

The *trishanku* gene and terminal morphogenesis in *Dictyostelium discoideum*

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SUMMARY Multicellular development in the social amoeba *Dictyostelium discoideum* is triggered by starvation. It involves a series of morphogenetic movements, among them being the rising of the spore mass to the tip of the stalk. The process requires precise coordination between two distinct cell types—presumptive (pre-) spore cells and presumptive (pre-) stalk cells. *Trishanku* (*triA*) is a gene expressed in prespore cells that is required for normal morphogenesis. The *triA*[−] mutant shows pleiotropic effects that include an inability of the spore mass to go all the way to the top. We have examined the cellular behavior required for the normal ascent of the spore mass. Grafting and mixing experiments carried out with tissue fragments and cells show that the upper cup, a tissue that derives from prestalk cells and anterior-like cells (ALCs), does

not develop properly in a *triA*[−] background. A mutant upper cup is unable to lift the spore mass to the top of the fruiting body, likely due to defective intercellular adhesion. If wild-type upper cup function is provided by prestalk and ALCs, *trishanku* spores ascend all the way. Conversely, Ax2 spores fail to do so in chimeras in which the upper cup is largely made up of mutant cells. Besides proving that under these conditions the wild-type phenotype of the upper cup is necessary and sufficient for terminal morphogenesis in *D. discoideum*, this study provides novel insights into developmental and evolutionary aspects of morphogenesis in general. Genes that are active exclusively in one cell type can elicit behavior in a second cell type that enhances the reproductive fitness of the first cell type, thereby showing that morphogenesis is a cooperative process.

INTRODUCTION

The social amoeba *Dictyostelium discoideum* is an excellent system for studying morphogenesis: unlike in most metazoans (Gilbert 2000), growth and development are separated during its life cycle (Bonner 1967; Kessin 2001). In *D. discoideum* starvation puts an end to growth and triggers a multicellular phase in which anywhere from 10²–10⁶ amoebae aggregate by chemotaxis (see <http://www.dictybase.org/Bonner%20paper.pdf> and Bonner 2009). The aggregate forms a mound that changes shape to become a cylindrical structure with a characteristic tip at the front called the slug. After a period of migration the slug “culminates” and forms a terminally differentiated fruiting body. The fruiting body contains three cell types. An erect columnar stalk and a basal disc that anchors the stalk are made up of dead cells. Live spore cells are organized as a lemon-shaped mass, the sorus, that sits on top of the stalk. The object of our study is the final and most characteristic aspect of morphogenesis, namely the ascent of the future spore mass.

Approximately the anterior 20% of the slug consists of presumptive stalk (prestalk) cells and the posterior 80%, of

presumptive spore (prespore) cells (Raper 1940; MacWilliams and Bonner 1979). The two can be distinguished in many ways, for example by using the vital dyes neutral red and Nile blue, both of which specifically stain anterior prestalk cells and cells in the slug’s posterior that contribute to the basal disc (Bonner 1952). There are cells scattered within the prespore zone, known as anterior-like cells (ALCs), that are also stained by neutral red and Nile blue (Sternfeld and David 1981; Sternfeld 1992). ALCs share many cytological and biochemical properties with prestalk cells even though they are located in the slug’s posterior and do not form part of the stalk proper (Devine and Loomis 1985). As culmination gets under way, some ALCs sort out to form two cellular sheets known as the upper and lower cups that cradle the ascending prespore mass above and below, respectively; other ALCs contribute to the outer part of the basal disc (Sternfeld and David 1982).

The *PsA* promoter is active in (posterior) prespore cells; different promoters are active in distinct sub-classes of prestalk cells and ALCs (sketched in Fig. 1; for details see Jermyn et al. 1987; Jermyn and Williams 1991; Early et al.

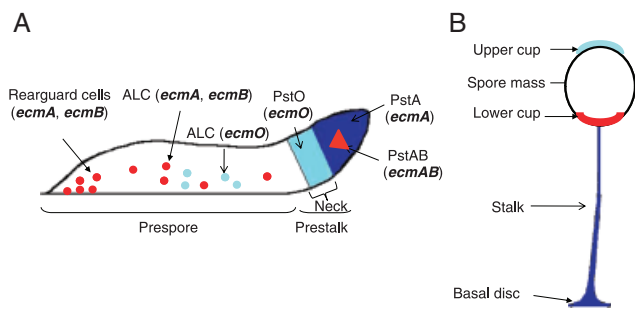


Fig. 1. Location of cell types in the slug (A) and fruiting body (B) as identified by staining and reporter expression patterns (based on Raper 1940, Abe et al. 1994, and Coates and Harwood 2001).

1993). The *ecmAO* promoter contains elements that are necessary for gene expression in different subpopulations of cells in the prestalk region of the slug. One part of the promoter (“*ecmA* promoter”) is active in the anterior 10% of the slug, the *pstA* region. It shows reduced expression in the rear half of the prestalk zone, known as the *pstO* region (which we will refer to as the neck of the slug). Another part of the *ecmAO* promoter (“*ecmO* promoter”) is active in the neck. The *ecmB* gene is expressed in a fraction of prestalk cells in the slug tip (which are therefore known as *pstAB* cells) and in some ALCs (Ceccarelli et al. 1991; Gaskell et al. 1992; Jermyn et al. 1996).

As the slug moves, prestalk and prespore cells remain restricted to its anterior and posterior, respectively. If transplanted to an ectopic location, or if they are disaggregated and allowed to reaggregate, they return to their original place (Raper 1940; Bonner 1952; Bühl et al. 1993). Once the slug stops migrating, ALCs scattered in the prespore region sort out into two groups (Sternfeld and David 1982; Dormann et al. 1996). Those that express the *ecmB* gene contribute to the lower cup and basal disc (Jermyn et al. 1991; Sternfeld 1992). *PstO*/ALCs from the posterior, together with *pstO* cells from the anterior, contribute to the upper cup (Jermyn and Williams 1991; Early et al. 1993; Dormann et al. 1996). The two cups cradle the spore mass and their cells remain amoeboid till the end of culmination, after which they die (Sternfeld and David 1982). The lower cup derives entirely from posterior-situated ALCs, whereas the upper cup is constructed by two subsets of cells: another set of posterior ALCs and anterior *pstO* cells (Early et al. 1993).

It has been proposed that the upward movement of the prespore mass could be similar to slug movement, but in a vertical direction (Bonner 1949; Matsukuma and Durston 1979; Traynor et al. 1992). But this cannot be the sole mechanism, at least not in *D. discoideum*, because prespore cells lose their amoeboid appearance and motility as they differentiate into spores (Bonner 1944; Raper 1984). Some observations point to an active role of prespore cells in moving up the stalk. Springer et al. (1994) and Chen et al. (1995) found that a lack of myosin II heavy chain or myosin essential light

chain function in prespore cells prevented the sorus from rising. Chen et al. (1998) further showed that when the myosin regulatory light chain (*mlcR*) was absent in prespore cells, some spores were found near the base of the stalk. Elegant experiments by Sternfeld (1998) make it likely that the upper cup plays an important role in elevating the sorus. Surgical removal of the upper cup shortly after the sorus had lifted off the substratum caused an arrest of further movement, but the stalk continued to rise upward (a similar removal of the lower cup was not reported). When the prespore mass was sucked out and substituted by oil, the oil drop was carried up too. Given these differences, it remains to be resolved whether some or all of prespore cells, upper cup cells, and lower cup cells are necessary for the ascent of the sorus. The *trishanku* (*triA*) gene is expressed in prespore cells and its absence has pleiotropic consequences including a destabilization of the differentiated state, aberrant cell-type proportioning and morphogenesis (Jaiswal et al. 2006). In particular, the sorus is subterminal (which is how the gene acquired its name). This made us look into what might prevent the full ascent of the sorus in the *triA*[−] mutant.

