Planta (1997) 202: 149–154

Planta

A positively gravitropic mutant mirrors the wild-type protonemal response in the moss *Ceratodon purpureus*

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Received: 4 November 1996 / Accepted: 26 November 1996

Abstract. Wild-type *Ceratodon purpureus* (Hedw.) Brid. protonemata grow up in the dark by negative gravitropism. When upright wild-type protonemata are reoriented 90°, they temporarily grow down soon after reorientation (“initial reversal”) and also prior to cytokinesis (“mitotic reversal”). A positively gravitropic mutant designated *wrong-way response* (*wwr-1*) has been isolated by screening ultraviolet light-mutagenized *Ceratodon* protonemata. Protonemata of *wwr-1* reoriented from the vertical to the horizontal grow down with kinetics comparable to those of the wild-type. Protonemata of *wwr-1* also show initial and mitotic reversals where they temporarily grow up. Thus, the direction of gravitropism, initial reversal, and mitotic reversal are coordinated though each are opposite in *wwr-1* compared to the wild-type. Normal plastid zonation is still maintained in dark-grown *wwr-1* apical cells, but the plastids are more numerous and plastid sedimentation is more pronounced. In addition, *wwr-1* apical cells are wider and the tips greener than in the wild-type. These data suggest that a functional *WWR* gene product is not necessary for the establishment of some gravitropic polarity, for gravitropism, or for the coordination of the reversals. Thus, the *WWR* protein may normally transduce information about cell orientation.

Key words: *Ceratodon* (mutant) – Gravitropism – Mutant (gravitropism, moss) – Plastid – Polarity

Introduction

In protonemata of the moss *Ceratodon*, the entire gravitropic signal transduction pathway, from receptor to response, occurs in the same apical cell (Sack 1993). *Ceratodon* protonemata are negatively gravitropic; when grown in darkness protonemata grow upright by oriented tip growth. After reorientation from the vertical to the horizontal, the protonemata grow back up to the vertical in approximately 24 h (Walker and Sack 1990).

The gravitropic response of *Ceratodon* protonemata following reorientation normally includes two instances when upward growth is temporarily reversed. First, there is an initial reversal of curvature (IR), temporarily downward, prior to the start of upward curvature (negative gravitropism; Young and Sack 1992). Second, a temporary downward curvature precedes nuclear envelope breakdown when mitosis occurs during upward curvature. This mitotic reversal (MR) has been observed during protonemal gravitropism in the mosses *Physcomitrella, Ceratodon* and *Funaria,* (Knight and Cove 1991; Young and Sack 1992; Schwuchow et al. 1995). Upward curvature either arrests or reverses during mitosis and resumes after cell plate formation. Thus, protonemal gravitropism is a complex net growth response.

Mutational analysis may be useful as a means to elucidate the mechanism of moss gravitropism. Jenkins et al. (1986) isolated gravitropic mutants of *Physcomitrella* protonemata that fell into at least three complementation groups. Two groups had reduced gravitropism where curvature following reorientation was absent, slow, or aborted. In a third class (*gtrC*), the protonemata grew down (positive gravitropism), a phenotype attributed to a single, recessive locus. Knight et al. (1991) hypothesized that positive gravitropism resulted from the loss of *GTRC* function and was the default response. The temporary downward growth of the wild-type during the MR was hypothesized to result from a normal, temporary, inactivation of the *GTRC* gene product (Knight and Cove 1991). If so, positively gravitropic protonemata would not show reversals of curvature during mitosis or following reorientation. However, it has not been technically feasible to study the *gtrC* mutant of *Physcomitrella* with time-lapse video microscopy to test this hypothesis.

Abbreviations: IR = initial reversal; MR = mitotic reversal

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Recently, we isolated a positively gravitropic mutant of *Ceratodon* designated *wrong-way response* (*wwr-1*). This mutant was examined to determine its cytology, kinetics of gravitropism, and whether it had an IR and MR. We report here that the gravitropic response of *wwr-1* mirrors the wild-type in all aspects, including the IR and MR.

Materials and methods

Plant material and culture. The wild-type strain (WT3) of *Ceratodon purpureus* (Hedw.) Brid. used for experiments and stock culture conditions is described by Walker and Sack (1990). For experiments, the wild-type and *wwr-1* protonemal cultures were grown on modified Knop's medium with 1% (w/v) agar, 5 mM ammonium tartrate, 10 mM sucrose, 1 mM $MgSO_4 \cdot 7H_2O$, 1.84 mM KH_2PO_4, 10 mM KNO_3, 45 μM $FeSO_4 \cdot 7H_2O$, 0.17 μM KI, 0.22 μM $CuSO_4 \cdot 5H_2O$, 10 μM H_3BO_3, 0.23 μM $CoCl_2 \cdot 6H_2O$, 0.1 μM $Na_2MoO_4 \cdot 2H_2$, 0.2 μM $ZnSO_4 \cdot 7H_2O$, and 2 μM $MnCl_2 \cdot 4H_2$.

