 2005).

Strains in which the genes encoding either AcbA or DhkA are disrupted sporulate poorly but can sometimes reach 60% of the wild-type level of spores, suggesting that there may be other sporulation signals. Disruption of the gene encoding another histidine kinase, DhkB, reduces the proportion of spores to about one-third when culmination is first completed (Zinda and Singleton, 1998). The number of viable spores in *dhkB*$^-$ strains decreases after 25 hours of development such that it is only 3% of wild-type levels by 72 hours, apparently because the spores germinate while still on top of the stalk. Zinda and Singleton (Zinda and Singleton, 1998) suggested that *dhkB*$^-$ mutant cells were unable to respond to the germination inhibitor, discadenine, to maintain dormancy. DhkB may also play a role in initiating sporulation as double mutants lacking both DhkA and DhkB are much more impaired in spore formation than either of the single mutants lacking only one of these histidine kinases (Wang et al., 1999). The almost complete lack of sporulation in *dhkA*$^-$ *dhkB*$^-$ strains suggests that sporulation may result from the combined activity of both histidine kinases. The adenylate cyclase AcrA is also required for sporulation and spore dormancy as the null strain is impaired in spore formation (Soderbom et al., 1999). Most of the spores that form in *acrA*$^-$ null strains fail to remain dormant and rapidly germinate. AcrA is a membrane associated adenylyl cyclase that carries a degenerate histidine kinase domain of unknown function. The region of the domain involved in phospho-transfer in other histidine kinases has multiple variations in key amino acids in AcrA and is probably not functional, whereas the two receiver domains in AcrA are well conserved and could accept a phosphate group transferred by one of the 13 functional histidine kinases existing in *Dictyostelium* (Anjard and Loomis, 2002).

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Cytokinins are N^6 substituted adenine derivatives that affect growth and development of plants by activating two-component phosphorelay pathways (Mok and Mok, 2001; Kakimoto, 2001; Kakimoto, 2003; Rashotte et al., 2006). There are three different receptor histidine kinases in the mustard *Arabidopsis thaliana*: AHK2, AHK3 and AHK4/CRE1 (Inoue et al., 2001; Nishumura et al., 2004; Suzuki et al., 2001; Yamada et al., 2001). Cytokinins bind to a conserved extracellular loop of about 200 amino acids found in each of these receptors, referred to as the CHASE domain (Anantharaman and Aravind, 2001; Heyl et al., 2007). Discadenine is a derivative of the cytokinin isopentenyl adenine, which is synthesized by condensation of isopentenylpyrophosphate and 5′AMP followed by removal of the ribose phosphate group (Abe et al., 1976; Taya et al., 1978). Both isopentenyl adenine and discadenine can be extracted from *Dictyostelium* fruiting bodies and shown to inhibit germination when added to washed spores at levels above 1 μM (Tanaka et al., 1978; Ihara et al., 1980) (D. Cotter, personal communication). Moreover, discadenine shows cytokinin activity in an assay using tobacco callus cells (Nomura et al., 1977). We have found that both discadenine and isopentenyl adenine, as well as other cytokinins, induce rapid sporulation in *Dictyostelium* in a process that is dependent on DhkB and AcrA. The cytokinin signaling pathway is independent of the SDF-2 pathway but both converge at the level of activation of PKA through an increase of intracellular cAMP.

MATERIALS AND METHODS

Chemicals

Synthetic SDF-1 and SDF-2 have previously been described (Anjard and Loomis, 2005). Zeatin and isopentenyl adenosine were purchased from Acros Organics (Geel, Belgium). Isopentenyl adenine, other cytokinins and adenine were purchased from Sigma (St Louis, MO). Synthetic DL-discadenine was a kind gift from Dr David Cotter. Labeled N^6-isopentenyl [2-^{3}H]adenine, 1 TBq/mmol, >97% pure, was purchased from Isotope Laboratory of the Institute of Experimental Botany (Prague, Czech Republic).

H89 and myristoylated PKI (14-22) were purchased from Calbiochem (San Diego, CA). The catalytic subunit of *D. discoideum* PKA is sensitive to these inhibitors (Anjard et al., 1993; Anjard et al., 1997).

Strains and bioassay

The wild-type strain AX4, the *pkaC* overexpressing strain KP and its derivative *dhkA*$^-$/K have been previously described (Anjard et al., 1992; Anjard et al., 1998a). The *acgA*$^-$ strain was a kind gift from Pauline Schaap (van Es et al., 1996). To generate the *dhkB*$^-$/K strain, KP cells were transformed with the construct used in the original disruption of *dhkB* (Zinda and Singleton, 1998). After selection with blasticidin and sub-cloning, *dhkB* disruptants were identified using specific primers. More than 90% of the clones presented the expected pattern for *dhkB* disruption.

In order to disrupt *iptA* (DDB0233672; GenBank XP 642693), a 1.5 kb fragment was amplified by PCR and cloned in the pGEMT vector (Promega A1360). The BSR cassette from pBSR519 (Puta and Zeng, 1998) was cloned into the unique *Eco*RI site located at codon 35 of the *iptA*. For gene disruption, 10 μg of the plasmid was linearized with *Not*I before electroporation into 10^7 AX4 cells. Disruption of the endogenous gene in transformants was confirmed by PCR using primers located outside the cloned sequences.

The bioassay was carried out on KP cells and their derivatives after 18 hours development in monolayers as previously described (Anjard et al., 1998a). Cells were incubated in buffer (20 mM MES pH 6.2, 20 mM NaCl, 20 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) at a density of 2×10^3 cells/cm^2 in the wells of a 24-well dish at 23°C. After 18 hours incubation, samples or defined products were added and the number of spores and undifferentiated cells were counted 1 hour (SDF-2) or 2 hours (SDF-1) later. The amounts of SDF-1 and SDF-2 activity were determined by serial dilution of the sample before addition to KP cells. One unit corresponds to the lowest dilution, giving full induction of spore formation. The number of units in the sample were standardized to 10^3 producing cells when applicable. The cell density of monolayers of *regA*$^-$ cells in the wells had to be reduced to 5×10^2 cells/cm^2 to reduce the level of spontaneous sporulation in the absence of added signals.

