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Plant reproduction during spaceflight: importance of the gaseous environment

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Abstract. Plant reproduction is a complex developmental process likely to be disrupted by the unusual environmental conditions in orbital spacecraft. Previous results, reviewed herein, indicated difficulties in obtaining successful seed production in orbit, often relating to delayed plant development during the long-term growth necessary for a complete plant life cycle. Using short-duration exposure to spaceflight, we studied plant reproduction in *Arabidopsis thaliana* (L.) Heynh. during three flight experiments: CHROMEX-03 on STS-54 (6 d), CHROMEX-04 on STS-51 (10 d), and CHROMEX-05 on STS-68 (11 d). Plants were 13–14 d old (rosettes) at time of launch and initiated flowering shoots while in orbit. Plants were retrieved from the orbiters 2–3 h after landing and reproductive material was immediately processed for in-vivo observations of pollen viability, pollen tube growth, and esterase activity in the stigma, or fixed for later microscopy. Plants produced equal numbers of flowers to those controls growing on the ground but required special environmental conditions to permit fertilization and early seed development during spaceflight. In CHROMEX-03, plants were grown in closed plant growth chambers (PGCs), and male and female gametophyte development aborted at an early stage in the flight material. In CHROMEX-04, carbon dioxide enrichment was provided to the closed PGCs and reproductive development proceeded normally until the pollination stage, when there was an obstacle to pollen transfer in the spaceflight material. In CHROMEX-05, an air-exchange system was used to provide a slow purging of the PGCs with filtered cabin air. Under these conditions, the spaceflight plants apparently had reproductive development comparable to the ground controls, and immature seeds were produced. In every aspect examined, these seeds are similar to those produced by the ground control plants. The results suggest that if the physical environment around the plant under spaceflight conditions meets the physiological demands of the plant, then reproductive development can proceed normally on orbit.

Key words: *Arabidopsis* – Fertilization (in spaceflight) – Ovule – Pollen – Pollination – Seed

Introduction

Investigation of plant responses to the environment of spaceflight has been a component of space life sciences research since its inception (Halstead and Dutcher 1987). Because of their well-characterized tropic responses to gravity, plants have been the vehicle of choice for addressing questions about the role of gravity in shaping basic life processes as we understand them. The advent of manned spaceflight provided the opportunity to observe plant growth and development in the near absence of gravity on spacecraft in the free-fall of low earth orbit (Krikorian and Levine 1991). Initially, long-duration Soviet missions included plants in on-board greenhouses to provide psychological relief to the crew (Nechitailo and Mashinsky 1993). Our understanding of the problems associated with growing plants in microgravity, particularly building suitable hardware to support plants, has grown from these experiences.

Plant growth for extended periods in microgravity was generally poor and plants frequently died in the transition from vegetative to reproductive stage (Halstead and Dutcher 1984, 1987; Nechitailo and Mashinsky 1993). Numerous attempts have been made to grow a plant through a complete life cycle in space (Kordyum et al. 1983; Mashinsky et al. 1994), but the only successful cycle was achieved in 1983 with *Arabidopsis thaliana* on board Salyut 7 in a miniature plant growth chamber called Phyton. The plants had grown from seed planted on orbit, and although development was delayed, they flowered and produced new seed. However,

Abbreviations: AES = air exchange system; PGC = plant growth chamber; PGU = plant growth unit

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reports on the returned material described a large proportion of empty seed, and a high number of embryonic lethals in the seeds that were produced (Merkys and Laurinavicius 1983), making this achievement a qualified success.

A summary of highlights of these previous studies on plant reproduction is given in Table 1. Flower formation also occurred on the US space shuttle in March 1989 on STS-29 during an experiment designed to study spaceflight effects on chromosomes using aseptically cultivated plantlets of *Haplopappus gracilis* (Nutt.) (Levine et al. 1990). The *Haplopappus* bud (Fig. 1) subsequently opened on earth and was checked for meiosis, which had occurred normally during the 5-d exposure to spaceflight (A.D. Krikorian, SUNY at Stony Brook, Stony Brook, NY, USA, personal communication).

In 1993 we began a series of experiments on the mid-deck of the Space Shuttle orbiter to investigate the apparent sensitivity of reproductive events to the spaceflight environment. Flight hardware called the Plant Growth Unit (PGU) was utilized. The PGU fits into a locker on the mid-deck of the Space Shuttle orbiter and provides light (approx. 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of photosynthetically active radiation) and nominal temperature control for the smaller Plant Growth Chambers (PGCs) located inside (Krikorian and Levine 1991). *Arabidopsis thaliana* was selected for use in these experiments because of its compact size, low light requirement, and short life cycle. Because flight opportunities on the shuttle are currently two weeks in duration or less, early events in reproductive development were studied: gametophyte development, pollination, fertilization, and early embryogenesis.