MATERIALS AND METHODS

Chemical reagents and growth media

Proteose peptone, yeast extract, and bacto-agar were from Difco Laboratories (Detroit, MI, USA), other standard media components from Ranbaxy (Ranbaxy, Haryana, India), and Himedia (Maharashtra, India); dyes (neutral red and Nile blue sulfate) were from Sigma Chemical Company (St. Louis, MO, USA) and Himedia. Antibiotics were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK) or Sigma Chemical Company.

Cell growth, staining, and development

Wild type (A×2) cells were grown in HL5 medium (Watts and Ashworth 1970) supplemented with penicillin–streptomycin (10 mg/ml of HL5). *triA*[−] cells were grown in HL5 supplemented with 5–10 μg/ml blasticidin. Neomycin-resistant strains were grown in HL5 supplemented with 20 μg/ml G418. Cells were grown at 22°C, spun at 800 g for 90 sec to remove the nutrients, washed twice with ice-cold KK2 buffer (16 mM KH₂PO₄/K₂HPO₄, pH 6.2) and developed on 1.5% bacto-agar in distilled water or in KK2 buffer. The plates were incubated in a dark, moist chamber at 22°C. Slugs obtained after 16–18 h were used for the grafting experiments.

For staining cells, neutral red (0.1% stock solution) or Nile blue sulfate (0.05% stock solution) was used. Stock solutions for both were made in sterile water and diluted 20–50 times in a thick cell suspension (approximately 10⁸ cells), incubated at room temperature for 1–2 min for neutral red and 5–6 min for Nile blue sulfate with constant shaking and then diluted to 15 ml with ice cold KK2 buffer. The cell pellet was washed once and spotted on water agar.

Microscopy

Fruiting body morphology was observed using an Olympus SZX12 microscope (Japan); a blue filter was used to observe GFP fluorescence and the pictures were recorded using an Olympus Camera C-5060 camera.

Slug grafting

Grafting experiments, in which an anterior portion of one slug (the donor) was grafted on to a comparatively larger posterior portion of another slug (the recipient), were carried out with slugs formed after 15–18 h of incubation. Donor and recipient were chosen to be of the same size and had migrated for roughly the same amount of time. The two slugs were gently lifted with a hair loop and placed close to each other on a water agar plate. Both were transversely cut at the same time using a single hair loop. The regions that were to be grafted were brought close using the hair till they touched each other, and the plate was incubated in the dark at 22°C for a minimum of 15–30 min. The plate was then observed to see if the graft was successful. Lack of success was inferred whenever the anterior fragment had moved away. “Successful” plates were transferred to a moist, dark chamber to encourage the slugs to migrate. The plate was exposed to light if fruiting had to be induced. The grafts made were (followed by a summary):

- (i) *PsA*-GFP grafts to study fruiting body morphology. The non-fluorescent anterior of a *PsA*-GFP slug was removed. GFP fluorescence in the remaining portion was observed using a blue filter; if a part of the nonfluorescent anterior was remaining, a further cut was made to remove it. At the same time the anterior one-fifth was cut from an unlabeled slug and the posterior portion was discarded. The GFP-positive posterior region of the first slug was pushed until it was continuous with this unlabeled anterior (as explained earlier) and the plate was incubated in the dark. Grafts involved an Ax2 unlabeled anterior region and Ax2 *PsA*-GFP posterior; and a *triA*⁻ unlabeled anterior with *triA*⁻ *PsA*-GFP posterior. In the latter case, as the *triA*⁻ *PsA*-GFP anterior region also shows some GFP-positive cells, the cuts were made in such a way that slightly more than the anterior one-fifth of the slug was removed, and the posterior region, which is more brightly fluorescent was used for the grafting. Cross-grafts were made as described above, except that an Ax2 anterior to a *triA*⁻ *PsA*-GFP posterior and vice versa. If the graft was successful, the slugs were exposed to light after 2–3 h and the fruiting body was observed for morphology and fluorescence.
- (ii) *Actin15*-GFP graft to study the rate of transdifferentiation. Using the method of Akiyama and Inouye (1987), the anterior one-fifth of an entirely fluorescent *Actin15*-GFP slug was grafted. The graft was incubated in a dark moist chamber for 2–4 days and there was no attempt made to induce fruiting. The distance between the site of the graft and the fruiting body that eventually formed was measured. Sori were removed with a needle, squashed on a glass slide, and the number of fluorescent and nonfluorescent spores counted. At least 1000 spores were counted.
- (iii) Neutral red and Nile blue grafts to look at the status of the upper cup. Slugs formed from neutral red or Nile blue stained cells were allowed to migrate for 3–4 h before using them

for grafting experiments because the red/blue color darkens on prolonged migration. A cut was made just behind the red anterior portion (approximately one-fifth of the slug); the posterior region was discarded. This was grafted to the posterior four-fifth of a colorless slug and incubated in the dark. After 2–3 h, grafted slugs were exposed to light and the fruiting bodies were observed. Both “self”- and “cross”-grafts (Ax2 colorless anterior to a *triA*⁻ neutral red/Nile blue posterior and vice versa) were carried out. In some cross-grafts a larger than usual portion of the posterior was combined with a correspondingly smaller anterior region. This was done by cutting a neutral red/Nile blue slug near the tip of the slug, much before the end of the red/blue prestalk region (approximately at about one-tenth of the slug length), and the large posterior fragment was combined with an anterior fragment of the other color (either one-fifth or one-tenth of the slug).

	Size of the anterior fragment (cell types included)	Size of the posterior fragment (cell types included)	Type of graft
1	Anterior one-fifth of the slug length of one genotype (PstA cells and PstO cells)	Posterior four-fifth of the slug length of the same genotype (prepsore cells and ALCs)	Self-graft
2	Anterior one-fifth of the slug length of one genotype (PstA cells and PstO cells)	Posterior four-fifth of the slug length of another genotype (prepsore cells and ALCs)	Cross-graft
3	Anterior one-tenth of the slug length of one genotype (PstA cells)	Posterior nine-tenth of the slug length of another genotype (PstO cells, prepsore cells and ALCs)	Cross-graft

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to check the expression of *lagC*

RNA was extracted from approximately 10⁸ cells using TRI reagent (Sigma Chemical Company) following the manufacturer’s instructions. The quality and quantity of RNA samples were assessed spectrophotometrically as well as on a 1.2% MOPS-formaldehyde agarose gel. Approximately 3 µg of total RNA from different developmental stages was used for the synthesis of the first strand of cDNA using the first-strand cDNA synthesis kit. Care was taken that exactly same amount of RNA was used for cDNA synthesis from different developmental stages. For synthesis of the second strand, cDNA corresponding to 50–75 ng RNA equivalents was used and amplified using primers specific to *lagC*. PCR amplification was carried out for different cycle numbers (15, 18, 21, 24, 27, and 30) from cDNA of a particular development stage. The cycle number, which showed an amplicon in the exponential phase was chosen and used to compare the level of expression of that transcript across different developmental stages. The same procedure was followed for a constitutively expressed gene, *IG7* (Hopper et al. 1993).

Semiquantitative RT-PCR for each transcript was done three times. To look at the expression pattern of a particular transcript across different developmental stages, a densitometric analysis was carried out. A graph of an average of the ratio of expression of the gene-specific transcript to *IG7* expression at each time point was plotted. Negative controls ("minus RT") were set up to check for the presence of genomic DNA contamination in the RNA samples.