The response of cells from strains that are not sporogenous to sporulation inducers was measured following dissociation of culminants that had developed on filters (Anjard and Loomis, 2005). Filters (25 mm diameter) were each spread with 10^7 cells and allowed to develop for 20 hours at 22°C. Each filter was then examined under a dissecting microscope. Only those filters where most of the structures were similar were used and any asynchronously developing culminants were removed from these filters with a needle. The cells were allowed to continue to develop and monitored every 15 minutes. When stalks became apparent under the rising sori, the cells were collected by vortexing the filters in 1 ml cAMP buffer followed by centrifugation at 4000 rpm for 1 minute in a microfuge. The cells were counted and diluted to 3.6×10^4/ml. Because the window of development during which induction of sporulation can be assayed is only 15-30 minutes, only preparations that contained between 10% and 20% spores were used. Suspension (500 μl) was added to each well of a 24-well plate, resulting into a cell density of 10^4/cm^2. Inducing compounds were added at various concentrations and the number of spores counted after 1 hour.

Expression of iptA in bacteria

The coding sequence of *iptA* was amplified by PCR using oligonucleotides that included a *Nco*I restriction site at the 5′ end and a *Xho*I site at the 3′ end. The coding sequence was cloned in pGEMT-EASY (Promega A1360) and sequenced. The *Nco*I-*Xho*I fragment was cloned into pET32a using the *Nco*I-*Xho*I restriction sites. This plasmid was introduced into *E. coli* BL21 DE3 and transformants selected. An overnight culture of BL21 DE3 transformed with pET32a-iptA or an empty vector were diluted 1/100 into 50 ml LB containing 50 μg/ml carbenicillin and grown to an OD_{600} of 0.4 before induction with 1 mM IPTG. The bacteria were incubated on a shaker at 37°C for another 4 hours. For the bioassay, 1 ml aliquots of the cultures were harvested and the bacteria pelleted by centrifugation at 14,000 rpm in an Eppendorf Microfuge for 1 minute. The supernatants were then tested for sporulation induction on the KP cells.

For determination of isopentenyl adenine production, 20 ml aliquots of the induced bacterial cultures were collected and mixed with 80 ml ethanol. Insoluble material was pelleted by centrifugation at 12,000 ***g*** for 30 minutes. The supernatants were dried under vacuum and resuspended in 10 ml water before addition of 1 ml of 50% Amberlite XAD-2 (Supelco, Bellafonte, PA). After 30 minutes incubation, the resin was spun down and washed twice with 10 ml water. Isopentenyl adenine was eluted by three successive additions of 2 ml of 30% ethanol. The eluates were pooled, dried under vacuum and resuspended in 100 μl methanol before analyses by HPLC/MS (see below).

Development and spore viability

Cell were grown in axenic media (HL5) at 22°C in shaking culture (Sussman, 1987). Development was initiated by harvesting exponentially growing cells at a density of 2-5 10^6/ml. Cells were washed with PDF buffer [20 mM Na/K phosphate (pH 6.5), 20 mM KCl, 1.2 mM $MgSO_4$], centrifuged again at 1000 rpm for 5 minutes and resuspended at a density of 1-2×10^8 cells/ml in PDF before being deposited on nitrocellulose filters placed on pads saturated with PDF (Anjard and Loomis, 2005).

For spore viability assays, 10^7 washed cells were deposited on a small filter and developed for 24 hours. Spores were collected by placing the filter in an Eppendorf tube with 1 ml PDF containing 0.5% Triton X-100 and briefly vortexed. Spores were incubated for at least 5 minutes in the buffer containing 0.5% Triton X-100 and then centrifuged at 6000 rpm for 1 minute and resuspended in 1 ml PDF. After counting and dilution, 50 spores were plated in triplicate on SM plates with a *K. aerogenes* suspension (Anjard and Loomis, 2005). The number of plaques, corresponding to the number of viable spores, was scored after 4-5 days of incubation at 22°C. Spore viability assays were repeated at least three times.

Isopentenyl adenine binding assay

Vegetative cells were centrifuged at 1200 rpm for 5 minutes and washed twice in 10 ml binding buffer [50 mM phosphate buffer (pH 7.5), 200 mM NaCl] per 10^8 cells. Developed cells were generated by depositing 5×10^7

to 10^8 cells on 4.5 cm diameter filters and incubating at 20°C until the fruiting bodies reached the early-mid-culmination stage (~22 hours). Fruiting bodies were collected on a spatula and washed three times in binding buffer before the cells were resuspended at density of 2.5×10^7/ml in binding buffer containing 1.25 mM adenine. Protein concentration of the suspension was determined using the BioRad reagent.

Aliquots of 400 μl of the cells suspension were incubated for 5 minutes at room temperature with the indicated concentrations of ^{3}H-isopentenyl adenine (^{3}H-iP) with and without a 10,000 fold excess cold iP, the final volume being 500 μl. Suspension (400 μl) was then filtered under vacuum through a GF/C filter (Whatman, Maidstone, England) on a manifold unit and washed three times with 2 ml binding buffer. The filters were counted in scintillation liquid, together with a standard for the amount of ^{3}H-iP used. Each point was carried out in duplicate (two tubes with ^{3}H-iP alone, two tubes with ^{3}H-iP and a 10,000 fold excess unlabelled iP) and repeated three independent times. Specific binding was taken as the difference between cell-associated counts in the absence and presence of unlabelled iP and was normalized to fmol ^{3}H-iP per mg of protein.