Materials and methods

Using an agar-based nutrient medium that had been developed to grow *Arabidopsis* on Biosatellite (Brown et al. 1976), we grew *Arabidopsis thaliana* (L.) Heynh. strain Columbia plants for 13 or 14 d in 7-ml polycarbonate centrifuge tubes that could be easily loaded into a phenolic foam supporting matrix in each PGC. At this age, the plants had formed rosettes and were ready to initiate flowering shoots (Kuang et al. 1995) when they were loaded into the PGCs less than 24 h prior to lift off. In Chromex-03 on STS-54 (Endeavour) in January 1993 (6-d mission) we had 6 PGCs, with a total of 36 plants. In Chromex-04 on STS-51 (Discovery) in September 1993 (10-d mission) we had 2 PGCs (12 plants), and in Chromex-05 on STS-68 (Endeavour) in September 1994 (11-d mission) we had 5 PGCs (30 plants).

Because of the rapid generation of flowers in *Arabidopsis*, we had a minimum of approximately 500 flowers to work with in each experiment (Table 2). Plants were retrieved from the orbiters 2–3 h after landing and reproductive material was immediately processed for in-vivo observations of pollen viability (Heslop-Harrison et al. 1984), pollen tube growth (Preuss et al. 1993), and esterase activity in the stigma (Shivanna and Rangaswamy 1992), or fixed for later microscopy (Kuang et al. 1995). Small portions of the vegetative material (leaves, stems and roots) were also fixed for microscopy, but the bulk of this tissue was frozen for later analyses (Musgrave et al. 1994). Ground controls were conducted in another PGU inside the Orbiter Environmental Simulator at the Kennedy Space Center, a growth chamber which provides a simulation of cabin temperature and gas composition during each mission.

Gas samples (1 ml) withdrawn from the headspace of the PGCs at the beginning (approx. 18 h prior to launch or initiation of ground control) and end (3 h after landing or termination of ground control) of the experiments were analyzed by gas chromatography to determine carbon dioxide and ethylene concentrations. In the STS-54 and STS-51 experiments, plants were grown in sealed PGCs while in STS-68, an Air Exchange System (AES; Krikorian and Levine 1991) was used with the PGCs to provide a slow exchange ($90 ml \cdot min^{-1}$) of chamber air with filtered air from the crew cabin.

Table 1. Highlights of previous experiments on plant reproduction during spaceflight

Plant type	Flight	Chamber	Duration (d)	Observation
Arabidopsis thaliana	Kosmos 1129	(described)[a]	18	Plants grown on earth to flowering stage formed seeds on orbit; 55% were fertile; 27% aborted; 18% had non-viable embryos[b]
	Salyut 6	Svetoblok-1	67	Plants flowered, but androecium and gynoecium were sterile; ovules degenerated (Kordyum et al. 1983)
	Salyut 7	Phyton	69	First and only complete plant life cycle in space to date; 33% of siliques produced contained aborted ovules; only half of the remaining seeds had viable embryos upon germination (Merkys and Laurinavicius 1983)
Epidendrum radicans	Salyut 6	Malachite	110	Flowers present at time of launch were lost immediately; no subsequent flowering occurred (Nechitailo and Mashinsky 1993)
Pisum sativum	Salyut 6	Oasis	40	Plants died at flowering stage[c]
Triticum aestivum	Mir	Svetoblok-M	167	Development delayed; heads were still in the boot when the plants were returned to earth and subsequently produced seeds (Mashinsky et al. 1994)

[a]Plants were housed in a Plexiglas beaker containing moist soil
[b]Results of other partial-life-cycle-duration experiments are reviewed here as well (Parfenov and Abramova 1981)
[c]Premature death was attributed to the open nature of the Oasis hardware (Nechitailo and Mashinsky 1993). For a review of other failed attempts at full plant life cycles see also Halstead and Dutcher (1984)