RESULTS

All manipulations were carried out with *D. discoideum* cells and slugs belonging to the trishanku (*triA*⁻) mutant or its parent, the wild type-derived axenic strain Ax2. In most experiments the anterior fragment of one slug was grafted to a (much larger) posterior fragment of another age- and size-matched slug (see "Materials and methods" for details and Fig. 1 for a visual description of the fragments). Usually the anterior fragment comprised slightly more than one-fifth of the slug by length, meaning that it included all anterior prestalk tissue including the pstO cells in the neck. When prestalk-specific vital dyes were used, this was ensured by paying heed to the extent of staining in the slug's anterior. Following grafting slugs were incubated in the dark for 15 min. If the graft had taken hold, the time was taken as our 0 h time point and the slug was photographed. Plates were incubated in the dark in a humid environment at 23°C thereafter and observations carried out as required. Postgrafting, slugs elongated as they migrated, more so in the case of Ax2 slugs. The results of the grafting experiments are summarized in Table 1.

Following grafting, cells cross the prestalk/prespore boundary in *triA*⁻ slugs but not in wild-type (Ax2) slugs

Movement of posterior cells

After an unlabeled anterior wild-type (Ax2) fragment was grafted to the posterior portion of a *PsA*-GFP slug of the same genotype, no GFP-positive cells were visible in the anterior. A similar procedure on *triA*⁻ slugs resulted in clusters of GFP-positive cells moving from the posterior to the anterior as the slug migrated (23/23 cases in each case). These conclusions were reinforced in the course of monitoring cell fates (text and figure, next section).

Movement of anterior cells

When a neutral red-stained anterior Ax2 fragment was grafted to an unstained Ax2 posterior, there was no cell movement initially from anterior to posterior until about 4 h, at which time the red region had expanded slightly and a few red cells were visible behind the graft surface but close to it. After 6 h of migration the posterior region had some more red cells, still concentrated near the graft border; also the intensity of the red color in the anterior had decreased (6/6 cases; Fig. 2, above). When a similar procedure was carried out with *triA*⁻ slugs, a streak of red cells were seen in the posterior as early as 2 h after grafting. By 4 h there were many more red cells in the posterior, extending almost to the back of the slug; their number increased further by 6 h. Again there

Table 1. Summary of grafting experiments

Graft	Anterior fragment	Posterior fragment	Location of spore mass	Upper cup status	Lower cup status	Stain status and phenotype of stalk	Successful cases/total (fig.)
1	Ax2	Ax2 (*)	Terminal	Not observed	Not observed	Not fluorescent, thin	15/15 (8a)
2	<i>triA</i> ⁻	<i>triA</i> ⁻ (*)	Subterminal	Not observed	Not observed	Fluorescent, thickened	15/15 (8b)
3	Ax2	<i>triA</i> ⁻ (*)	Subterminal	Not observed	Not observed	Fluorescent, thickened	21/24 (8c)
4	<i>triA</i> ⁻	Ax2 (*)	Subterminal	Not observed	Not observed	Not fluorescent, thin	20/25 (8d)
5	Ax2	Ax2 (C)	Terminal	Cradling the spore mass	Cradling the spore mass	Colorless, thin	15/15 (10a)
6	<i>triA</i> ⁻	<i>triA</i> ⁻ (C)	Subterminal	Detached from the spore mass	Cradling the spore mass	Colored, thickened	18/18 (10b)
7	Ax2	<i>triA</i> ⁻ (C)	Subterminal	Detached from the spore mass	Cradling the spore mass	Colored, thickened	20/26 (11a)
8	<i>triA</i> ⁻	Ax2 (C)	Subterminal	Detached from the spore mass	Cradling the spore mass	Colored, thin	21/26 (11b)
9	<i>triA</i> ⁻ (NR)	Ax2 (NB)	Terminal	Cradling the spore mass	Cradling the spore mass	Colorless, thin	12/12 (12)
10	Ax2 (NB)	<i>triA</i> ⁻ (NR)	Subterminal	Detached from the spore mass	Cradling the spore mass	Thickened with colored cells	10/10 (13)

In grafts number 1–8, anterior slug fragments were not labeled; posterior fragments were either labeled with *PsA*-GFP (*; grafts number 1–4) or stained with neutral red or Nile blue (C for either color; grafts number 5–8). The anterior fragment in graft number 9 was stained with neutral red (NR) and the posterior fragment was stained with Nile blue (NB). In graft number 10, the anterior fragment was stained with Nile blue (NB) and the posterior fragment was stained with neutral red (NR). In all cases the genotype of the spores corresponded to that of the posterior fragment (column 2). This table does not include the experiments in which cell movement was followed after grafting.

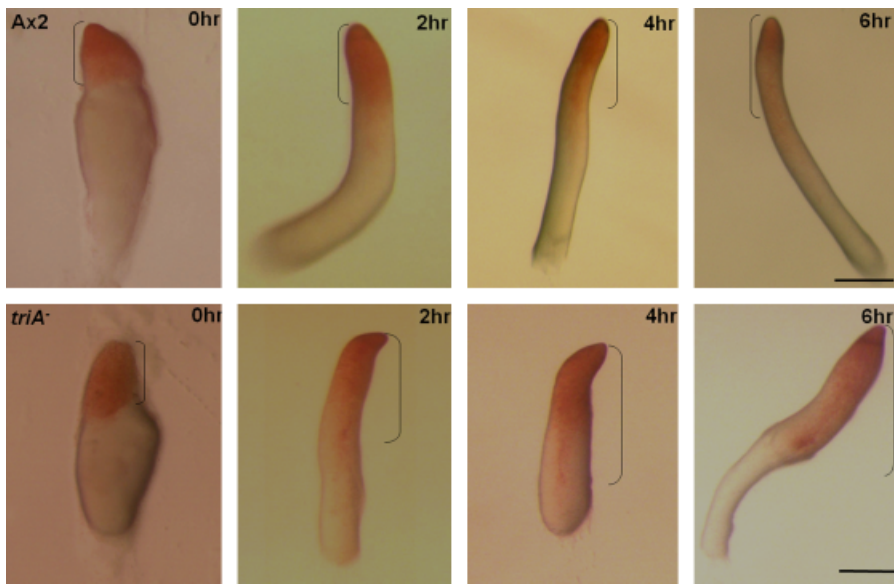


Fig. 2. Grafts of neutral red-stained anterior prestalk fragment onto a colorless prespore fragment. Ax2 on Ax2 (upper panel) and *triA*⁻ on *triA*⁻ (lower panel). The same slug was photographed at different time points for both the genotypes. Occasionally a weak streak of red is seen in the posterior region of the Ax2 graft, but not to a much lesser extent than in the *triA*⁻ graft. The bracket depicts the extent of movement of red cells into the psp region. Scale bar: 100 μ m.

was a decrease in the intensity of the red color in the anterior (6/6 cases, Fig. 2, below).

In short, in *triA*⁻ slugs posterior cells cross over to the anterior and vice versa. The anterior–posterior boundary is maintained in Ax2 slugs.

Cells that cross the boundary in *triA*⁻ slugs change their fate

Again these grafts involved unlabeled anterior fragments and fluorescent (*PsA*-GFP) posterior fragments, 20 in each set. The GFP reporter that was used had a half-life of >24 h (Fey et al. 1995); therefore posterior *PsA*-GFP cells that moved into the unlabeled anterior and changed their cell fate would show up as GFP-positive stalk cells. In the case of Ax2 on Ax2 grafts, none of the slugs showed GFP-positive cells in the anterior region even after 6 h (Fig. 3A, upper panel) and GFP fluorescence in the resulting fruiting bodies was restricted to

the spore mass (Fig. 3A, lower panel). In the case of *triA*⁻ on *triA*⁻ grafts, the anterior region of the slug was soon populated by GFP-positive cells (Fig. 3B, upper panel). Fruiting bodies contained GFP-positive cells both in the spore mass and in the stalk and basal disc (Fig. 3B, lower panel). It was verified by observation that the GFP-positive cells in the stalk were indeed stalk cells, not undifferentiated amoebae. Thus *triA*⁻ GFP-positive cells that moved from the posterior to the anterior of *triA*⁻ slugs changed their fates in accordance with their altered positions.