Isopentenyl adenine and discadenine quantitation

Isopentenyl adenine and discadenine were purified from developed *Dictyostelium* cells using a simplification of the protocol of Taya et al. (Taya et al., 1980). Cells (10^8) of each strain were developed on filters for the indicated times, harvested with a spatula and resuspended in 10 ml water. Cells were pelleted by centrifugation at 2000 rpm for 5 minutes and the supernatants were incubated for 10 minutes with 1 ml of Amberlite XAD-2 resin. The resin was collected by centrifugation at 1000 rpm for 5 minutes and washed twice in 10 ml water. Cytokinins were eluted from the resin by three successive additions of 1 ml 30% ethanol which were pooled and dried under vacuum before being resuspended in 100 μl of methanol. Between 2.5 and 25 μl of the samples were loaded on a Majic C-18 Column (ID 1 mm × 150 mm) column using an ultrafast HPLC apparatus (Microm BioResources) coupled to a LCQdeca-Mass spectrometer with electrospray ionization source (ESI) under positive ion mode (ThermoFinnigan). The LC mobile phase A was 2.5% methanol in water and the LC mobile phase B was pure methanol. The LC flow rate was 50 μl/minute, and the LC gradient was 10% B to 95% B in 20 minutes then held at 95% B for three minutes. Isopentenyl adenine gives a characteristic $[M+H^+]$ peak at m/z 204 and is eluted after about 17 minutes in this gradient. Discadenine gives a $[M+H^+]$ peak at m/z 305 and is eluted after about 3 minutes. The product identities were further confirmed by ESI-MS/MS analysis. Upon ESI-MS/MS fragmentation, isopentenyl adenine gives characteristic daughter peaks at m/z 136 and m/z 148 respectively, while discadenine gives a daughter peak at m/z 204 (data not shown). Known amounts of isopentenyl adenine and discadenine standards were run under identical conditions for quantitation of the samples using select ion monitoring.

RESULTS

Cytokinins induce rapid sporulation

Addition of discadenine or isopentenyl adenine results in rapid encapsulation of developing KP cells (Fig. 1A). Maximum activity was observed at concentrations of 10 nM, considerably less than the levels required for inhibition of germination of wild-type spores (Tanaka et al., 1978; Taya et al., 1980). It appears that discadenine and isopentenyl adenine not only function to maintain spore dormancy but also act earlier to induce sporulation.

Zeatin, a plant cytokinin not found in *Dictyostelium*, induces rapid encapsulation but only when added to about 100-fold higher concentration (Fig. 1A). The artificial cytokinins, thidiazuron, kinetin and 6-benzyl-aminopurine, which are not found in either plants or *Dictyostelium* but are as effective as the natural cytokinins in plants, also induce rapid sporulation in *Dictyostelium* when present at more than 10 μM (Fig. 1B). Induction of sporulation by cytokinins appears to act through PKA as addition of either of the specific inhibitors, H89 or myristoylated PKI, blocks sporulation in response to isopentenyl adenine (Fig. 1A).

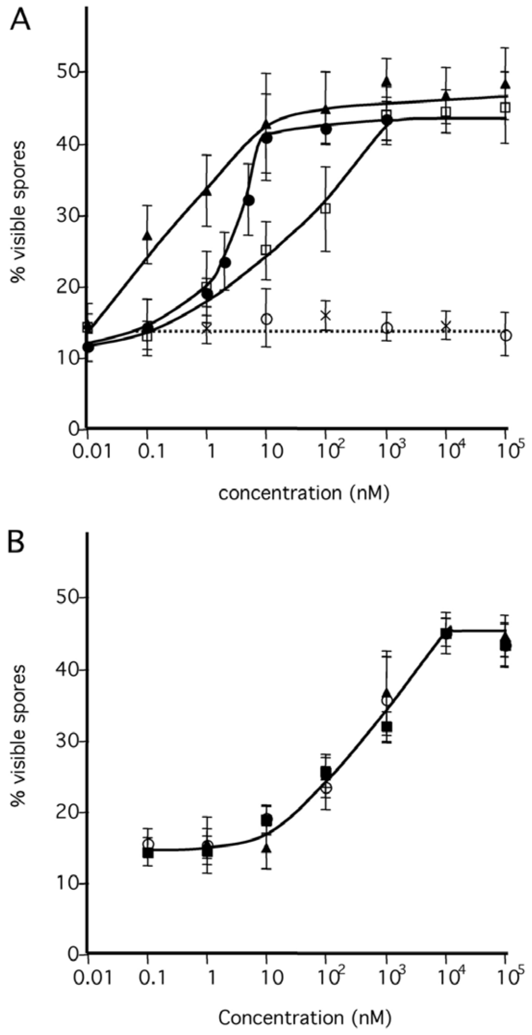

Fig. 1. Sporulation induced by cytokinins. (**A**) Developed KP cells were incubated with various concentrations of isopentenyl adenine (triangles), discadenine (filled circles) or zeatin (squares). Cells were treated with PKA inhibitors 10 μM H89 (open circles) or 5 μM myristoylated PKI (crosses) 45 minutes prior to addition of isopentenyl adenine. Spores were counted after 1 hour. (**B**) Developed KP cells were incubated with various concentrations of the artificial cytokinins, thidiazuron (squares), kinetin (open circles) and 6-benzyl-aminopurine (triangles), and the number of spores determined after 1 hour. Each experiment was repeated three to five times.

