Optospectroscopic Detection of Primary Reactions Associated with the Graviperception of *Phycomyces*. Effects of Micro- and Hypergravity[1]

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The graviperception of sporangiophores of the fungus *Phycomyces blakesleeanus* involves gravity-induced absorbance changes (GIACs) that represent primary responses of gravitropism (Schmidt and Galland, 2000). GIACs ($\Delta A_{460-665}$) of sporangiophores were measured in vivo with a micro-dual wavelength spectrometer at 460 and 665 nm. Sporangiophores that were placed horizontally displayed an instant increase of the GIACs while the return to the vertical position elicited an instant decrease. The GIACs are specific for graviperception, because they were absent in a gravitropism mutant with a defective *madJ* gene. During parabola flights hypergravity (1.8*g*) elicited a decrease of the GIACs, while microgravity ($0 \pm 3 \times 10^{-2}g$) elicited an instant increase. Hypergravity that was generated in a centrifuge (1.5–6.5*g*) elicited also a decrease of the GIACs that saturated at about 5*g*. The GIACs have a latency of about 20 ms or shorter and are thus the fastest graviresponses ever measured for fungi, protists, and plants. The threshold for eliciting the GIACs is near $3 \times 10^{-2}g$, which coincides numerically with the threshold for gravitropic bending. In contrast to gravitropic bending, which requires long-term stimulation, GIACs can be elicited by stimuli as short as 20 to 100 ms, leading to an extremely low threshold dose (acceleration × time) of about $3 \times 10^{-3}g$ s, a value, which is four orders of magnitude below the ones described for other organisms and which makes the GIACs of *Phycomyces blakesleeanus* the most sensitive gravi-response in literature.

Classical research on the graviperception of plants and fungi has been largely restricted to investigating bending responses, to studies of the requisite statoliths, the involvement of hormones, and the concomitant modulation of cell wall growth (for reviews, see Volkmann and Sievers, 1977; Björkmann, 1988; Sack, 1991). In more recent years, the role played by the cytoskeleton (e.g. Baluska and Hasenstein, 1997; Braun, 1997; Braun and Sievers, 1994) and by ion channels and transport has received closer attention (Machemer and Bräucker, 1992; Lebert and Häder, 1996; Scott and Allen, 1999; Plieth and Trewawas, 2002). A shortcoming of classical and even modern research has been the lack of rapid in vivo assays for detecting the primary responses associated with graviperception. This problem persists despite the application of the tools of molecular biology that has led to the identification of several genes that play essential roles in graviperception (Kiss et al., 1989; Fukaki et al., 1998).

We have tackled this problem by applying a novel technique, i.e. rapid-scan spectrometry (Schmidt, 1997; Schmidt and Galland, 1999), to the unicellular fungus, *Phycomyces blakesleeanus*, which has served in the past mainly as a model system of phototropism (Galland, 2002) and to a lesser extent also of gravitropism (Dennison, 1961; Dennison and Shropshire, 1984; Schimek et al., 1999a, 1999b; Grolig et al., 2000). As a microorganism, *P. blakesleeanus* possesses several features, such as a short life cycle, amenability to classical and molecular genetics, and the availability of many phototropism and gravitropism mutants, which render it an ideal system to investigate photo- and graviorientation even in the confinements of limited space and time. The giant sporangiophore of 2 to 5 cm length emerges two days after spore inoculation from the mycelium and grows upright at a constant velocity of about 2 to 3 mm h^{-1}. It is extremely sensitive to blue light, to which it bends phototropically, and reacts to the earth's gravitational field. The gravitropic threshold is near $3 \times 10^{-2}g$ (Galland et al., 2004). In microgravity of a satellite the growth direction of the sporangiophore is completely random (Parfyonov et al., 1979).

The negative gravitropism of the sporangiophores of *P. blakesleeanus* is under the redundant control of several distinct stimuli. These are: (1) bending force (flexure) of the sporangiophore (Dennison, 1961), (2) the sedimentation of statoliths (Schimek et al., 1999a, 1999b), and (3) the buoyance of apical lipid globules (Schimek et al., 1999a; Grolig et al., 2003). The statoliths of *P. blakesleeanus* are identical with the previously described vacuolar protein crystals that display a paracrystalline structure (Ootaki and Wolken, 1973). The protein crystals are made up of

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of three proteins that contain noncovalently bound pterin- and flavin-like pigments (Eibel et al., 2000; Fries et al., 2002). Stage-1 sporangiophores (without sporangium) possess an apical aggregate of large lipid globules, which migrate in the subsequent developmental stages 2 to 4 into the columella (the central part of the sporangium, which does not contain spores). When stage-1 sporangiophores are inclined horizontally, the aggregate of lipid globules floats approximately 10 μm upward, generating a cap-like structure (Schimek et al., 1999). Sporangiophores that are raised at low temperature (5°C) possess fewer lipid globules and display a substantially reduced gravitropism (Grolig et al., 2003).

On a formal level the gravitropism of the fungus *P. blakesleeanus* and that of higher plants such as the *Avena* coleoptile share many similarities. The absolute gravitropic thresholds are similar (near $10^{-2}g$; Galland et al., 2003, in press), and the gravitropic bending rates are slow (near 0.1–0.4 deg min^{-1}; Dennison and Shropshire, 1984). Both organisms obey the so-called sine law, and the interaction between gravi- and photostimulation display adherence to a novel exponential law for photogravitropic equilibrium (Galland et al., 2002; Galland, 2002). The similarities in gravitropic behavior exist despite the fact that the cell organelles eliciting gravitropic bending and the cell walls of fungi and plants are very different.

To detect primary responses that are associated with the phototropism and the gravitropism of the sporangiophore of *P. blakesleeanus* we took advantage of a novel instrument, the rapid-scan spectrometer (RSS; Schmidt 1997; Schmidt and Galland, 1999). We have found light-induced absorbance changes (LIACs) that occur in the growing zones of sporangiophores in response to blue-light stimulation. These LIACs are specifically associated with the blue-light receptor of *P. blakesleeanus* and indicate the generation of flavosemiquinone from the oxidized flavin receptor (Schmidt and Galland, 1999). When we analyzed with the same method the gravitropism of the sporangiophore we found in earth-bound experiments gravity-induced absorbance changes (GIACs) that differed substantially from the LIACs (Schmidt and Galland, 2000). While the LIACs are characterized by distinct maxima near 460 and 610 nm, the GIACs display a major maximum near 420 nm and a steady increase above 600 nm (Schmidt and Galland, 2000). These results showed that phototropism and gravitropism possess distinct transduction chains, which are characterized by different absorbance changes. It is very likely that the GIACs are associated with primary responses of gravitropism. This assumption is supported by the observations that the GIACs begin before the gravitropic bending is detectable, and that gravitropism mutants show abnormal GIACs (Schmidt and Galland, 2000).