Because the quality of fluorescence in an anterior cell type-specific transformant (*ecmA0*-GFP) was poor and variable, the corresponding experiment with anterior fragments was carried out by grafting *Actin15*-GFP anteriors to unstained posterior fragments and observing fruiting bodies after 2 days as described in details in “Materials and methods.” Four out of 15 Ax2 grafts did not migrate and culminated in the same place where the grafts were made. The remaining 11 grafts

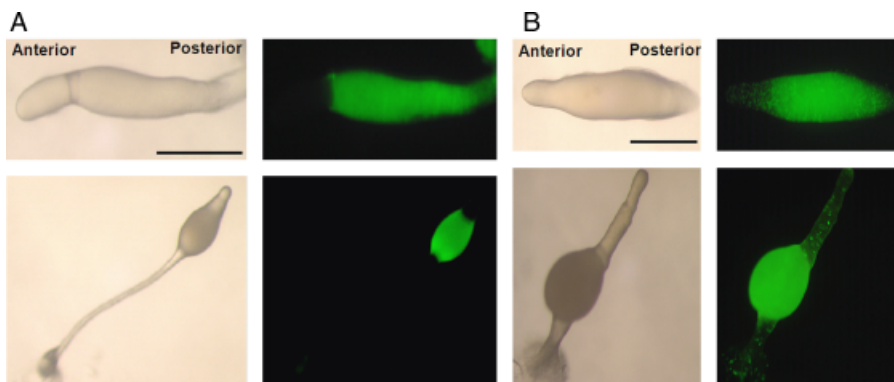


Fig. 3. Grafts of colorless anterior slug fragment to a *PsA*-GFP posterior fragment (paired bright-field and fluorescent images). (A) Ax2 on Ax2; (B) *triA*⁻ on *triA*⁻. Upper panel, grafted slug after 6 h of migration; lower panel, fruiting body.

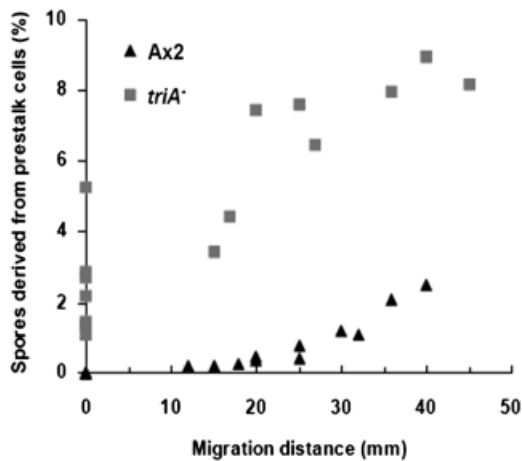


Fig. 4. Extent of transdifferentiation between Ax2 and *triA*⁻ slugs (abscissa, distance of migration after grafting; ordinate, percentage of spores that showed GFP fluorescence). Each symbol represents one graft (and so one fruiting body). In all cases, an Actin15-GFP prestalk region was grafted to a colorless prespore region of the same genotype.

migrated from 12 to 40 mm. Slugs that had not migrated contained no GFP-positive spores. When slugs had migrated before fruiting, the proportion of fluorescent spores went up with the extent of migration (range, 0.2–2.5%; Fig. 4). Seven out of 15 slugs from *triA*⁻ grafts did not migrate. The other eight culminated after migrating 15–45 mm. GFP-positive spores were found in all 15 fruiting bodies. They ranged from 1.11% to 5.26% of all spores in those fruiting bodies that had formed without migration and, depending on the extent of migration, from 3.46% to 8.2% in the remaining eight.

Thus in *triA*⁻ on *triA*⁻ grafts there is significant cross-boundary cell movement and transdifferentiation; no migration is required. In Ax2 on Ax2 grafts the small amount of transdifferentiation seen in the last set of experiments may be due to some anterior to posterior (but not posterior to anterior) cell movement. Or, it may be an artifact due to the fact the *Actin15* promoter is expressed in all cells.

The ascent of prespore cells requires wild-type function in other cells

Next we asked, could the subterminal position of the *triA*⁻ spore mass be explained by a defect in *triA*⁻ prespore cells? To find out, an unlabeled Ax2 anterior slug fragment was grafted to a *triA*⁻ *PsA*-GFP posterior fragment or vice versa and the morphology of the fruiting body was observed. As expected, fruiting bodies in the control (Ax2 on Ax2 and *triA*⁻ on *triA*⁻) “self”-grafts had the expected terminal and subterminal spore masses, respectively (15 grafts each; Fig. 5, A and B).

When an Ax2 anterior was grafted to a *triA*⁻ *PsA*-GFP posterior, the sorus was subterminal and brightly fluorescent

(21/24 cases; Fig. 5C). The stalk and basal disc contained some GFP-positive cells too; the stalk was noticeably thicker than in the case of an Ax2 “self”-graft and the basal disc was prominent (in three experiments the graft did not take). To our surprise, when an unlabeled *triA*⁻ anterior fragment was grafted to an Ax2 *PsA*-GFP posterior, the spore mass was again subterminal and GFP positive (Fig. 5D, 20/25 cases; the remaining five were unsuccessful). The stalk was much thinner than in *triA*⁻ “self”-grafts. Neither it nor the (prominent) basal disc showed any fluorescence (compare Fig. 5, D with B).

This means that for prespore cells to rise to the top, it is not sufficient that they alone are wild type with regard to *triA*; wild-type function is also required in some or all of the remaining cells.

In *triA*⁻ the lower cup is normal but the upper cup is not

Evidence for what might be occurring came from observing the behavior of cells that are expected to constitute the upper and lower cups.

Ax2 fruiting bodies formed by neutral red- or Nile blue-stained cells had appropriately colored (red or blue) upper and lower cups above and below the sorus (Fig. 6, first and third panels; 30/30 cases). *triA*⁻ fruiting bodies showed a difference (Fig. 6, second and fourth panels; 30/30 cases). With neutral red or Nile blue staining an elongated streak of colored cells, varying in extent, was seen higher up the stalk. A normal-looking colored lower cup cradled the spore mass from below. In sum, all *triA*⁻ fruiting bodies had a normal-looking lower cup (i.e., as in the wild type) and an aberrant upper cup. The upper cup was absent (and stained cells were scattered along the stalk, often concentrated at the top) or, took the form of a dispersed thin band on top of the spore mass.

To find out whether the stained cells that were concentrated at the top of the stalk in *triA*⁻ fruiting bodies were really upper cup cells that were located inappropriately or were part of the stalk, an unstained anterior slug fragment was grafted to a stained posterior fragment. We expected that the part of the upper cup that derived from pstO/ALCs located in the posterior would be colored and therefore distinguishable from the stalk proper and tip which would be colorless. Ax2 “self”-grafts showed exactly that: during culmination there was a band of clearly visible colored tissue above the prespore cells of the culminating mass along with a colored lower cup and colorless tip (Fig. 7A, first and third panels). After the fruiting body had formed one could see colored upper and lower cups cradling the spore mass; the papilla and stalk were colorless and the basal disc was colored (Fig. 7A, second and fourth panels; 15/15 cases). In *triA*⁻ “self”-grafts, a colored upper cup was apposed to the spore mass at the early culminant stage; unlike the case of the Ax2 “self”-grafts, the papilla was colored too (Fig. 7B, first and