The number of spores started to increase within 10 minutes of addition of discadenine and reached maximum at 45 minutes (Fig. 2). There was no response to 1 mM adenine which is not a cytokinin. Simultaneous addition of discadenine and SDF-2 did not increase the proportion of spores (data not shown).

iptA encodes isopentenyl-transferase which generates cytokinin

The first step dedicated to the biosynthesis of cytokinins is catalyzed by isopentenyl-transferase and results in the condensation of isopentenyl pyrophosphate with AMP, ADP or ATP (Taya et al., 1978; Ihara et al., 1984). After dephosphorylation, the ribose moiety is removed to generate isopentenyl adenine. Isopentenyl adenine is also generated by post-transcriptional addition of an isopentenyl group to adenosine groups in tRNA. Most bacteria and

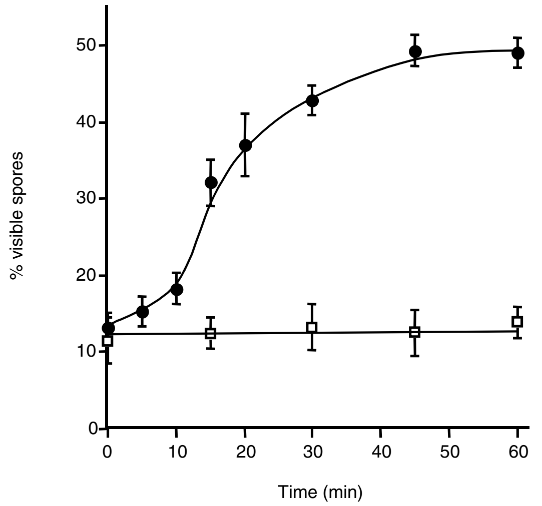

Fig. 2. Time course of spore induction by discadenine. KP cells that had developed at low density in buffer containing cAMP were treated with 100 nM discadenine (circles) or 1 mM adenine (squares). The proportion of cells that became ellipsoid phase-bright spores was determined microscopically over the following hour. The experiments were repeated three times.

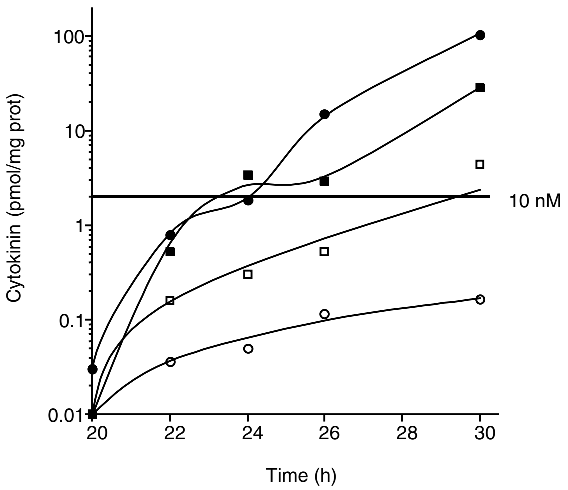

Fig. 3. Isopentenyl adenine and discadenine production in wild-type and *iptA*-null strains. Isopentenyl adenine (squares) and discadenine (circles) recovered from wild-type cells (filled symbols) and mutant cells (open symbols) at the indicated times of development. The horizontal line indicates the approximate level of cytokinin required to induce spore formation fully.

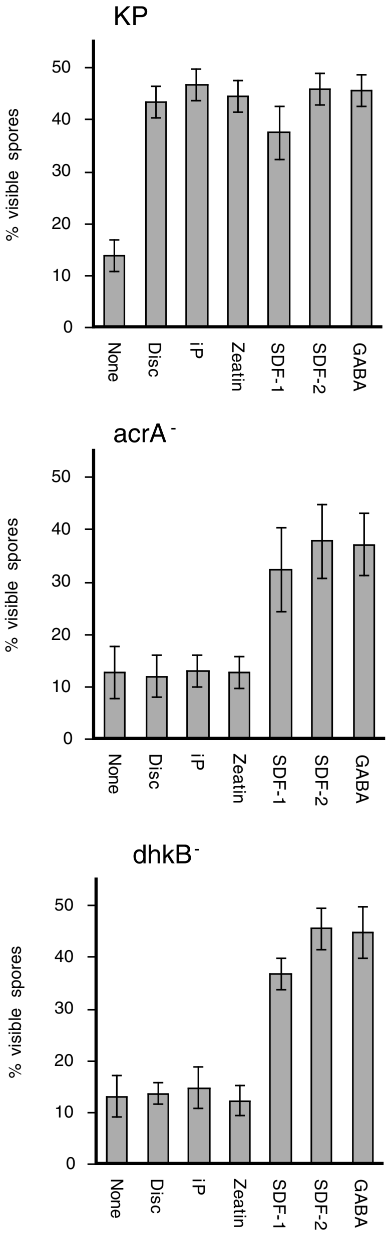

Fig. 4. Response of sporogenous strains to spore inducers. Mutant strains overexpressing the catalytic subunit of PKA as the result of being transformed with the KP construct were developed as monolayers at 2×10^3 cells/cm^2 for 20 hours. Cells of the indicated mutant strains were then treated with no addition (none), 100 nM discadenine (disc), 100 nM isopentenyl adenine (iP), 1 μM zeatin, 10 pM synthetic SDF-1, 10 pM synthetic SDF-2 or 1 μM GABA. Spores were counted after 1 hour. Cells treated with SDF-1 were scored after 90 minutes. Each experiment was repeated three times. Error bars correspond to one standard deviation.

animals have a single gene encoding isopentenyl-transferase to modify their tRNAs. Plants, however, have multiple isopentenyl-transferases (Takei et al., 2001; Miyawaki et al., 2006). *Dictyostelium* has three isopentenyl-transferases genes, *iptA*, *iptB* and *iptC*. A phylogenic analysis of the corresponding proteins showed that IptB and IptC are closely related to the eukaryotic and bacterial isopentenyl-transferases that modify tRNAs, while IptA clusters with enzymes involved in cytokinin synthesis (data not shown).