To analyze the gravitropic primary responses in further detail and to better exploit the technical potential of in vivo spectroscopy we employed the technique of dual-wavelength spectroscopy rather than relying exclusively on rapid-scan spectroscopy. Dual-wavelength spectroscopy has the advantage of elevated sensitivity, which exceeds that of absorption spectroscopy by two to three orders of magnitude. For our specific purposes we employed a recently constructed instrument, the micro-dual-wavelength spectrometer (MDWS), which measures in vitro and in vivo reflection changes at a resolution corresponding to 10^{-2} mOD (Schmidt, 2004). Our results from earth-bound and parabola-flight experiments show that the graviperception of *P. blakesleeanus* is associated with absorbance changes that represent the fastest gravi-reactions ever measured and that are characterized by the lowest threshold doses for short-term stimulation that are presently known.

RESULTS

Generation of GIACs by Tilting and Centrifugation of Sporangiophores

Sporangiophores were placed vertically into the MDWS and were then adapted in this position for 30 min. During this period the sporangiophores were exposed unilaterally via fiber optics to the alternating irradiation generated by two light-emitting diodes at 460 and 665 nm. The MDWS measures the light that is reflected from the irradiated sporangiophores and monitors the relative reflection changes occurring at the two wavelengths. It should be emphasized that the MDWS is employed in the reflectance mode so that it measures physically reflection and reflection changes rather than absorbance as is suggested by the name GIAC. The relative absorbance, A, is calculated from the reflected light according to the following definition: $A = R_{460}/R_{665}$, where R_{460} is the reflectance at 460 and R_{665} the reflectance at 665 nm. If $\Delta R << R$, this formula replaces the correct definition $A = \log R_{460}/R_{665}$ (see "Materials and Methods"; Schmidt and Galland, 1999).

When the sporangiophores were tilted horizontally they generated GIACs ($\Delta A_{460-665}$) that occurred instantaneously (Fig. 1A) and that corresponded to a decrease of the absorbance at 665 nm (or increase of the absorbance at 460 nm). When the sporangiophores were placed again vertically, the GIAC signals reverted instantaneously to the prestimulus level. A mutant with abnormal gravitropism, A909 *madJ*, was lacking the tilt-induced GIACs (Fig. 1B). To show that the GIACs were specific for graviperception further control experiments were done with (1) card board of various colors, (2) white plastic-coated wires, (3) agravitropic mycelia of *P. blakesleeanus*, and (4) with sporangiophores of the wild type that had been killed by dunking them for 2 min in ethanol. In none of these control experiments did we obtain a GIAC signal after tilting (data not shown).

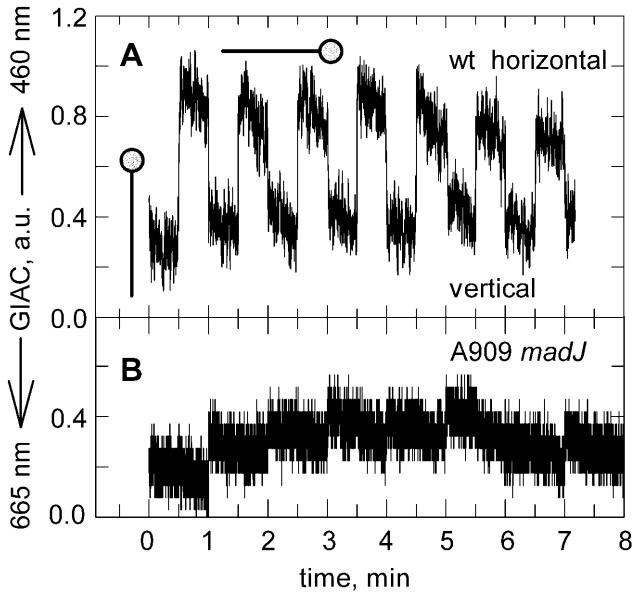

Figure 1. GIACs ($\Delta A_{460-665}$) of sporangiophores of *P. blakesleeanus* occurring in response to a change from the vertical to the horizontal position. Sporangiophores were tilted up and down every 30 s. A, Wild-type NRRL1555. B, Gravitropism mutant A909 *madJ*. Arrows indicate the relative absorbance changes (which were measured through reflection changes, see "Materials and Methods"). Upward arrow, Relative increase of the absorbance at 460 nm or decrease of the absorbance at 665 nm. Downward arrow, Relative increase of the absorbance at 665 nm or decrease of the absorbance at 460 nm. Units for the GIACs are arbitrary.

GIACs occurred also in response to gravitropic stimulation by centrifugal acceleration. Sporangiophores were placed horizontally into the MDWS and were then centrifuged together with the MDWS and the aluminum housing in the human centrifuge of the DLR at Köln-Porz (see "Materials and Methods"). The sporangiophores were positioned horizontally, i.e. parallel to the floor of the swing-out cabin of the centrifuge. The centrifugal stimuli were increased stepwise every 30 s by 0.5*g* until a stimulus level of 6.5*g* was reached (Fig. 2A). The results displayed in Figure 2B show that the GIAC signals ($\Delta A_{460-665}$) of wild-type sporangiophores decreased at the moment when the centrifugal stimuli were increased. The decrease of the GIAC signal corresponds to an increase of the absorbance at 665 nm. The GIACs showed no further decrease near 5*g*; apparently the response was saturated at these high stimulus levels. As was the case with the tilting experiments (Fig. 1B), the gravitropism mutant, A909 *madJ*, lacked GIACs even at elevated centrifugal accelerations (Fig. 2C). The GIACs that were obtained for wild-type sporangiophores did not change in magnitude when the centrifugal acceleration was maintained for prolonged time. Even for elevated *g*-values (1.8–3*g*) that lasted several minutes the GIACs maintained a constant magnitude, i.e. the GIACs did not adapt during this time to the prestimulus level (data not shown).