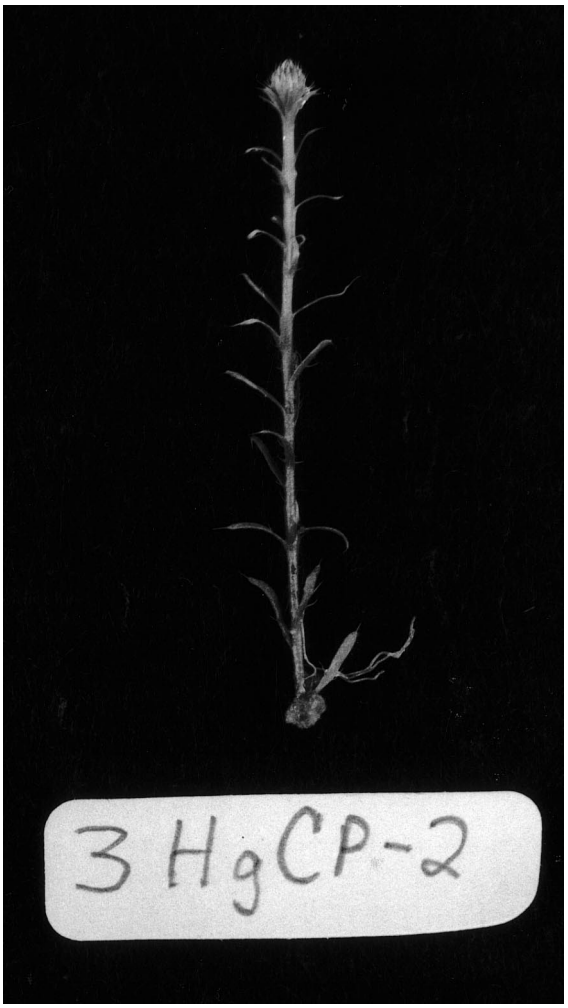


Fig. 1. Flower bud formed by a *Haplopappus gracilis* explant during a 5-d exposure to spaceflight on STS-29. The hardware used for this experiment was the PGU equipped with the AES. Photograph courtesy of A.D. Krikorian

Table 2. Comparison of flower production in spaceflight and ground control treatments[a]

Exp.	Mission duration (d)	Total plants (*n*)	Flowers per plant	
			Flight	Control
STS-54	6	36	16.1	16.1
STS-51	10	12	44.1	39.8
STS-68[b]	11	24	79.5	77.3

[a]No difference between treatments was detected by *t*-test for the number of flowers per plant
[b]The AES has only four positions on its manifold so that one of the five PGCs in this experiment was not supplied with airflow. The rate of flowering that occurred in the closed chamber was not significantly different from the STS-51 result. Therefore, data from this chamber have not been included in the STS-68 analysis

For reasons to be discussed later, the headspaces of the PGCs in STS-51 and STS-68 were enriched with carbon dioxide at the start of the experiment (Kuang et al. 1996a). No significant differences in headspace gas composition were found between the flight and ground control treatments in any of the experiments. Mean ethylene levels were below 100 nl · l^{-1}. Mean carbon dioxide concentrations for the three experiments are summarized in Table 3.

Table 3. Comparison of initial and final carbon dioxide concentrations in the PGC headspaces in three experiments (mean with SE in parentheses)

Exp.	Carbon dioxide (μl · l^{-1})	
	Initial	Final
STS-54	446 (96)	117 (8)
STS-51	8001 (1521)	76 < X < 114[a]
STS-68	5745 (522)	302 (43)

[a]Values from two of the PGCs were below the detection limit of the gas chromatograph (75 μl · l^{-1}), so the mean could not be determined beyond the accuracy indicated here

Results and discussion

Before considering the course of floral development in spaceflight, it was necessary to determine whether or not floral initiation rates were comparable to those in the ground-based controls. In these experiments, flowers were initiated at the same rate whether plants were growing on the ground or in the orbiter. No significant difference in numbers of flowers in each size class was found between ground control and flight material on STS-54 (Fig. 2A), STS-51 (Fig. 2B), or STS-68 (Fig. 3). The longer duration explains the difference in number of

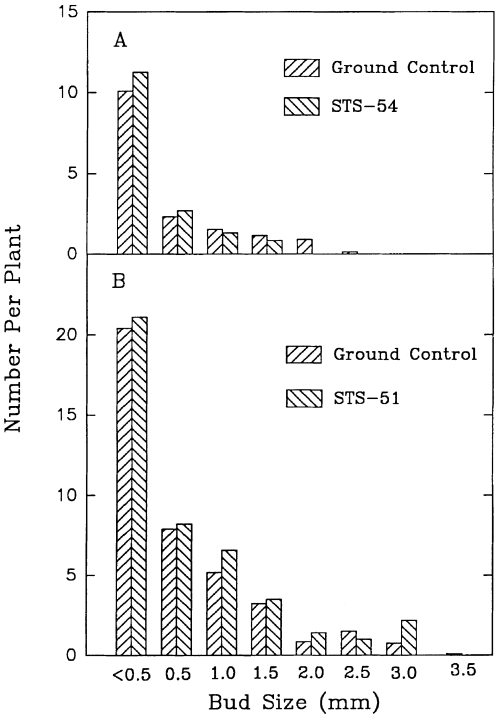


Fig. 2A,B. Distribution of *Arabidobsis thaliana* flowers by length of bud (pre-anthesis) or pistil (post-anthesis) for the STS-54 (**A**) and STS-51 (**B**) experiments. No statistically significant differences were found in numbers of flowers in a size class between ground control and flight material