We constructed a plasmid for expression of *iptA* in *E. coli* and found that culture supernatants of bacteria transformed with this construct contain material that induces rapid sporulation in KP cells, whereas supernatants from cultures of untransformed cells have no effect (data not shown). Cytokinins were purified from supernatants of *iptA*-expressing bacteria by extraction with ethylacetate and absorption on the hydrophobic resin Amberlite XAD-2. The recovered material was analyzed by Mass Spectroscopy after HPLC on C-18 column and compared with authentic compounds. We found 210 pmol/ml isopentenyl adenine in the supernatants of

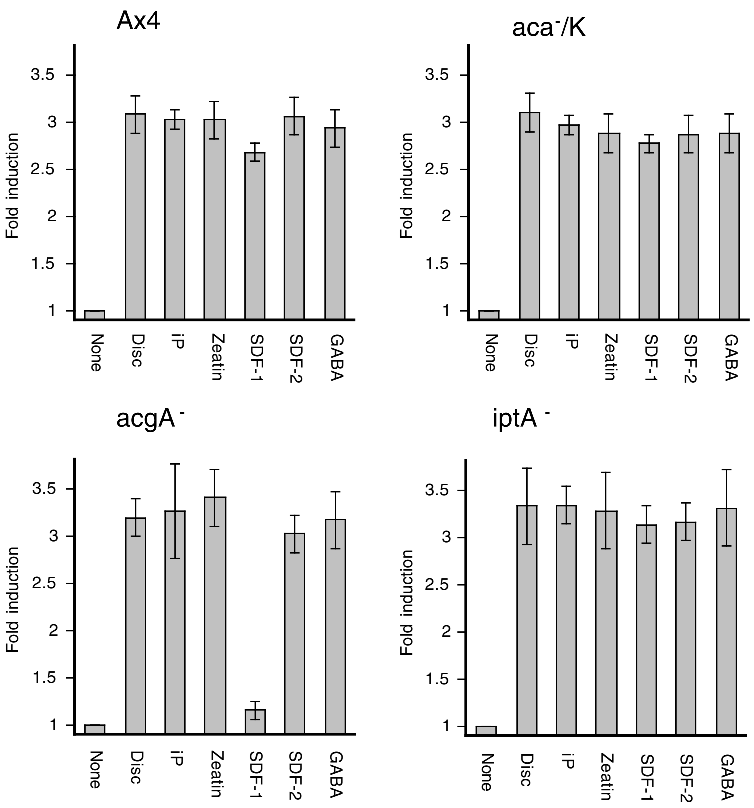

Fig. 5. Response to spore inducers in culminants. Cells of the indicated wild-type and mutant strains were developed on filters and harvested at the mid-culmination stage. The fruiting bodies were dissociated and the cells washed before plating a density of 10^4 cells/cm^2 in cAMP buffer. The cells were then treated with no addition (none), 100 nM discadenine (disc), 100 nM isopentenyl adenine (iP), 1 μM zeatin, 10 pM synthetic SDF-1, 10 pM synthetic SDF-2 or 1 μM GABA. Spores were counted after 1 hour for most factors. Cells treated with SDF-1 were scored after 90 minutes.

transformed bacteria and less than 10 pmol/ml in the supernatants of mock transformed cells (data not shown). It appears that *iptA* encodes the isopentenyl-transferase responsible for synthesis of isopentenyl adenosine.

Reduction of cytokinin production impairs spore viability

We disrupted *iptA* in wild-type AX4 cells by introducing a construct in which a blasticidin resistance cassette was inserted near the start of the gene and selected for blasticidin resistant transformants. Strains in which *iptA* was disrupted by homologous recombination were found to grow and develop well, but to form many round wrinkled spores rather than the normal ellipsoid shaped spores. Cytokinins were purified from wild-type and mutant fruiting bodies at different times in development and quantitated following separation by HPLC. No isopentenyl adenine or discadenine could be detected before 20 hours of development. Thereafter, the cytokinins accumulated rapidly although isopentenyl adenine leveled off for 2 hours at 24 hours of development in wild-type cells perhaps as the result of rapid conversion to discadenine at this time (Fig. 3). The rate of accumulation of the cytokinins was reduced >90% in the *iptA*$^-$ cells (Fig. 3). The amount of isopentenyl adenine and discadenine isolated from wild-type fruiting bodies was comparable with the levels observed by Ihara et al. (Ihara et al., 1980), whereas the levels in *iptA*$^-$ cells were less than 10%. Assuming that the cytokinins are uniformly distributed, we calculated that the threshold concentration of 10 nM cytokinin, sufficient to fully induce spore formation, corresponds to about 2 pmol/mg protein in our assay. Wild-type cells reach this level between 22 and 24 hours of development, whereas *iptA*$^-$ cells only reach it after 30 hours of development, long after encapsulation is usually completed. As less than half of the *iptA*$^-$ spores were viable after detergent treatment, whereas wild-type spores are completely detergent resistant (see Table S1 in the supplementary material), it appears that these cytokinins play significant roles in triggering efficient sporulation.

The defect in *iptA*$^-$ cells appears to be non-cell autonomous as developing them together with an equal number of wild-type cells resulted in improved sporulation (see Table S1 in the supplementary material). However, developing them together with an equal number of *acbA*$^-$ cells, which cannot make the SDF-2 precursor, resulted in only a modest increase in the number of spores. All of these strains except *acbA*$^-$ accumulated comparable levels of SDF-2 in their sori (see Table S1 in the supplementary material).

Induction of sporulation by cytokinins depends on DhkB and AcrA

When spores are collected from wild-type fruiting bodies and incubated at high density, they are inhibited from germinating by the presence of the germination inhibitor (Nomura et al., 1977). Disruption of the gene encoding the histidine kinase DhkB was found to result in spores that germinate in the presence of discadenine (Zinda and Singleton, 1998). We found that *dhkB*$^-$ K cells, developing at low density, fail to differentiate into spores in response to discadenine or other cytokinins but are still able to respond to SDF-1, SDF-2 and GABA just as well as wild-type KP cells (Fig. 4).