Generation of Micro- and Hypergravity during Parabola Flights

Micro- and hypergravity were generated during parabola flights with the Airbus ZERO G stationed at the International Airport at Bordeaux/Merignac, France, and operated by the company Novespace (Merignac, France). The flights were organized by the European Space Agency (32nd ESA Campaign, March 2002) and the Deutsches Zentrum für Luft- und Raumfahrt, respectively (DLR Campaigns, October 2002 and June 2003). The trajectory of a flight parabola and the flight characteristics are shown in Figure 3; at the height of about 6,000 to 7,000 m the Airbus A300 ZERO G flies horizontally at a maximum speed of 850 km h^{-1}. The plane is then pulled up at a pitch angle of 47 degrees. This phase of the flight lasts about 20 s during which hypergravity of 1.8*g* is generated. The trajectory of the airplane in the pull-up and the pull-out phase is adjusted in such a way that the 1.8*g* vector is always pointing vertically on the floor of the airplane, i.e. parallel to the plumbline; as a result, experimenters can stand upright during the 1*g* and the 1.8*g* phases without requiring additional support or body adjustment. During the subsequent 22 to 25 s the plane flies the actual parabola; in this phase the plane

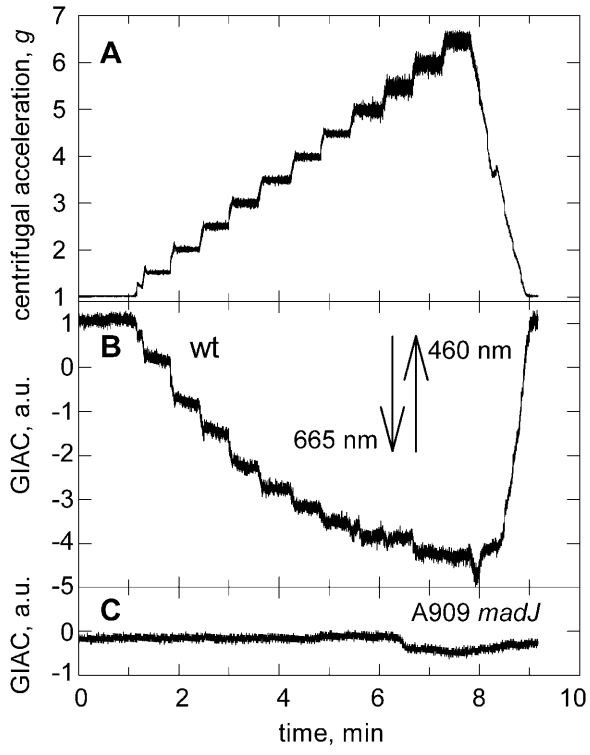

Figure 2. GIACs ($\Delta A_{460-665}$) of sporangiophores of *P. blakesleeanus* occurring in response to centrifugal acceleration. Sporangiophores were placed horizontally relative to the swing-out cabin of the centrifuge and were then subjected to increasing centrifugal accelerations that were every 30 s increased stepwise by 0.5*g*. A, Centrifugal acceleration. B, Wild-type NRRL1555. C, Gravitropism mutant A909 *madJ*. The stimulus program of the mutant was identical to the one of the wild type and is omitted here. Arrows as in Figure 1.

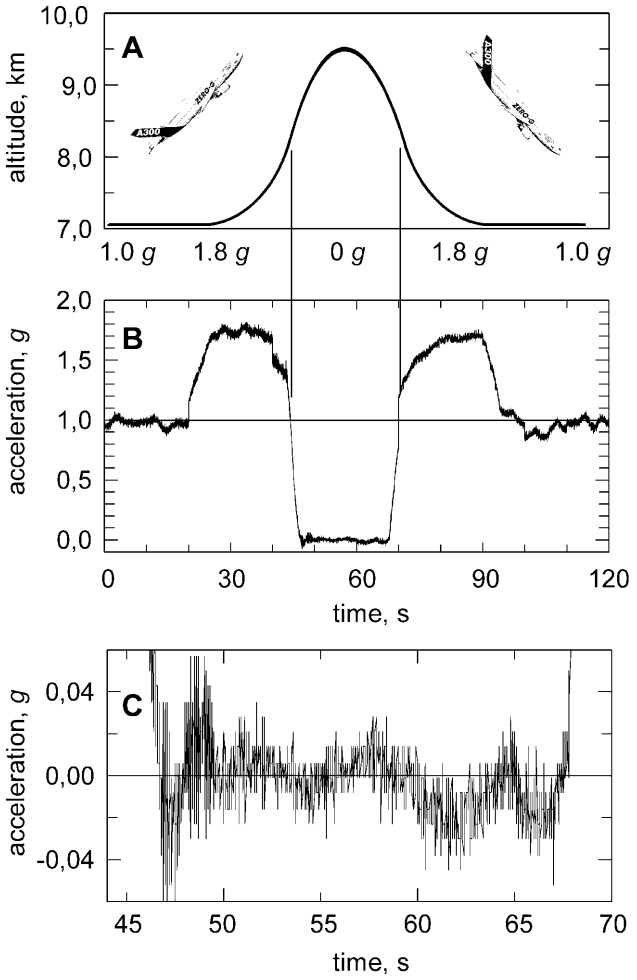

Figure 3. A, Trajectory of the airbus A300 ZERO G during a parabola flight. The vectors of the 1*g* and the 1.8*g* accelerations are always parallel to each other and to the plumb line, i.e. 90° relative to the floor of the airplane. B, Gravitational acceleration during flight parabolas expressed as multiples of *g* (9.82 m s^{-2}). C, *g*-values during microgravity (blow up of Fig. 3B).

is in engine-controlled free fall (i.e. compensating for the drag) and one experiences thus weightlessness, i.e. microgravity (Fig. 3, A and B). During the subsequent pull-out phase the plane is subjected to another 20 s of hypergravity (1.8*g*) and approaches again the horizontal flight path. Between flight parabolas the airplane picks up speed for about 3 min of horizontal flight, during which time one experiences normal earth gravitational acceleration (1*g*; Fig. 3B). We monitored during parabola flights the actual accelerations as multiples of the earth acceleration, *g* (9.82 m s^{-2}). It can be seen from Figure 3C that during the microgravity phase the residual acceleration was in the order of $0 \pm 3 \times 10^{-2}g$.