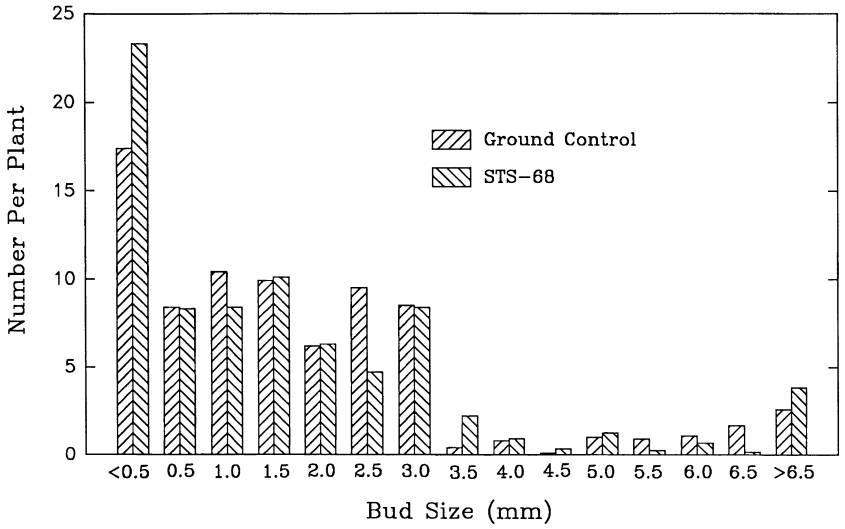
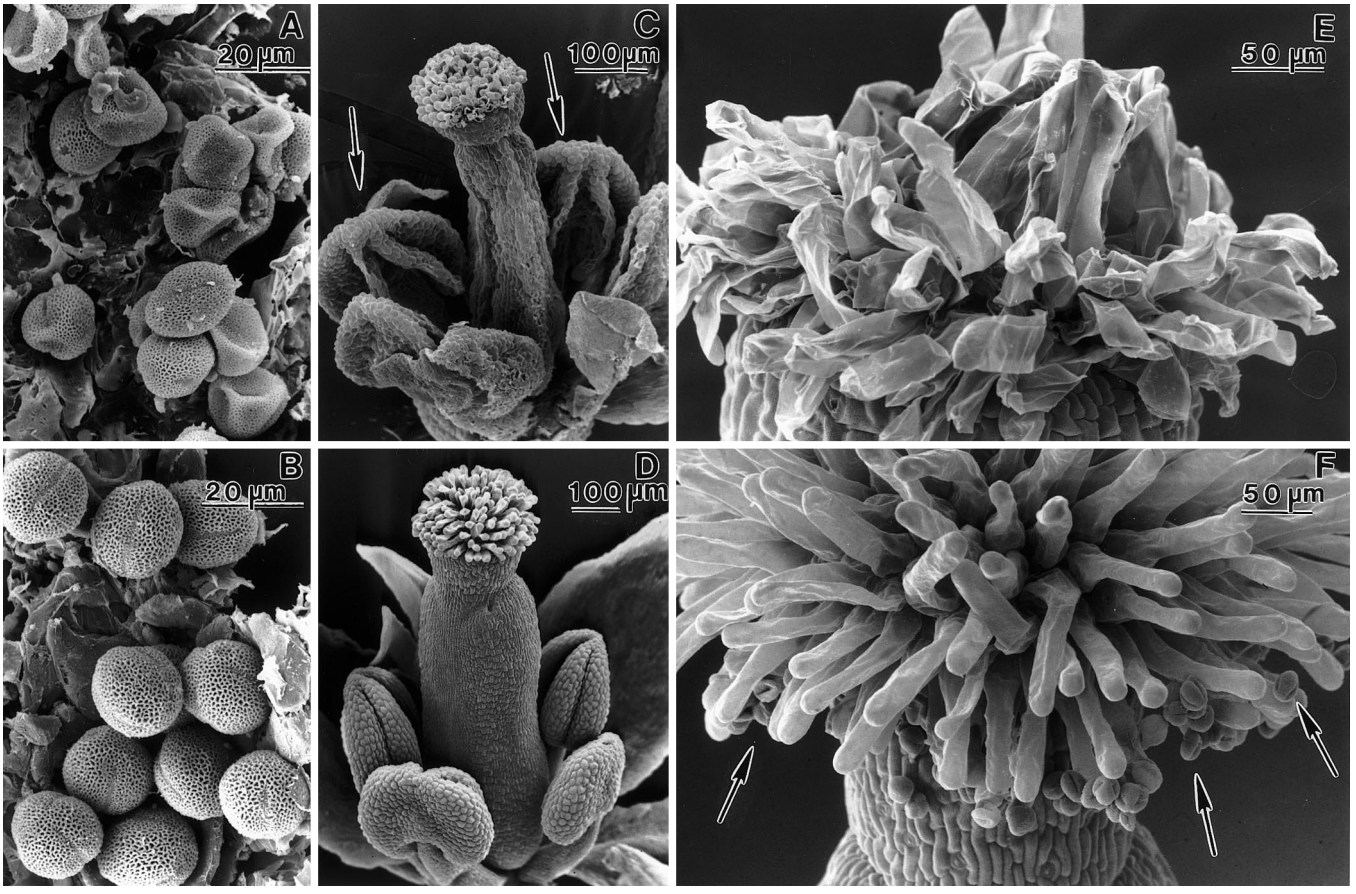