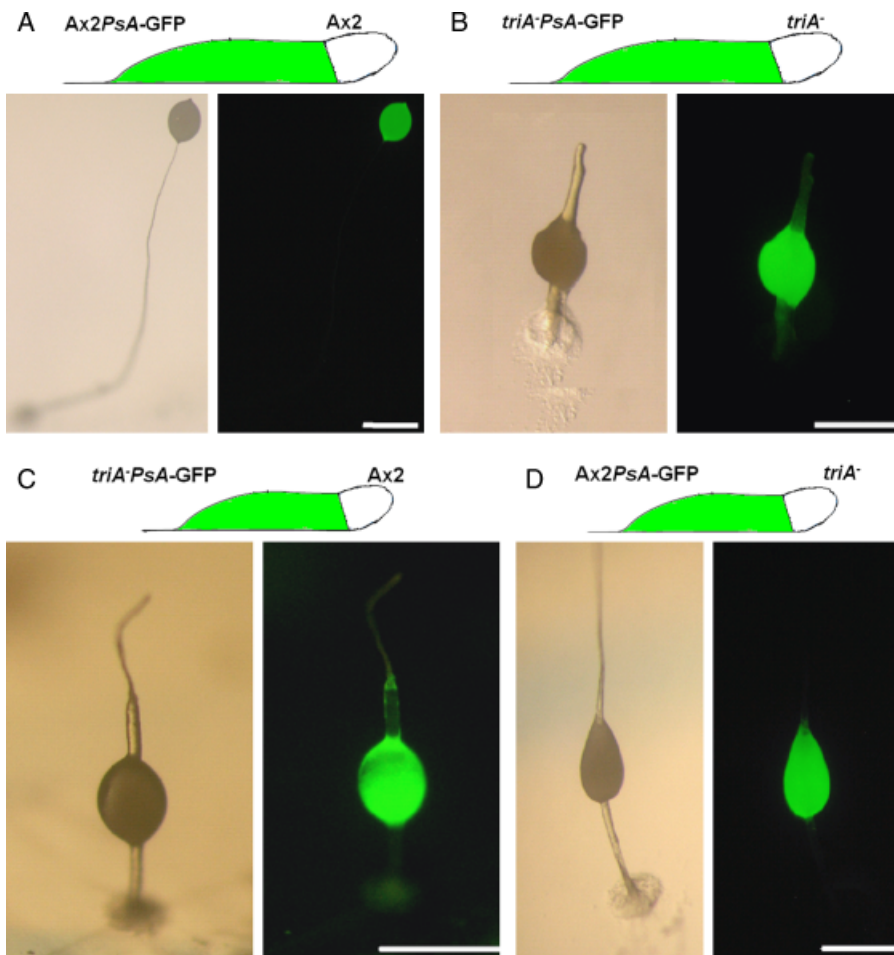


Fig. 5. Role of prespore cells in the formation of a terminal spore mass. All grafts involve an unlabeled anterior fragment and a *PsA*-GFP-labeled posterior fragment. (A), Ax2 on Ax2; (B), *triA*⁻ on *triA*⁻; (C), Ax2 on *triA*⁻; (D), *triA*⁻ on Ax2. Left hand picture, bright field image; right hand picture, fluorescent image of the same fruiting body. Scale bar: 100 μ m

third panels). In the fruiting body, a thin colored band was partially attached to the top of the sorus; besides, stained cells were seen all through the stalk above the spore mass and concentrated at its tip. A colored, normal-looking lower cup and an enlarged, colored basal disc were present (Fig. 7B, second and fourth panels; 18/18 cases).

These observations indicate that in a *triA*⁻ condition the spatial distribution of cells which would normally form the upper cup is deranged. Could it be because of this that the spore mass fails to rise in Ax2-*triA*⁻ grafts (of both sorts)? An answer required that the status of the upper cup in these grafts be examined.

A normal upper cup is missing also in Ax2-*triA*⁻ cross-grafts (of both types)

In order to determine the status of the upper cup in Ax2-*triA*⁻ cross-grafts, an unstained Ax2 (or *triA*⁻) anterior slug fragment was grafted to a neutral red or Nile blue-stained posterior fragment from a *triA*⁻ (or Ax2) slug. Twenty out of 26 grafts in which an Ax2 anterior was grafted to a *triA*⁻

posterior were successful. In early culminants one could see a colored upper cup apposed to the prespore mass; the tip of the stalk showed colored cells (Fig. 7C, first and third panels; Fig. 7B shows that this was also the case with *triA*⁻ “self”-grafts). In the fruiting body the upper cup was partially attached to the sorus but stained cells extended upwards beyond the spore mass all through the stalk (Fig. 7C, second and fourth panels). The neutral red-stained graft clearly shows a thin band of upper cup cells attached to the spore mass and the stalk above the spore mass consists of colored cells which are concentrated toward the tip. In the fruiting bodies from these unstained Ax2 anterior-stained *triA*⁻ posterior grafts, the stalk was thickened and there was a prominent basal disc with colored cells. The lower cup looked normal in all cases.

In the reverse situation, that is when a colorless *triA*⁻ anterior slug fragment was grafted to an Ax2 colored posterior, 21/26 grafts took hold. Later, early culminants displayed a band of colored upper cup cells attached to the top of the prespore mass and a colorless tip (Fig. 7D, first and third panels). By the time fruiting was complete, the cells that formed the upper cup had separated from the spore mass and occupied the top of an

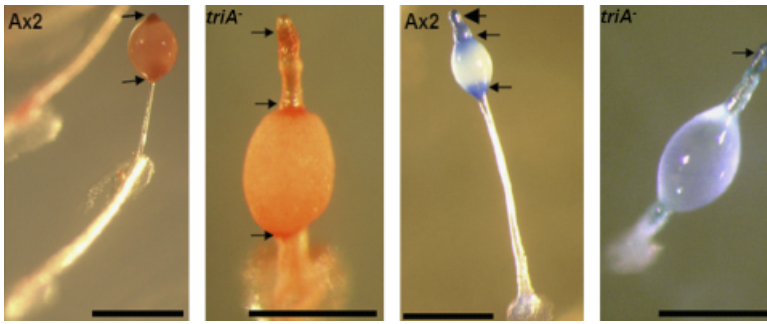


Fig. 6. Neutral red and Nile blue staining pattern in Ax2 and *triA*⁻ fruiting bodies. The neutral red stained (first and second panel) or Nile blue stained (third and fourth panel). In Ax2 the upper and lower cups are seen attached to the spore mass (shown by arrows); with Nile blue staining the papilla on top of the upper cup is clearly seen (arrowhead). In the case of *triA*⁻ (neutral red staining) some upper cup cells are attached to the sorus (shown by arrow) and others form a band below the tip of the stalk (arrow). In the *triA*⁻ Nile blue stained fruiting body, blue cells are seen concentrated at the tip of the stalk (shown by an arrow). The *triA*⁻ lower cups look normal. Scale bar: 100 μm.