GIACs Elicited during Parabola Flights

During three flights we monitored in parallel the actual *g*-values experienced during the flight parabolas and also the GIACs ($\Delta A_{460-665}$) of *P. blakesleeanus* sporangiophores that were elicited as a result of the changing *g*-values. The GIACs were detected with the same MDWS that was employed for the experiments described above. To detect substantial GIACs it was necessary to place the sporangiophores horizontally, i.e. parallel to the floor of the airplane. We found that hypergravity induced an sudden decrease of the GIACs ($\Delta A_{460-665}$), which corresponds to an increase of the absorbance at 665 nm. In contrast, microgravity induced an increase of the GIACs ($\Delta A_{460-665}$), which corresponds to a decrease of the absorbance at 665 nm. (Fig. 4B). A blow-up of the graphic of one of these parabola experiments shows that the responses occurred practically instantaneously (Fig. 5). The latency for these GIACs was in the order of 20 ms or shorter (the present detection limit of the MDWS).

In contrast to horizontally placed sporangiophores, vertical ones displayed GIACs that were only barely detectable (Fig. 6B). GIACs were again absent in the gravitropism mutant, A909 *madJ* (Fig. 6C), irrespective of whether horizontal or vertical sporangiophores were employed.

Threshold Determination for GIACs and Hysteresis

The threshold for gravitropic bending of sporangiophores of *P. blakesleeanus* is near $3 \times 10^{-2}g$ (Galland et al., 2004; Fig. 7A). If the GIACs represent primary

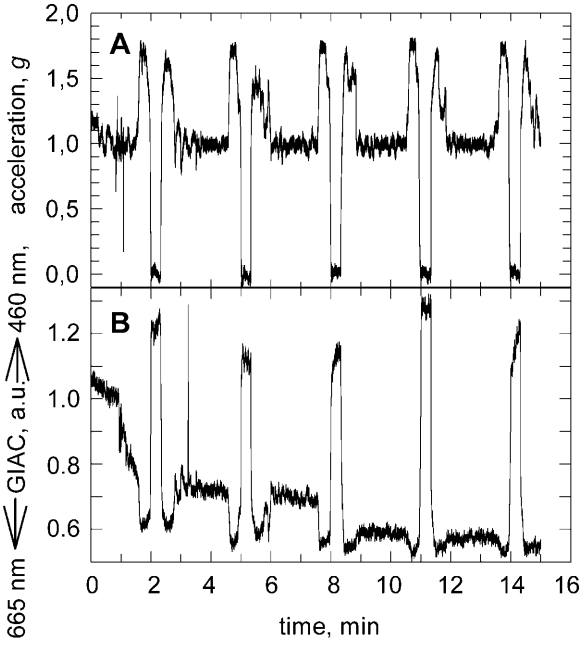

Figure 4. GIACs ($\Delta A_{460-665}$) of sporangiophores of the wild type of *P. blakesleeanus* occurring during flight parabolas. Sporangiophores were placed horizontally, i.e. parallel to the floor of the airplane. A, Change of the gravitational acceleration (*g*) during five flight parabolas. B, GIACs ($\Delta A_{460-665}$) elicited by hyper- and microgravity. ESA campaign March 2002. Arrows as in Figure 1.

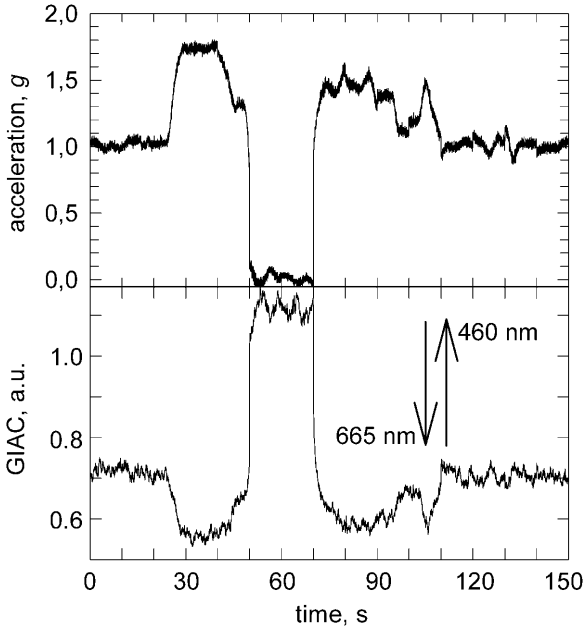

Figure 5. GIACs ($\Delta A_{460-665}$) of sporangiophores of the wild type of *P. blakesleeanus* occurring during one flight parabola (blow-up of the second parabola shown in Fig. 4B). Arrows as in Figure 1.

responses for the graviperception then the threshold for eliciting them should be in the same order of magnitude as that for the gravitropic bending or even lower. The threshold for the GIACs were obtained from the kinetic data shown in Figures 2 and 4 by plotting the GIACs in dependence of the corresponding *g*-values and by omitting the parameter time. Figure 7B shows the results obtained during one flight parabola. The black symbols show the GIAC values for the first one-half of the flight parabola, which includes the first period of hypergravity (20 s) and the first one-half of the microgravity period (12 s). The white symbols display the GIAC values for the second one-half of the parabola, i.e. 12 s of microgravity followed by 20 s of the second hypergravity period. The shapes for the two resulting curves are substantially different and possess the typical feature of hysteretic behavior. The curve for the transition from hypergravity to microgravity indicates a threshold near $3 \times 10^{-2}g$, which is close to the threshold for gravitropic bending (Fig. 7A). The curve for the transition from microgravity to hypergravity has an ill-defined threshold; relative to the other curve it is, however, clearly shifted to elevated *g*-values.

When the same plotting procedure is applied to the data from the centrifuge experiments (Fig. 2) one obtains a similar hysteris curve (Fig. 7C). The GIAC values for the transition from 1 to 6.5*g* (white symbols; corresponding to the stepwise increase of *g* in Fig. 2A) are shifted relative to the curve that was generated from the transition from 6.5 to 1*g* (black symbols; corresponding to the monotonous decrease of *g* in Fig. 2A).