Fig. 3. Distribution of *A. thaliana* flowers by length of bud (pre-anthesis) or pistil (post-anthesis) for the STS-68 experiment. No statistically significant differences were found in numbers of flowers in a size class between ground control and flight material

Fig. 4A–F. Scanning electron micrographs of flowers of *A. thaliana* produced during spaceflights. **A** Collapsed pollen grains from an STS-54 flower. **B** Pollen grains from STS-51 plants appeared normal. **C** Flower from STS-54 plant. Pistil and anthers (*arrows*) had collapsed at an early stage. **D** Flower from STS-51 plant. Pistils and anthers were plump and developing normally. **E** Stigma from post-anthesis flower on STS-51. Papillae were flattened and no pollen grains were visible on the stigma. **F** Stigma from STS-68 post-anthesis flower. Pollen grains (*arrows*) were present on the stigma

flowers per se and the larger sizes obtained in STS-51 relative to the first experiment on STS-54. With air flow provided from the AES and an additional day of development in the third experiment on STS-68, significant numbers of large siliques were obtained (Fig. 3). Because no differences in flower numbers were seen in the spaceflight material compared to the ground controls, we concluded that our comparative developmental



study would not be subject to problems encountered in previous experiments, in which substantial delays in plant development occurred in the flight material (Merkys and Laurinavicius 1983; Halstead and Dutcher 1987; Mashinsky et al. 1994).

In the first experiment on STS-54, both male and female reproductive development aborted at an early stage (Kuang et al. 1995) in the spaceflight plants. Pollen from the returned flowers had less than 1% viability as assessed by fluorescein diacetate staining (Heslop-Harrison et al. 1984) and appeared collapsed when viewed by Scanning electron microscopy (Fig. 4A). Scanning electron microscopy of whole flowers revealed withered anthers and pistils (Fig. 4C). Carbohydrate analysis of the foliage indicated very low levels of fructose in the spaceflight material and total carbohydrate, including starch, was only 61% of the ground control (Musgrave et al. 1994). We suspected that one possible cause of reproductive failure on STS-54 was the low carbohydrate status of the plant, and in the subsequent experiments on STS-51 and STS-68 we supplemented the medium with additional sucrose (2%) and enriched the gases in the PGC headspace with carbon dioxide (Table 3). On STS-51, these countermeasures resulted in morphologically normal pollen production (Fig. 4B), and the general morphology of the flowers (Fig. 4D) was very similar to the ground controls (Kuang et al. 1996a). Nevertheless, reproductive development failed to continue beyond the production of normal male and female gametophytes. Reproduction was apparently blocked at the pollination or fertilization stage.

Arabidopsis generally is self-pollinating. As the stamens elongate, the anthers dehisce and discharge pollen on the stigmatic papillae as they grow alongside the pistil. None of the pistils examined immediately postflight on STS-51 or fixed for scanning electron microscopy (Fig. 4E) had pollen grains visible on the stigmatic papillae, leading us to believe that pollination had failed in this material.