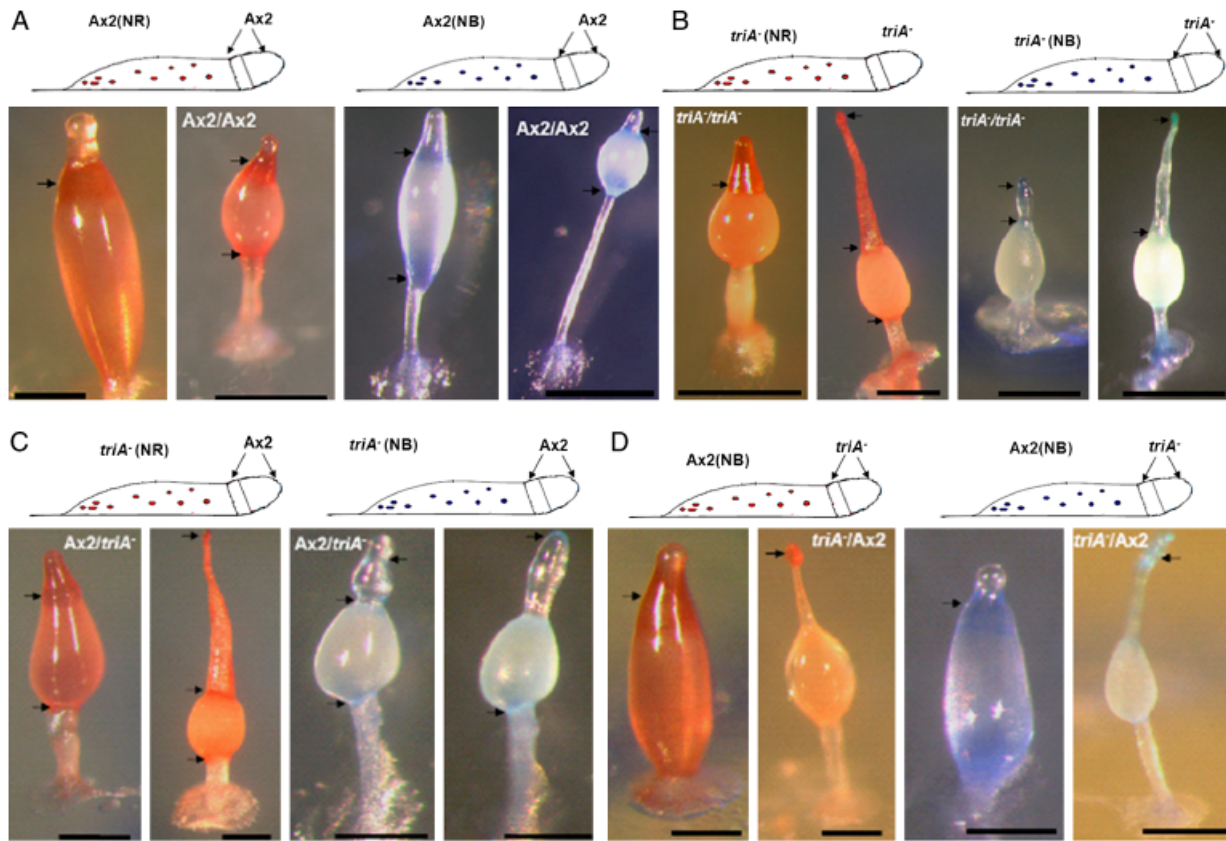


Fig. 7. Position of upper and lower cups in fruiting bodies resulting from self- or cross-grafts. In all cases the anterior fragment was unstained; cells in the posterior fragment had been stained with neutral red or Nile blue. (A), Ax2 on Ax2; (B), *triA*⁻ on *triA*⁻; (C), Ax2 on *triA*⁻; (D), *triA*⁻ on Ax2. The position of the upper and the lower cups in a mid-to-late culminant (first and third panels) and fruiting body (second and fourth panels) is shown by arrows. Scale bar: 100 μm in (A) and (B), 50 μm in (C) and (D).

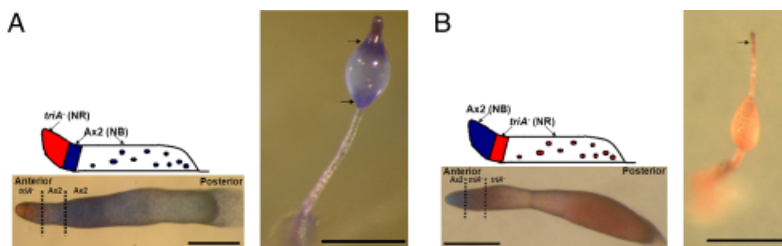


Fig. 8. Final location of sorus when the upper cup is made up of (A) wild-type or (B) *triA*⁻ cells. The conditions result from grafting a neutral red-stained *triA*⁻ anterior fragment to a Nile blue-stained Ax2 posterior fragment (A) or a Nile blue-stained Ax2 anterior fragment to a neutral red-stained *triA*⁻ posterior fragment. In both cases the posterior fragment includes both the prespore region and the slug neck. In (A) the spore mass is cradled on either side by blue upper and lower cups (as shown by the arrows). In (B) the upper cup cells are detached from the sorus and are present at the tip of the stalk. Scale bar: 100 μm.

otherwise colorless stalk. The lower cup appeared normal and a prominent basal disc was present (Fig. 7D, second and fourth panels). The stalk in these grafts was invariably thinner than the one seen in the *triA*⁻ “self”-graft (compare Fig. 7, D and B).

In short, a proper upper cup fails to form and spore mass remains subterminal in all successful Ax2-*triA*⁻ grafts. This strengthened the hypothesis that a defective upper cup was behind the inability of the *triA*⁻ sorus to fully ascend.

Wild-type upper cup function is necessary and sufficient for *triA*⁻ prespore cells to rise to the top of the stalk

In the wild type, the cells that make up the upper cup come partly from the slug’s anterior (*ecmO*-positive cells from the neck of the slug) and partly from its posterior (pstO/ALCs scattered through the prespore region; Jermyn and Williams 1991; Early et al. 1993). In all our cross-grafts the presumptive upper cup cells must have been genetically mosaic, that is, composed of both Ax2 and *triA*⁻ genotypes. Could an upper cup that was largely or wholly wild type in origin make the sorus climb all the way to the top, whatever its own genotype?

First, anterior–posterior grafts were carried out with the difference that this time the posterior fragment was significantly longer than the one used in earlier grafts. It now included the pstO “neck” region in addition to posterior prespore tissue, whereas the anterior fragment did not (Fig. 8A, left panel; see “Materials and methods” for details). When the anterior fragment of a neutral red-stained *triA*⁻ slug was grafted to a Nile blue-stained Ax2 posterior fragment, the fruiting body contained a terminal sorus in every case (12/12 grafts; Fig. 8A, right panel). A thick blue band of upper cup cells was apposed to the top of the spore mass and a somewhat thinner blue band of lower cup cells cradled it from below. The stalk and basal disc appeared similar to those seen in the earlier Ax2 “self”-grafts. In the reciprocal experiment (the anterior fragment of a Nile blue-stained Ax2 slug grafted to a neutral red-stained *triA*⁻ posterior that contained both pstO and prespore regions), in every case the position of the sorus was subterminal. Upper cup cells had detached from the spore mass and were at the tip of the stalk (10/10 grafts; Fig. 8B). The stalk was much thicker below the sorus than above and the basal disc was prominent.

Next, we followed morphogenesis in well-mixed Ax2-*triA*⁻ chimeras. We used the knowledge that when mixed with Ax2 cells in a 1:1 ratio, *triA*⁻ cells sort out to the posterior of the genetically mosaic slugs that resulted; in particular, they are absent from the neck of the slug (Jaiswal et al. 2006). A minority (20%) of freshly starved *triA*⁻ *Actin15*-GFP cells was mixed with a majority (80%) of unlabeled Ax2 cells. The cells co-aggregated and the GFP-tagged *triA*⁻ cells indeed sorted out to the slug posterior (Fig. 9A). This permitted wild-type cells from the anterior and the posterior to contribute to the upper cup. Gratifyingly, in the chimeric fruiting body the spore mass was right at the top; the stalk and the basal disc

were devoid of fluorescence (Fig. 9B). We confirmed that when Ax2 and *triA*⁻ cells were mixed in the same ratio as before (80:20) but only a minority of Ax2 cells had been labeled with GFP, GFP-tagged Ax2 cells were indeed at the neck of the slug, from where one would expect part of the upper cup cells to be derived (Fig. 9C). The labeled Ax2 cells contributed to forming normal-looking upper and lower cups (Fig. 9D). The sorting pattern shown by the GFP-tagged Ax2 cells in these chimeras is not due to a nonspecific effect of the GFP reporter: when GFP-labeled and -unlabeled Ax2 cells were mixed with each other, labeled cells were distributed uniformly in the slug and the late culminant (Fig. 9E and F).