DISCUSSION

The optospectroscopic detection of gravity-induced absorbance changes represents a convenient technique to probe into the early molecular events that are associated with graviperception (Schmidt and Galland, 2000). The technique is noninvasive and thus very well suited for in vivo experiments. Because GIACs are by no means restricted to *P. blakesleeanus* but also occurring in coleoptiles of *Avena* and seedlings of Arabidopsis (data not shown), it is likely that this technique can be applied to a wide range of different organisms.

Specificity of GIACs and Directionality of the Gravitational Stimulus

The fact that the GIACs are absent in the gravidefective mutant A909 *madJ* and in dead sporangiophores of the wild type indicates that they are specific for graviperception and that they are not spurious byproducts of gravistimulation or instrumental artifacts. Also the observation that the GIACs are expressed well only in horizontal sporangiophores, not however, in vertical ones (Figs. 4 and 6B) supports the notion that the GIACs are specific for the transduction chain of gravitropism. The residual GIACs that were detected in vertical sporangiophores during flight parabolas (Fig. 6B) are best explained by the observation that the sporangiophores deviate to some extent from the plumb line so that a gravistimulation should take place in accordance with the classical sine-law

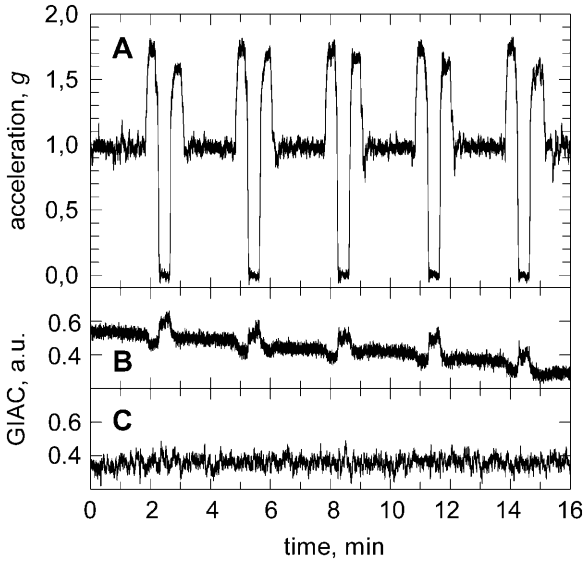

Figure 6. GIACs of sporangiophores of the wild type of *P. blakesleeanus* and mutant A909 *madJ* occurring during flight parabolas. A, Change of the gravitational acceleration (*g*) during five flight parabolas. B, GIACs of sporangiophores of wild-type NRRL1555 that were placed vertically relative to the floor of the airplane. C, GIACs of sporangiophores of mutant A909 *madJ* that were placed vertically relative to the floor of the airplane. ESA campaign March 2002.

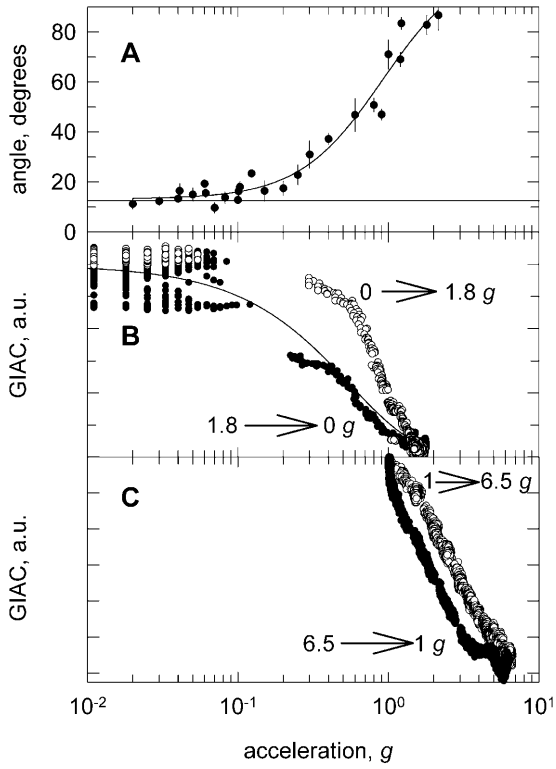

Figure 7. Determination of the threshold for GIACs elicited by hyper- and microgravity. The data of Figures 2 and 4 were replotted by showing the size of the GIACs in dependence of the gravitational acceleration *g* during centrifugal acceleration and parts of one flight parabola, respectively. A, Threshold of gravitropic bending as determined with a clinostat-centrifuge. Fitted to the function: $y = y_0 + a\,[1 - \exp(-bx)]$ (modified after Galland et al., 2004). B, GIACs occurring in a flight parabola (data replotted from parabola 3 shown in Fig. 4). Fitted to the function: $y = y_0 + a\,\exp(-bx)$. C, GIACs occurring during centrifugal acceleration between 1 and 6.5 g (data replotted from Fig. 2B). Arrows as in Figure 1.

according to which the gravitropic stimulus is proportional to the sine of the inclination angle (deviation from the vertical) (Sachs, 1882). We have shown recently that the sporangiophores of *P. blakesleeanus* obey the sine law for inclination angles ranging from 0° to 90° (Galland et al., 2002).

Very pertinent for understanding the nature of the gravitropic signal is the observation that GIACs elicited by tilting differ with respect to their sign from those that are elicited by centrifugation or acceleration changes during flight parabolas. Tilting sporangiophores from the vertical to a horizontal position causes an increase of the GIACs (equivalent to a decrease of A_{665} and an increase of A_{460}; Fig. 1), while an increase of acceleration elicits in horizontal sporangiophores a decrease of the GIACs (equivalent to an increase of A_{665} and a decrease of A_{460}; Figs. 2, 4, and 5). Even though both of these types of gravitropic stimulation cause negative gravitropic bending, the signs of the GIAC signals are reversed relative to each other. The puzzling behavior indicates then that sporangiophores discriminate between stimuli that involve a change of stimulus direction and those that involve the maintenance of stimulus direction. If one assumes that vertical sporangiophores perceive a symmetric gravitropic stimulus, then the tilting experiment implies that sporangiophores were subjected to a change from symmetric (vertical) to asymmetric gravitational stimulation (horizontal). If one assumes on the other hand that vertical sporangiophores do not perceive a gravitropic stimulus, then the tilting experiments imply that sporangiophores underwent a change from no stimulation (vertical) to asymmetric gravitational stimulation (horizontal). The question thus arises how one can distinguish between the two models and whether the sporangiophore in the vertical position perceives a symmetric gravitational stimulus or none at all.