On STS-68, the PGU was equipped with an Air Exchange System (AES) (Krikorian and Levine 1991) which provided a slow exchange of chamber air with filtered air from the crew cabin (90 ml · min^{-1}). Each chamber received separate flow via a manifold. During the 11-d mission, plants developed from the rosette stage at loading (Fig. 5A) to the reproductive stage, with numerous flowers and developing siliques (seed pods) (Fig. 5B). Flowers had a normal appearance (Fig. 5C). Scanning electron microscopy of pistils from this material also detected the remains of germinated pollen grains on the stigmatic papillae (Fig. 4F). Esterases, important components of the stigma surface proteins, were localized, cytochemically, based on the hydrolysis of the substrate α-naphthyl acetate to form a reddish

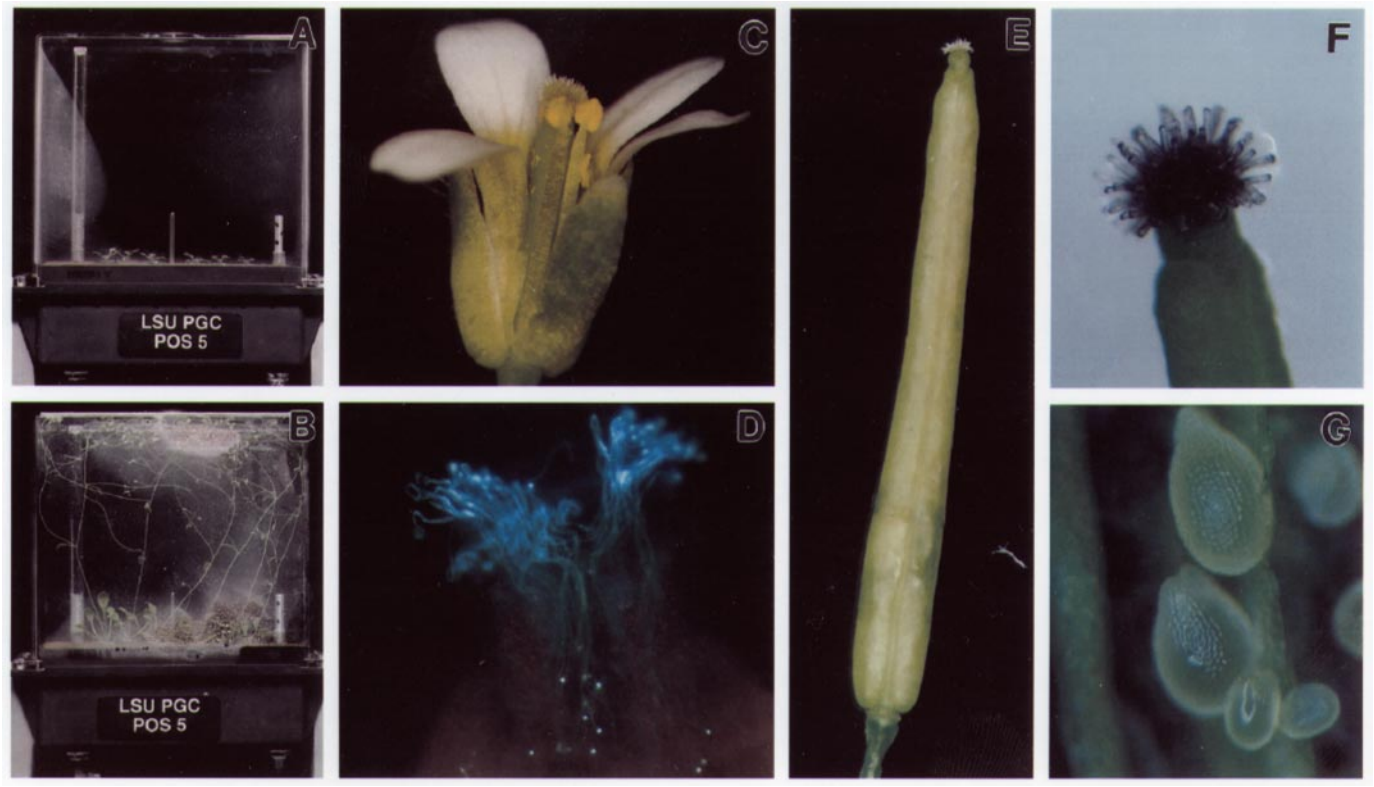


Fig. 5A–G. Reproductive development in *A. thaliana* on STS-68. **A** Loaded plant growth chamber, 18 h prior to launch. **B** Plant growth chamber as in **A** 2 h after landing. **C** Flowers from STS-68 plants were normal in appearance. **D** Fluorescing callose stained with aniline blue shows that pollen germinated and the tubes were growing down towards the style. Bright dots are callose plugs of pollen tubes. **E** An 11-mm-long silique (seed pod) formed during spaceflight. **F** A positive esterase reaction was observed in pistils of STS-68 flowers. The dark purple color of the stigmatic papillae means that esterases were present and active. **G** Dissected silique showing ovules inside. Note that some ovules developed while others remained small. ×22 (**C**), ×113 (**D**), ×12 (E), ×52 (**F**), ×64 (**G**)