Histidine kinases relay phosphate to proteins carrying response regulator regions resulting in modulation of activity. The adenylyl cyclase of late development, AcrA, carries two response regulatory regions that might be targets for DhkB (Anjard et al., 2001). In support of this notion, we found that cells lacking AcrA failed to respond to discadenine, isopentenyl adenine or zeatin, although they responded normally to the other sporulation inducers (Fig. 4). If the adenylyl cyclase activity of AcrA is stimulated when its response

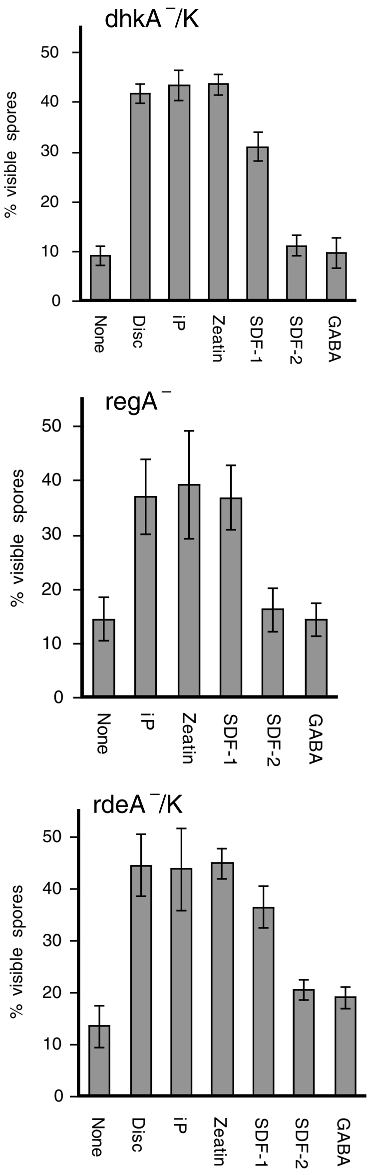

Fig. 6. Consequences of loss of components in the SDF-2 pathway. Cells of the *dhkA*⁻/K, *rdeA*⁻/K strains were developed as monolayers at 2×10^3 cells/cm^2 for 20 hours. *regA*⁻ cells were developed as monolayers at 5×10^2 cells/cm^2 for 20 hours. They were then treated with no addition (none), 100 nM discadenine (disc), 100 nM isopentenyl adenine (iP), 1 μM zeatin, 10 pM synthetic SDF-1, 10 pM synthetic SDF-2 or 1 μM GABA. Spores were counted after 1 hour. Cells treated with SDF-1 were scored after 90 minutes. Each experiment was repeated three times. Error bars correspond to one standard deviation.

regulatory regions are phosphorylated, the resulting increase in cAMP would lead to high levels of PKA activity necessary for rapid encapsulation.

We also determined whether the other two adenylyl cyclases, ACA and ACG, were essential for cytokinin induction of sporulation. As cells lacking ACA are unable to aggregate or proceed through development (Pitt et al., 1992), we introduced a construct that results in partially constitutive PKA activity into an *acaA*⁻ strain. The resulting *acaA*⁻/K strain is able to make fruiting bodies but does not have a sufficiently sporogenous phenotype that we could test cells of this strain developing at low cell density

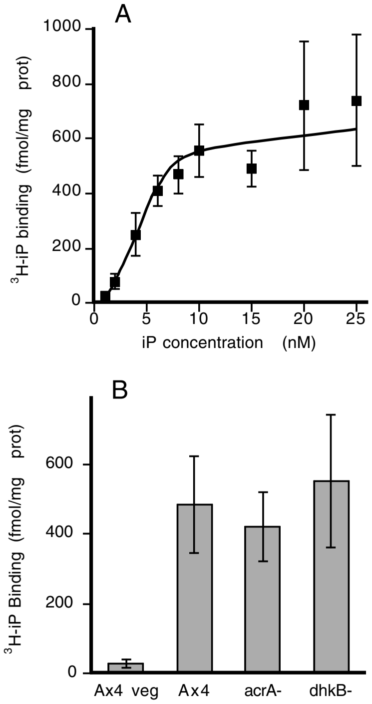

Fig. 7. Isopentenyl adenine binding to whole cells. (**A**) Cells of strain AX4 were developed on filters for 22 hours, dissociated, washed and suspended at 2.5×10^7/ml in binding buffer containing 1.25 mM adenine. ^{3}H-isopentenyl adenine was added to the indicated concentrations and the amount of specific binding determined after 5 minutes at 20°C. (**B**) Vegetative and 22 hour developed AX4, *acrA*⁻ and *dhkB*⁻ cells were suspended at 2.5×10^7/ml in binding buffer containing 1.25 mM adenine. ^{3}H-isopentenyl adenine (10 nM) was added and the amount of specific binding determined after 5 minutes at 20°C. All experiments were repeated at least three times.

for responses to cytokinins. Therefore, we dissociated cells from early culminants that had developed on filter supports and stimulated them with different spore inducing factors. Cells lacking ACA responded to discadenine, zeatin, SDF-2 and GABA just as well as cells dissociated from early culminants of wild-type AX4 cells (Fig. 5). Likewise, cells of the *acgA*⁻ strain dissociated from early culminants responded well to cytokinins (Fig. 5). It appears that DhkB-dependent induction of encapsulation by cytokinins acts exclusively through the specific adenylyl cyclase that carries response regulatory regions, AcrA, while SDF-2 induction of encapsulation is not dependent on any specific source of cAMP. The response to SDF-1, however, is dependent on ACG (Fig. 5).