In contrast to the tilting experiments, centrifugation and flight parabolas provided gravitational stimulation for sporangiophores that had been in a horizontal position even prior to the stimulation so that no further change of stimulus direction but only a change of stimulus size was involved. With respect to their sign the GIACs obtained by tilting (vertical to horizontal, Fig. 1) are equivalent to those that are elicited by a decrease of acceleration, for example, from 1.5 to 1*g* (Fig. 2) or from 1.8 to 1.0 and from 1.8 to 0*g* (Figs. 4 and 5). Tilting from vertical to horizontal is, therefore, tantamount to a step-down stimulus. From this observation one can conclude that vertical sporangiophores are experiencing permanently a symmetric gravitational stimulation that exceeds that experienced in the horizontal position. The paradoxical sign reversal associated with the two types of gravitropic stimulations indicates then that a vertical positition is subjectively perceived as symmetric gravitational stimulation rather than no stimulation at all.

Primary Response and Latency

The GIACs ($\Delta A_{460-665}$) described in this work represent the fastest gravi-induced responses so far reported for plants, fungi, and protists. Because the time resolution of the MDWS is presently 20 ms, it is quite possible that the true latency of the GIACs is even shorter than this value. The GIACs found in sporangiophores of *P. blakesleeanus* are thus even faster than the change of the swimming velocity of *Paramecium* when it is subjected to microgravity; the latency for this reaction (gravikinesis) is in the order of 250 ms (Bräucker et al., 1998). The GIACs of *P. blakesleeanus* are also faster than electrical responses that have been measured upon gravistimulation in plant roots (Weisenseel and Meyer, 1997; Monshausen and Sievers, 2002). The fastest electrophysiological signals that are associated with graviperception were observed in roots of *Lepidium* after 8 (Behrens et al., 1985) and 30 s (Behrens et al., 1982), respectively. Some of the electrophysiological responses elicited by gravistimulation, e.g. in roots of maize (*Zea mays*; Björkman and

Leopold, 1987) or *Lepidium* (Sievers et al., 1995) and in bean (*Phaseolus vulgaris*) epicotyls (Shigematsu et al., 1994), display substantially longer latencies in the range of minutes. The gravitropism of Arabidopsis roots is associated with cytosolic pH changes in columella cells that occur within 55 s (Scott and Allen, 1999).

The fact that the GIACs occur quasi-instantaneously in *P. blakesleeanus* indicates that they occur very early in the transduction chain and that they represent primary responses of graviperception. The latency of sporangiophores of *P. blakesleeanus* for gravitropic bending is in the order of 10 to 30 min (Dennison and Shropshire, 1984). The latencies for the GIACs of *P. blakesleeanus* that we measured previously with the RSS were in the order of some 10 to 15 min (Schmidt and Galland, 2000). The huge apparent discrepancy is explained by the fact that the MDWS employed in this work is about 100 to 1,000 times more sensitive than the RSS employed previously by us (see introduction).

Primary Response and Thresholds

A criterion for a primary response is the notion that its threshold should be equal to or be even lower than the threshold of the physiological response that it is mediating. The threshold for the GIACs during parabola flights was near $3 \times 10^{-2}g$ (Fig. 7A). The real value may be even lower, because the microgravity aboard the airplane is near $0 \pm 3 \times 10^{-2}g$ (Fig. 3) and thus possibly masking lower thresholds. However, even if we accept for the moment the estimate of $3 \times 10^{-2}g$, one needs to take into account that gravitropic thresholds can be determined in two fundamentally different ways.

The threshold for gravitropic bending (Fig. 7A) was determined with a clinostat centrifuge that provided gravitropic long-term stimulation for 5 h (Galland et al., 2003, in press). In such an experiment the response system has come into an equilibrium and the gravitropic stimulus is time independent, which is demonstrated by the fact that an 8-h stimulation generates practically the same response curve and threshold as a 5-h stimulation (Galland et al., 2003, in press). The situation is very different when short gravitropic stimuli in the order of seconds or minutes are applied. For such short-term experiments one can define a gravitropic dose, D, which is the product of acceleration, g (9.82 m s^{-2}) and stimulus time, t: $D = g \times t$ (Volkmann and Sievers, 1979). In experiments done with *Avena* coleoptiles reciprocity is valid between 0.2 and 1g and stimulation times between 2 and 65 min (Johnsson et al., 1995). Broader ranges for g-values between 0.09 and 12.5g have also been reported (Johnsson, 1965; Shen-Miller, 1970). Dose-response curves that are generated for gravitropic bending yield a threshold dose, which represents the gravitropic sensitivity (1/threshold dose). For the gravitropism of *Avena* coleoptiles the threshold dose was found in earth-bound experiments to be between 30 to 120g s (Johnsson, 1971; Johnsson et al., 1995); for roots of Arabidopsis values of 30g s were determined (Caspar and Pickard, 1989). Space experiments involving centrifugation in microgravity yielded for *Avena* coleoptiles threshold doses between 36 to 94g s (Brown et al., 1995; Johnsson et al., 1995) and for lentil seedling roots one of 26g s (Perbal and Driss-Ecole, 1994). For the gravitropism of *P. blakesleeanus*, similar data for the threshold dose are presently not available. It is, however, very likely that the threshold dose for the gravitropism of *P. blakesleeanus* would exceed about 10-fold that of *Avena*. This can be inferred from the observation that *Avena* coleoptiles display for long-term stimulation on the clinostat-centrifuge a threshold that is 10-fold lower than that of *P. blakesleeanus* (P. Galland, unpublished results), i.e. *Avena* is gravitropically 10 times more sensitive than *P. blakesleeanus*.