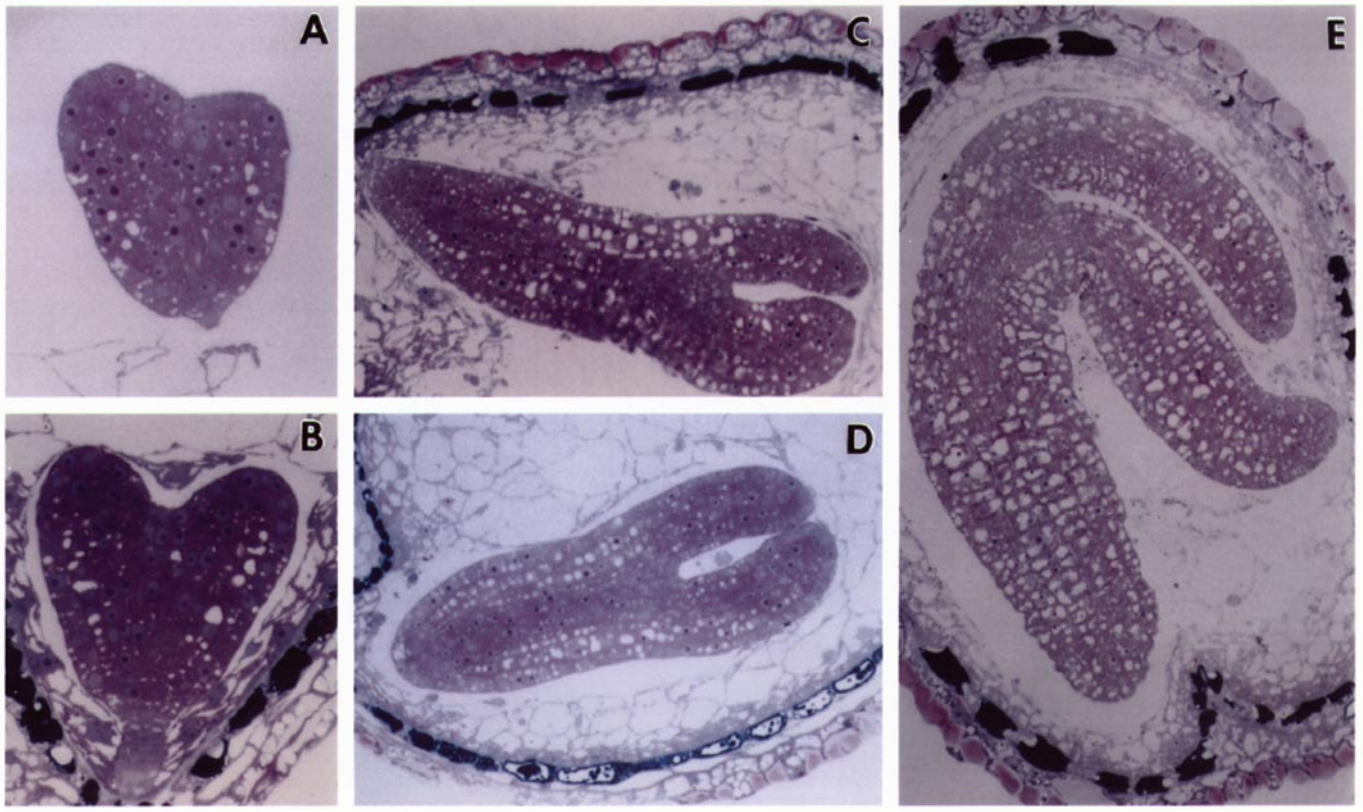


Fig. 6A–E. Embryogenesis in *A. thaliana* on STS-68 (**A, C, E**) or in the ground control (**B, D**). **A, B** Heart-shaped embryo; **C, D** Cotyledons and radical have developed; **E** tissues such as protoderm, proapical meristem and provascular tissue have developed in this immature seed. ×1069 (**A, B**), ×535 (**C–E**)

insoluble complex upon treatment with fast blue B (Fig. 5F) (Shivanna and Rangaswamy 1992). The esterase reaction obtained in the flight material was indistinguishable from that of the ground control.