Induction of sporulation by cytokinins is independent of DhkA, RdeA and RegA

SDF-2 induces rapid encapsulation by inhibiting the relay of phosphate from DhkA to the cAMP phosphodiesterase RegA via the H2 intermediate RdeA (Thomason et al., 1999; Anjard and Loomis, 2005). Cytokinins might be indirectly inducing sporulation by stimulating SDF-2 production, much as GABA induces sporulation by triggering the release of AcbA and its processing to SDF-2 (Anjard and Loomis, 2006). The resulting SDF-2-dependent inhibition of RegA, rather than activation of the GABA signal transduction pathway, is responsible for the rapid increase in PKA activity that leads to encapsulation. To determine whether cytokinins

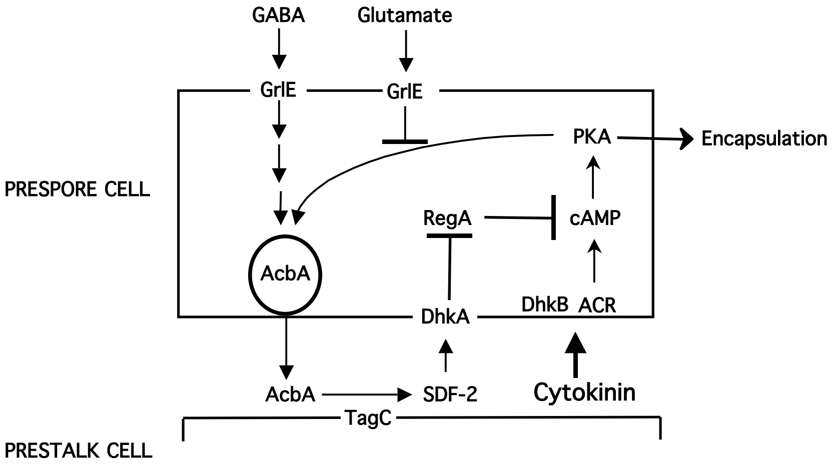

Fig. 8. Model for induction of sporulation by cytokinin and SDF-2. Cytokinins activate the histidine kinase DhkB and the adenylyl cyclase AcrA in prespore cells, while SDF-2 binds to the histidine kinase DhkA, such that the cAMP phosphodiesterase RegA is no longer activated. As a result, cAMP accumulates and activates PKA, which triggers encapsulation. PKA activity also leads to the release of the SDF-2 precursor AcbA which is processed by the prestalk specific protease TagC. Glutamate blocks the effect of PKA on AcbA. GABA competes with glutamate for the receptor GrlE. The kinases PI3K and PKBR 1 are essential components of the GABA signal transduction pathway leading to release of AcbA [adapted, with permission, from Anjard and Loomis (Anjard and Loomis, 2006)].

also induce rapid sporulation by the SDF-2 pathway, we used *dhkA*$^{-}$ KP cells in the low density monolayer assay. The mutant cells lack the SDF-2 receptor and so fail to encapsulate in response to either SDF-2 or GABA, although they do respond to SDF-1 (Anjard and Loomis, 2005; Anjard and Loomis, 2006). The responses to discadenine, isopentenyl adenine and zeatin were normal in *dhkA*$^{-}$ KP cells, indicating that the cytokinins induce rapid sporulation independently of SDF-2 signaling (Fig. 6).

To determine whether cytokinin activation of the histidine kinase DhkB results in phosphorelay through the unique H2 intermediate RdeA, we analyzed *rdeA*$^{-}$ cells that carry the construct resulting in partially constitutive PKA activity. Cells of the *rdeA*$^{-}$ KP strain developed in monolayers were found to respond to addition of cytokinins, although they failed to respond to SDF-2 or GABA, which depend on phosphorelay through RdeA (Fig. 6). Likewise, we found that *regA*$^{-}$ cells responded well to the cytokinins but failed to respond to SDF-2 or GABA (Fig. 6). Strains impaired in the GABA pathway (*gadA*$^{-}$, *grlE*$^{-}$, PKBR1^{-} and PI3K 1^{-}, 2^{-}) were found to respond well to cytokinins (see Table S1 in the supplementary material). It appears that the cytokinin signal transduction pathway is independent of the GABA and SDF-2 signal transduction pathways.

Neither DhkB nor AcrA is responsible for binding isopentenyl adenine

Either DhkB or AcrA could be a cytokinin receptor as they are large proteins with several potential transmembrane domains. AcrA has two predicted extracellular loops of about 35 amino acids each flanked by transmembrane domains near the N terminus that might bind cytokinins. However, DhkB has no predicted extracellular loops of more than 10 amino acids separating the transmembrane domains (Zinda and Singleton, 1998). We determined the ability of whole cells to bind ^{3}H-isopentenyl adenine in the presence of 1 mM adenine to reduce non-specific background binding. Cells collected from wild-type culminants were incubated at 20°C for 5 minutes with various concentrations of ^{3}H-isopentenyl adenine in the absence or presence of 10,000 fold excess unlabelled isopentenyl adenine. The cells bound ^{3}H-isopentenyl adenine with an apparent K_d of 6 nM (Fig. 7A). The concentration dependence for binding was very similar to the concentration dependence in the bioassay (Fig. 1). We then tested vegetative and developed wild-type cells, as well as developed *acrA*$^{-}$ and *dhkB*$^{-}$ cells at 10 nM ^{3}H-isopentenyl adenine (Fig. 7B). Vegetative cells bound very little of the cytokinin, indicating that the receptor is developmentally controlled. However, the *acrA*$^{-}$ and *dhkB*$^{-}$ mutant cells bound as much ^{3}H-isopentenyl adenine as wild-type cells, indicating that neither DhkB nor AcrA is likely to be the isopentenyl adenine receptor.