The upper cup in *triA*⁻ is weakly attached to the ascending prespore mass

Why does the *triA*⁻ upper cup get detached from prespore cells? Defective cell–cell adhesion is a possibility. The adhesion system behind the attachment of the upper cup to prespore cells is currently unknown. However, it has been shown by *in situ* hybridization that the cell adhesion molecules *lagC* and *lagD* are specifically localized to the neck of the slug and the upper cup in the fruiting body (Kibler et al. 2003).

We used semiquantitative RT-PCR to examine the expression of *lagC* during development (Fig. 10). In Ax2 cells, the *lagC* transcript began to appear after aggregation, by 12 h; its level gradually increased thereafter and reached a plateau, where it remained till the end of development. In *triA*⁻ cells on the other hand, the *lagC* transcript was expressed by 9 h, that is, much earlier than in Ax2. The transcript level peaked at 12 h and then declined by 21 h. The decreased expression of *lagC* during late development could be the reason why *triA*⁻ upper cup cells do not adhere to the spore mass as well as Ax2 upper cup cells do and so fail to lift up the spore mass fully. This needs further investigation.

DISCUSSION

Transdifferentiation in *triA*⁻ slugs

Cells move from the prespore to the prestalk region and vice versa in *triA* but not in Ax2; concomitantly, there is a change in cell fate (Figs. 2–4). Does transdifferentiation precede or follow movement to the “wrong” zone? We are unable to reach a definite conclusion. Two likely consequences of the absence of *triA* function merit consideration. (i) The differentiated state becomes unstable; prespore cells transdifferentiate into prestalk and vice versa. Cells that find themselves in the wrong environment sort out to the zone that is appropriate to their new identity. (ii) The functional barrier that prevents intermixing between the prestalk and prespore zones becomes weakened. This permits prespore cells to move to the slug anterior and prestalk cells to the posterior. Cells that

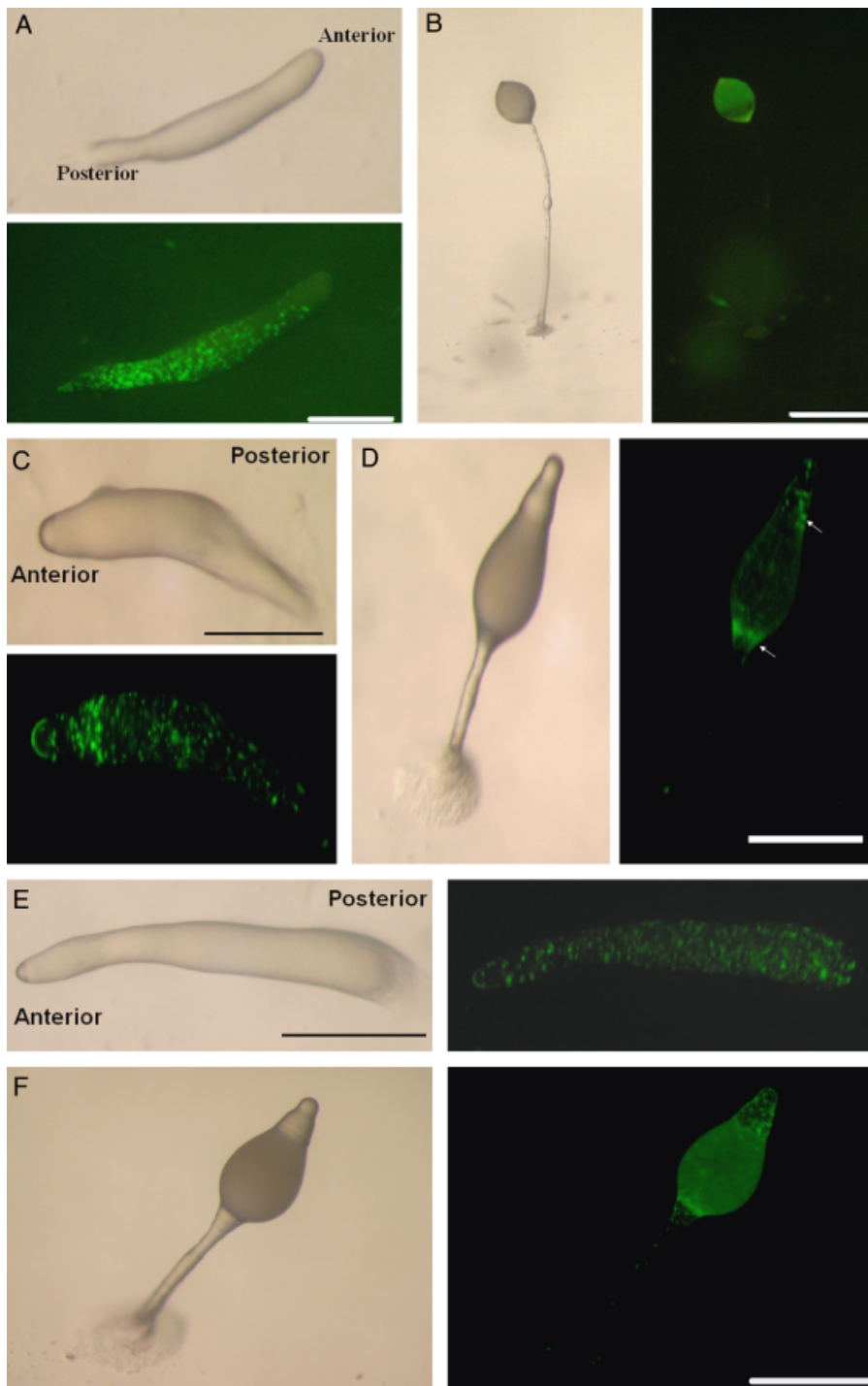


Fig. 9. Sorting out in chimeras. Chimeric slug (A) and fruiting body (B) formed by mixing 80% Ax2 cells (unlabeled) with 20% *triA*⁻ cells (labeled with *Actin15*-GFP). (C), slug and (D) early culminant from a similar mix except that a minority of Ax2 cells are labeled with *Actin15*-GFP and *triA*⁻ cells are unlabeled. (E) and (F) are controls in which Ax2 cells are mixed with Ax2 *Actin15*-GFP cells in a 80:20 ratio. The GFP tagged *triA*⁻ cells sort out to the posterior region of the chimeric slug; (A) and form spores which occupy a terminal position on the stalk (B); the stalk and basal disc are devoid of fluorescence. Note the presence of Ax2 cells in the neck of the slug in (C) and in the upper and lower cups in (D). Scale bar: 100 μ m.

have moved to the “wrong” zone switch their state of differentiation in accord with their new location.

In relation to possibility (i), a *triA* gene product could be part of a mechanism for stabilizing the states of prestalk and prespore differentiation once they have been attained. On the

other hand, if possibility (ii) is valid, the increased level of intermixing between the anterior and posterior regions in *triA*⁻ slugs could result from a relatively higher chemotactic ability of mutant cells toward cAMP (which is produced by the tip and less so by the rest of the slug; see Bonner 1949 and