The thresholds that we determined in the parabola-flight experiments were obtained on the basis of short-term stimuli which lasted only 20 s (hyper- and microgravity). Because the GIACs elicited during flight parabolas occur practically instantaneously, a gravitropic stimulus lasting for a duration as short as 0.1 s (minimum 20 ms) is sufficient to elicit a response. On the basis of the data shown in Figure 7B one obtains for *P. blakesleeanus* a threshold dose for the GIACs of $3 \times 10^{-3}g$ s ($3 \times 10^{-2}g \times 0.1$ s). This threshold dose is four orders of magnitude smaller than that for gravitropism of *Avena* coleoptiles and five orders of magnitude smaller than the inferred threshold dose for the gravitropic bending of *P. blakesleeanus*. Apparently, short gravitropic stimuli that elicit GIACs are nevertheless subliminal with respect to gravitropic bending. The huge difference between the threshold dose for the GIACs and that for the concomitant gravitropic bending requires that the subliminal stimuli are summated over time to reach the critical threshold dose for gravitropic bending. The capacity of plant shoots and roots for response summation is a phenomenon that is well-established since the beginnings of gravitropism research (Fitting, 1905; Günther-Massias, 1928; Volkmann and Sievers, 1979; Kiss et al., 1989). In *Avena* coleoptiles, for example, short stimuli of 0.5 s duration can be summated to generate a stimulus eliciting gravitropic bending, an observation that led to the prediction that graviperception involves a process that starts nearly instantly after gravitropic stimulation (Pickard, 1973). We believe that the GIACs represent this instant process that previous authors have predicted on the basis of experiments with intermittent stimuli.

The extremely small threshold dose of $3 \times 10^{-3}g$ s that we determined for *P. blakesleeanus* is by far the smallest ever detected to date. The question thus arises whether or not the known gravisusceptors of *P. blakesleeanus* could possibly provide the force and the potential energy to account for such a great sensitivity. The buoyancy of lipid globules and the sedimentation of vacuolar protein crystals are contributing to the graviperception of *P. blakesleeanus* (see introduction).

The force that is generated at 1g by these particles is in the order of 10^{-10} to 10^{-11} *N*, and the potential energy is about 10^{-16} to 10^{-17} J and thus four to five orders of magnitude above the thermal noise (3/2 kT = 6.21×10^{-21} J at 300 K; Schimek et al., 1999; Grolig et al., 2004). At the threshold for gravitropic bending near $3 \times 10^{-2}g$ (Fig. 7A) these cell organelles generate a potential energy that is still about two orders of magnitude above thermal noise. The force and potential energy are, however, generated instantaneously and are, therefore, available even during the short stimulus durations prevailing during the flight parabolas (Fig. 7B).

The gravitropic threshold of *P. blakesleeanus* (in response to long-term centrifugation) is about 5 times lower than that of the phytoflagellate, *Euglena gracilis*, which has an absolute threshold of about $1.6 \times 10^{-1}g$ (Häder et al., 1996, 1998). Substantially more sensitive are roots of *Lepidium*, having a threshold of about $10^{-3}g$ (Sobick and Sievers, 1979). The acceleration-sensitivity threshold for the modification of rhythmic contraction of the slime mold *Physarum polycephalum* is about $10^{-1}g$ (Block et al., 1996). The gravitaxis of the ciliate, *Paramecium biaurelia*, has a threshold of 1.6 to $3 \times 10^{-1}g$ (Hemmersbach et al., 1996). The intermediate gravitropic sensitivity of *P. blakesleeanus* seems nevertheless to provide for an adequate ecophysiological adaptation to its natural environment. The fact that the threshold dose (acceleration × time) for the GIACs of *P. blakesleeanus* is several orders of magnitude below that of gravitropic bending is suggesting that the primary responses of other gravi-sensitive organisms too may possess threshold doses for the respective primary responses that are far lower than those for gravi-orientation (bending or swimming).

The threshold curves for the GIACs that were obtained between 10^{-2} and $1.8g$ (Fig. 7B) and 1 and $6.5g$ (Fig. 7C) show a hysteretic pattern. The branches of the curves that were obtained for decreasing stimulation (6.5–1*g*, and 1.8–$10^{-2}g$) are displaced along the *x* axis to smaller *g*-values relative to those branches obtained for increasing stimuli (1–6.5*g*, and 10^{-2} to $1.8g$). In formal terms, a displacement to lower *g*-values indicates sensitization while a displacement to higher *g*-values indicates desensitization. At the moment it remains an empirical but interesting observation that the graviperception system for the GIACs is most sensitive after a strong gravitropic stimulus, i.e. after 6.5 or $1.8g$. The observation is pertinent in the context of light perception, because it is well established that strong light stimuli decrease the light sensitivity of *P. blakesleeanus* or to that effect of most light-sensitive organisms (Galland, 1989, 1990).

Synergism between Photo- and Graviperception: A Model for the Generation of GIACs

To be able to measure GIACs it is essential to employ measuring light emitting diodes (LEDs) that include blue light. When green and red LEDs were employed as measuring lights, no GIACs or only very minute ones could be detected (data not shown). A further prerequisite for the detection of GIACs is that the irradiances provided by the blue (460 nm) and red (665 nm) LEDs exceeded a critical value of about 1 μmol m^{-2} s^{-1} (data not shown). Both of these observations indicate that the gravitational stimulus modulates a pool of pigments that are under the control of blue light. Blue, not however red, light elicits absorbance changes (LIACs) in sporangiophores of *P. blakesleeanus* that occur at the level of the blue-light photoreceptor. The reaction scheme of the LIACs implies a blue-light induced photoreduction of the oxidized flavin photoreceptor to the flavo-semiquinone state (half-reduced state; Schmidt and Galland, 1999).

The data presented in this work can be best explained by the assumption that gravistimulation modulates the pool of these flavo-semiquinones that need to be present in darkness and that are substantially boosted by blue-light irradiation. Evidence for the presence of such flavo-semiquinones in darkness and particularly after blue-light irradiation has been presented recently for *P. blakesleeanus* (Galland and Tölle, 2003). We thus propose that graviperception involves and largely depends on the blue-light photoreceptor system. The observation that the gravitropism mutant A909 *madJ* lacks GIACs provides further support for this notion. A909 *madJ* is not only defective for gravitropism but possesses in addition a phototropic threshold that is elevated by three orders of magnitude (Grolig et al., 2000) and a very abnormal phototropic action spectrum that is indicative for a greatly altered blue-light photoreceptor (Campuzano et al., 1996). The gravitropic deficiency of this mutant appears thus to be caused be the defective photoreceptor system.