DISCUSSION

Spore differentiation is finely tuned to occur at the right time and place through a series of negative and positive signals. Two different signaling pathways involving the peptide SDF-2 and cytokinins converge to induce spore formation through the activation of PKA. Cytokinins are hormones that affect many different processes in plants but are not known to function in any animal. In plants, histidine kinases bind and mediate the responses to cytokinins. Although histidine kinases are prevalent in bacterial and plant signal transduction pathways, they are not found in metazoa (Anjard and Loomis, 2002). Nevertheless, *Dictyostelium* uses a histidine kinase, DhkB, in the cytokinin signal transduction pathway leading to encapsulation of spores. The use of cytokinins as hormones apparently predates the divergence between *Dictyostelium* and plants over a billion years ago.

The cyanobacteria *Anaebena species* genome includes two isopentenyl transferases, one being similar to IptA. Moreover, *Anaebena* encodes an adenlylyl cyclase, *cyaC*, with the same unusual domain organization as AcrA, starting with a receiver domain followed by a histidine kinase domain and another receiver domain to finish with the adenylate cyclase domain (Katayama and Ohmori, 1997). This suggests that components of the cytokinin pathway might have appeared in bacteria that predated eukaryotes but were lost in animal and fungal lineages.

As the cytokinin response in *Dictyostelium* is mediated by the histidine kinase DhkB and the adenylyl cyclase AcrA to the exclusion of other histidine kinases or adenylyl cyclases, these proteins may have a close association. Moreover, considering that the only known H2 component, RdeA, is not required for the cytokinin response, DhkB may directly relay phosphate to the aspartates in the response regulator regions of AcrA. Histidine kinases are known to form dimers in which the phosphate on the active histidine is transferred to an aspartate in the receiver domain (Posas et al., 1996; Wang et al., 1999). DhkB and AcrA may form a heterodimer in which DhkB directly activates adenylyl cyclase by phosphorelay. AcrA has a pseudo-histidine kinase domain in which the replacement of the active histidine by aspartate precludes it from autophosphorylation (Soderbom et al., 1999). However, this domain may have been retained on the basis of its ability to form a heterodimer with DhkB.

The cytokinin receptors are known in plants to be histidine kinases that bind cytokinins through an extracellular CHASE domain (Anantharaman and Aravind, 2001). This domain is found in only two *Dictyostelium* proteins, DhkA and ACG, but neither appears to be a cytokinin receptor as null mutants lacking these proteins respond normally to cytokinins (Figs 5, 6). Because neither DhkB nor AcrA appear to account for isopentenyl adenine binding, further studies will be required to recognize the *Dictyostelium* cytokinin receptor.

The cyctokinin response could be an artifact of the bioassay as we used cells that overexpress PKA developed as monolayers or cells dissociated from culminants to assess rapid sporulation. However, *iptA*$^{-}$ cells, in which synthesis of isopentenyl adenine is compromised in an otherwise wild-type background, are impaired in sporulation, indicating the importance of cytokinin signaling during normal development. Moreover, DhkB has been shown to play a direct role in determining the intracellular levels of cAMP during culmination as cAMP levels have been found to be significantly reduced in *dhkB*$^{-}$ spores (Zinda and Singleton, 1998).

Cytokinins can be produced either through a dedicated pathway or from the degradation products of tRNA (Kakimoto, 2003). Primitive plants like the moss *Physcomitrella patens* seem to use only the tRNA-IPT pathway for cytokinin production (Yevdakova and von Schwartzenberg, 2007). In *Dictyostelium*, the inactivation of *iptA* results into a 5- to 10-fold reduction in isopentenyl adenine during culmination. The remaining isopentenyl adenine is probably generated from degraded tRNA. Discadenine levels are even lower in *iptA*$^{-}$ cells (Fig. 3). However, when *iptA*$^{-}$ cells are allowed to form fruiting bodies within clearings of a bacterial lawn, they can accumulate 25% as much discadenine as wild-type spores (data not shown). As these cells had been actively ingesting bacteria under these conditions, discadenine may have been derived from the degradation products of bacterial tRNA.

SDF-2 stimulates spore formation at about the same time as cytokinins (Anjard et al., 1998b). However, SDF-2 acts by removing the activating phosphates from RegA in the pathway involving RdeA and DhkA (Fig. 8). As the RegA cAMP phosphodiesterase activity drops, cAMP can accumulate and stimulate PKA. At the same time cytokinins activate an independent pathway such that cAMP is rapidly synthesized by AcrA. This dual control is similar to pushing the accelerator and releasing the brake on cAMP accumulation (Fig. 8).

As both SDF-2 and cytokinins are released at about the same time of development (*t*=22 hours), it is possible that their production is coordinated. However, cytokinins do not seem to play an essential role in SDF-2 production as *iptA*$^{-}$ cells accumulate SDF-2 normally (see Table S1 in the supplementary material). Moreover, addition of cytokinins to developed KP cells does not result in the accumulation of SDF-2. Likewise, SDF-2 is not essential for cytokinin release as *acbA*$^{-}$ cells produce as much cytokinin as wild-type cells (data not shown). Cytokinin production is dependent on the activity of isopentenyl-transferase, which appears only during culmination (Ihara et al., 1980). Thus, the timing of cytokinin production may be mediated by the regulation of *iptA*. The timing of SDF-2 production may be set by the time of accumulation of GABA, which triggers release of the SDF-2 precursor AcbA (Anjard and Loomis, 2006). The enzyme that synthesizes GABA, GadA, accumulates only in prespore cells late in development and its regulation may determine the time of appearance of SDF-2. No matter how accumulation of SDF-2 and cytokinin are coordinated, both pathways need to be activated at about the same time to obtain maximal efficiency of sporulation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/5/819/DC1

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