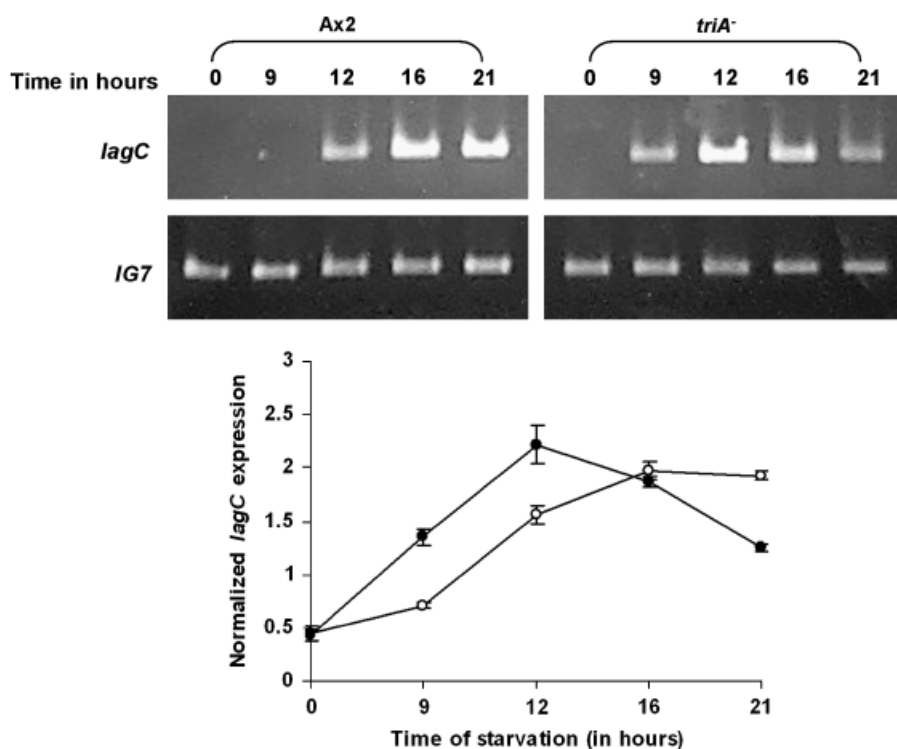


Fig. 10. The expression pattern of the gene encoding the EDTA-resistant cell adhesion molecule *lagC* during development. (A) A representative gel picture of semiquantitative reverse transcriptase-polymerase chain reaction shows that there is a precocious expression of *lagC* in *triA*⁻ cells. The expression of *lagC* is absent in at 0 h in both *Ax2* (○) and *triA*⁻ (●). The level of the constitutively expressed gene *IG7* remains unchanged through development. (B) Ratios of mean band intensities of *csA* to *IG7* at each time point (from three independent experiments).

Siegert and Weijer 1992) or lowered sensitivity to suppression of chemotaxis by ammonia (Sternfeld and David 1982; Feit et al. 1990, 2001, 2007). Note that extensive movement and changes in cell fate occur over vastly different time scales. *triA*⁻ cell movement from the posterior to anterior is well under way within minutes of grafting (not shown), whereas a histochemical change in cell type takes significantly longer (Bonner et al. 1955; Sakai 1973). When considered along with the observation that labile gene-reporter constructs invariably show a clear anterior–posterior demarcation in *triA*⁻ slugs (Jaiswal et al. 2006), the difference in time scales appears to favor the hypothesis that transdifferentiation precedes movement.

Culmination in the absence of *triA* function

Granted that wild-type *triA* function in prespore cells is not sufficient for the full ascent of the spore mass, might it nevertheless be necessary? An answer would require us to monitor chimaeric slugs in which prespore cells are mutant but prestalk cells and ALCs are wild type. Because the upper cup derives from cells in both the anterior and posterior zones (Fig. 1), such slugs will not be easy to generate.

TriA is expressed only in the slug posterior; this makes it likely that it is a prespore-specific gene (Jaiswal et al. 2006). However, the absence of *triA* function can—among other things—lead to aberrant behavior in a subset of prestalk cells,

namely those that will form the upper cup. A likely reason is that the aberrant upper cup in *triA*⁻ is derived from former ALCs in which the “*ecmO*” part of the *ecmA*O promoter is inactive during culmination (even though it used to be active in the past; see Fig. 5E in Jaiswal et al. 2006). One way of interpreting what goes wrong with the ascent of the spore mass in *triA*⁻ fruiting bodies is by saying that the *trishanku* gene mediates a form of cooperation between distinct cell types that is disrupted in the mutant. This interpretation is strengthened by the morphology of the stalk in cross-genotype grafts: it seems to reflect the genotype of the posterior fragment rather than that of the anterior (Fig. 5, C and D). The reason why fruiting bodies formed from *Ax2* anterior-*triA*⁻ posterior grafts (or for that matter *triA*⁻ fruiting bodies) form a thick stalk might be that in both cases the stalk contains *triA*⁻ cells that have differentiated incompletely from a prespore state. A lack of full transdifferentiation may also be responsible for the relatively lower viability of *triA*⁻ spores (72% as against 91% for the wild type); it may be that in a formal sense some of them retain a memory of their prestalk origin.

The upper cup and the ascent of the sorus

This study confirms the contention of Sternfeld (1998) that it is the upper cup, and not the lower cup, that plays an active role in the ascent of the sorus. The lack of full ascent of

the sorus in grafts has to do with the nature of the upper cup (Fig. 7, A–D). When a neutral red-stained *triA*[−] anterior is grafted to a larger than usual Nile blue-stained Ax2 posterior fragment (which includes the pstO and prespore regions): the upper cup contains only wild-type cells and remains apposed to the sorus as it rises to a terminal position (Fig. 8A). Mixing experiments strengthen this inference (Fig. 9, A and B). The lower cup, which is normal in all these cases, may play a passive supportive role: Saito et al. (2008) have found that *D. discoideum* cells lacking DIF-1 fail to make a lower cup, with the result that the sorus often slips down the stalk.

Genes that influence the proper positioning of the sorus

The cell adhesion molecule lagC is a possible intermediary between the action of *triA* and the rise of the spore mass (Fig. 10), and it would be worth overexpressing *lagC* (or even some other gene that encodes a cell–cell adhesion molecule) in pstO cells to see if the *triA*[−] defect can be overcome. There are other mutants of *D. discoideum* in which the spore mass remains subterminal. The list includes *regA*[−] (which shows an uncoupling of sporulation with normal morphogenesis; Shaulsky et al. 1998), *SP85*[−] (which shows accelerated spore maturation; West et al. 2002) and *dmnA*[−] (which is a knock out of a DNA methyltransferase; Katoh et al. 2006). Also, in some conditions prespore cells are unable to rise at all and remain at the base of the stalk. They include *dmtA*[−], *dimA*[−], and *dimB*[−] (Thompson and Kay 2000; Thompson et al. 2004; Huang et al. 2006). The mechanisms behind aberrant terminal morphogenesis in these mutants are not known, and it remains to be seen whether the relevant genes act independently of *triA* or in concert with it. The predicted protein sequence of *triA* indicates the existence of putative protein–protein interaction domains (Jaiswal et al. 2006), but we have no information as yet about what the interacting partners may be. What one can conclude is that the proper positioning of the spore mass in *D. discoideum* is a complex process in terms of the number of genes involved.

Concluding remarks

Efficient spore dispersal appears to be the main function of the fruiting body (Bonner 1967). This alone must provide strong selective pressure for maintaining wild-type *triA* function. With regard to the rise of the sorus, the tissue whose role is compromised in the absence of *triA* (the upper cup) is different from the tissue in which the gene is expressed normally (prespore tissue). One may say that the presence of wild-type gene activity in prespore cells elicits a form of upper cup behavior that in turn benefits prespore cells. There are precedents from *D. discoideum* development in which the genetic constitution of one cell type is essential for the proper

functioning of the other. In the case of the genes *sdf2* (Anjard et al. 1998) and *comD* (Kibler et al. 2003), gene activity in prestalk cells is required for prespore cells to differentiate into spores. Such observations show that in situations involving cooperative behavior in groups, natural selection may act indirectly—by favoring a trait that enables an individual (here, cell) to stimulate another individual to help it (Kawli and Kaushik 2001; Kaushik and Nanjundiah 2003). Finally, it should be noted that there are other cellular slime molds (e.g., *Polysphondylium violaceum*, *Polysphondylium pallidum*, and *Dictyostelium rosarium*) in which a portion of the sorus is periodically left behind as the rest continues to climb upwards (Bonner 1967). It would be interesting to see whether this can be related to periodic variations in the strength of upper cup–sorus adhesion, possibly under the mediation of a *trishanku*-like gene.

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