MATERIALS AND METHODS

Strains

The standard wild-type strain of *P. blakesleeanus* (Burgeff) NRRL1555 (−) was originally obtained from the Northern Regional Research Laboratories (Peoria, IL; Bergman et al., 1973). The mutant A909 *madJ407* (−) was derived from NRRL1555 after mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Campuzano et al., 1994); it is defective in phototropism, gravitropism, and the avoidance response (Campuzano et al., 1996).

Growth Conditions

Sporangiophores were grown and employed for experiments in glass shell vials (1 cm diameter × 4 cm height; Flachbodengläser, AR Klarglas, Münnerstädter Glaswarenfabrik, Münnerstadt, Germany) on a synthetic solid medium (Sutter, 1975). Until the appearance of stage-4b sporangiophores (i.e. with sporangium) of 2.5 cm length the material was kept in transparent plastic boxes at ambient temperature (19–21°C) under white incandescent light fluence rate (0.5 Wm^{-2}).

Human Centrifuge of the DLR (Köln-Porz)

To elicit GIACs in sporangiophores of *P. blakesleeanus* at hypergravity (1.5–6.5*g*) we employed the so-called human centrifuge of the DLR at Köln-Porz (Germany). The centrifuge is devised for hypergravity experiments with

human subjects. The centrifuge possesses a horizontally rotating arm of 5 m length and a swing-out cabin (1.6 m × 1 m × 1.5 m height) that assures that the floor of the cabin is always oriented at a 90° angle relative to the vector of centrifugal acceleration (similar to a swing-out rotor in commercial centrifuges). For measuring GIACs of sporangiophores under continuous stimulation we mounted the MDWS in the aluminum expedition box (see above) on the floor of the cabin. Sporangiophores were tilted horizontally so that they were positioned parallel to the floor of the expedition box and the floor of the cabin. This way the vector of centrifugal acceleration was always perpendicular to the long axis of the sporangiophores. The centrifuge was operated under remote control according to preselected parameters determining the time course and stimulus levels.

Micro-Dual Wavelength Spectrometer

The MDWS is an improved version of an earlier dual-wavelength spectrometer (Schmidt, 1980). The set up for measuring GIACs and the novel version of the MDWS has been published in detail elsewhere (Schmidt, 2003). It should be emphasized that the MDWS is employed in the reflectance mode so that it measures physically reflection and reflection changes rather than absorbance as is suggested by the name. The relative absorbance, A, is calculated from the reflected light according to the following definition: $A = \log R_{460}/R_{665}$, where R_{460} is the reflectance at 460 nm and R_{665} the reflectance at 665 nm.

The components of the MDWS and the sporangiophores of *P. blakesleeanus* were kept in a conventional aluminum expedition box (56 cm legth × 36 cm width × 41 cm height). One glass shell vial containing the solid growth medium and about 5 to 10 stage-4 sporangiophores (with sporangium) was mounted vertically within a light-tight black plastic box (10 cm × 10 cm × 14 cm height) that could be tilted and fixed either in a vertical or a horizontal position. A trifurcated mixed glass fiber entering the black plastic box provided the measuring lights at wavelengths 460 and 665 nm generated by two rectangularly alternating LEDs operating at 1 kH. The photon-fluence rates generated by the LEDs at the site of the sporangiophores could be adjusted between 0 to 6.5 μmol m^{-2} s^{-1} (460 nm) and 0 to 7.4 μmol m^{-2} s^{-1} (665 nm). The irradiated area had a diameter of about 20 mm and included the upper half of the sporangiophores and thus their growing zones. The light that was reflected from 10 to 15 irradiated sporangiophores was received by the glass fiber and guided to the mini-photomultiplier. The signal was processed by a lock-in amplifier that obtained its reference voltage from the electronics that generated the alternating voltage operating the LEDs. After conversion to a DC-signal by a low-pass filter the signal was fed to a miniaturized AD-converter card in a notebook.

The triple-branched light fiber (Schölly, Denzlingen, Germany) of 1 m length (material SUV) transmits light in the range from 400 nm to 870 nm (60%). Its individual light fibers are mixed to ensure homogeneous light distribution on sample and photomultiplier cathode. The adapters for connecting the light fibers to the LEDs and the miniature photomultiplier module were custom made by our machine shop, allowing easy exchange of LEDs (colors). The miniature photomultiplier module H5784 by Hamamatsu includes a red extended photomultiplier with 8 mm diameter photocathode (02-type). It requires a moderate voltage supply of ±11.5 to 15.5 V and a high voltage (HV) control voltage of only ±0 to 1.0 V (resulting in an internal HV of 0–1,000 V). The voltage output impedance of the preamplifier is maximally 100 Ohm.

The single board miniature lock-in amplifier LIA-BV-150 by FEMTO (Berlin) has a working frequency ranging from 5 Hz to 10 kHz. The control electronics chopping the LEDs by alternating rectangular pulses capable of covering the whole frequency range acceptable by the LIA was operated at 2,000 Hz. This frequency optimizes the signal to noise ratio in the current optical reflection experiment (lock-in process versus 1/f noise). The time constant of the low pass filter of the LIA was set to 0.03 s. The specific program for acquiring, displaying, and storing GIAC-data was written by Kay Dörnemann (Philipps-Universität, Marburg, Germany) in the programming language JAVA (SUN, Santa Clara, CA) Files were stored in text file format to be analyzed by common evaluation programs such as Excel, Origin, or SigmaPlot.

To monitor during parabola flights accelerations in the vertical and in the flight direction the aluminum housing of the MDWS was equipped with two 2-*g* sensors (ADXL202, Analog Devices, Edinburgh). The signal output of these sensors was handled by the same software and stored on the hard disc of the notebook.

Measurement of Irradiances

Fluence rates were determined with a UV-enhanced photodiode (BN-9102-4, Gigahertz-Optik, Puchheim, Germany) and a calibrated readout instrument (Optometer P-9201, Gigahertz-Optik).

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