Germination of pollen on the stigma surface results in the formation of tubes that carry sperm cells to the ovules. These tubes contain a high concentration of callose which fluoresces when stained with aniline blue (Preuss et al. 1993). Using this test on fresh post-flight material, we saw that pollen tubes had grown down through the style (Fig. 5D). The plump appearance of the numerous elongated siliques (Fig. 5E) suggested immediately that pollination had occurred successfully. Inside the ovary the ovules were developing rapidly (Fig. 5G).

Developing seeds were dissected from this material, fixed and embedded for microscopy. All stages of embryo development present in the ground control were also found in the flight material (Fig. 6A–D) and the most advanced stage was an immature seed with developing cotyledons and radical (cotyledonary stage, Fig. 6E). Further detailed analysis of the developing seeds has confirmed through cytochemical localization techniques that storage reserves had been deposited similarly in the spaceflight and ground control material (Kuang et al. 1996b).

Conclusions

Given the constraints of a short time frame, reproduction had proceeded normally in *Arabidopsis* on STS-68 through the stage of an immature seed. In comparison to this success which used the PGU hardware equipped with an AES, the prior failures in closed PGCs point to the importance of enhancing gas exchange for plants growing in the spaceflight environment. In the absence of buoyancy-driven convective air movement, there is a limitation on the rate of movement of metabolic gases. Evidence of such a limitation (hypoxia) was found in the roots of STS-54 plants (Porterfield et al. 1994), and levels of fixed carbohydrate were low in the spaceflight leaves compared to the ground controls despite equal supplies of carbon dioxide (Musgrave et al. 1994). Previous reports mentioned alteration of carbohydrate metabolism in plants growing in microgravity (Johnson and Tibbitts 1968; Volkmann et al. 1986). In our system, providing the plants with extra carbon through medium supplementation and carbon dioxide enrichment of the atmosphere in the PGCs on STS-51 allowed early reproductive development to occur normally. Flow-through of filtered cabin air was necessary for reproduction to proceed through pollination, fertilization, and early seed development on STS-68.

Many physical changes will occur in the plant's environment due to microgravity. The stagnant air layers that form in the absence of buoyancy-driven convection may challenge plants with potential stresses of localized high humidity, diminished availability of

metabolic gases, build-up of potentially harmful gases, and high temperatures in the canopy. Furthermore, water and nutrient delivery become problematic in an environment in which water's cohesive and adhesive properties differ from those on earth. Static charge could become an important factor in microgravity during processes such as pollination (Fig. 4F). Gravity-dependent physical processes may also impinge on cellular processes (Todd 1989).

Because microgravity changes the behavior of fluids and gases, protocols established for successfully growing plants on the ground do not provide the same physical environment for plants growing in orbit. The growth conditions provided in CHROMEX-03 allowed ground control plants to complete their early reproductive development properly; however, the same protocol resulted in aborted development at an early stage in the spaceflight material. Because subsequent modifications of the gaseous environment in CHROMEX-04 (carbon dioxide enrichment) and CHROMEX-05 (gas flow-through) allowed reproduction to proceed normally during spaceflight, we hypothesize that the actual gaseous environment occurring immediately around the spaceflight plants in CHROMEX-03 was in fact inadequate to support continued reproductive development.

Future plans for plant experimentation hold the promise of on-board centrifuge facilities that will add an important 1-*g* spaceflight control to studies such as those reported herein. Even with this advance, however, we will still have to come to grips with the thorny problem of how to produce physiologically equivalent plants in 1 *g* and microgravity. This will entail introducing specialized monitoring devices [such as the substrate moisture probes that have been integrated into the improved Svet greenhouse on Mir (Bingham et al. 1995)] that will be part of a controlling system that regulates the environment the plant actually experiences. The old concept of using phytometers (Daubenmire 1959) will be useful as we struggle with these design issues and performance measures such as canopy gas exchange, leaf area increments, and chlorophyll fluorescence may provide additional information.

The quality of the vegetative growth and development of the plant prior to the transition to reproduction will probably be the most important factor in determining plant reproductive success. Long-term experiments with plants started on orbit from dry seeds have been plagued by delayed development and anomalies in reproductive processes (Table 1). On the other hand, our experiments with pre-grown *Arabidopsis* rosettes have shown that normal reproductive development can occur during spaceflight. Much additional work is needed in this important area of research. As reproduction is studied in other species and over extended periods, attention must be given to quantifying and controlling the plant microenvironment.

With the availability of longer-duration missions and improved plant growth hardware it should be possible to explore seed-to-seed cycling in more detail on the shuttle, on the Mir space station (Salisbury et al. 1995), and in the future on the International Space Station. Plant reproduction must be successful in space if man is to rely on plants for food, as well as atmosphere and water regeneration, in a closed ecological life support system (Olson et al. 1988) on very long-duration missions.

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