Mutant isolation. The positively gravitropic mutant (*wwr-1*) was isolated in a screen designed to identify phototropic mutants (Lamparter et al. 1996). In brief, 7-d-old WT3 *Ceratodon* protonemal fragments were irradiated for 5 min with UV at 0.4 $W \cdot m^{-2}$, a dose estimated to allow the survival of about 5% of the cells. After recovery, the tissue was transferred to darkness and incubated with the surface of the agar vertical for 7 d. Out of approximately 3×10^6 cells screened, one positively gravitropic filament was identified, transferred to a new petri dish, and cultured until the clone was phenotypically pure.

Kinetics. Protonemata were grown vertically in sterile plastic dished for 5–7 d in darkness because gravitropism is inhibited by most wavelengths of visible light (Young and Sack 1992). The dishes were then rotated 90° for 0–24 h and protonemata were chemically fixed in place for 1 h (Walker and Sack 1990). Curvature was measured from prints as the angle between the tip axis and the horizontal.

Time-lapse video microscopy. Protonemata were grown on a thin layer of agar medium in an autoclavable culture chamber (Adams and List, Westbury, N.Y., USA) attached to a microscope slide with a mixture of Vaseline, lanolin, and petrolatum. The chambers were placed vertically and kept in darkness for 5–7 d, then placed on a horizontally-mounted Photomicroscope I (Zeiss, Oberkochen, Germany) with a rotating stage. Protonemata were visualized with infrared light and a video camera as described in Young and Sack (1992). Sequences were recorded using time-lapse video recorders with real time compressed 300 or 506 times. Photographs were taken from the monitor every 10 min for the first hour following a 90° reorientation to determine if and when the IR occurred. Data on the MR were gathered directly from the monitor by observing the recorded sequences and by analyzing photographs taken from the monitor.

Results

Wild-type vs. wrong-way response (*wwr-1*) *gravitropism*. In darkness wild-type *Ceratodon* protonemata are negatively gravitropic, that is, they grow up the vertical surface of the agar (Fig. 1). In contrast, protonemata of the *wwr-1* mutant grow down (positive gravitropism, Fig. 1).

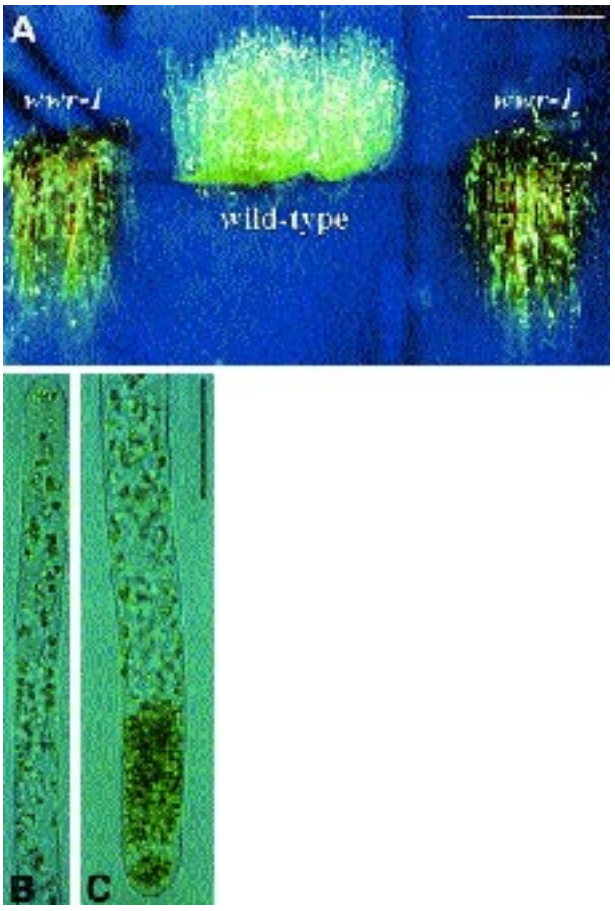

Fig. 1. **A** Cultures of wild-type and *wwr-1 Ceratodon* protonemata grown vertically on the same dish in darkness for 7 d. The wild-type protonemata grow up while the *wwr-1* protonemata grow down. The gravity vector is towards the bottom in this and all subsequent micrographs. **B** Apical cell of an upright wild-type protonema. **C** Downward growing *wwr-1* protonema. Note the increased cell width, shortened plastid-free zone, more numerous plastids, and the greener tip in *wwr-1* compared to the wild-type. 3.6, bar = 5 mm (**A**); 325, bar = 50 μm (**B, C**)

The time-course of downward curvature following a 90° reorientation was determined for populations of *wwr-1* and wild-type protonemata (Fig. 2). The kinetics for *wwr-1* protonemata were comparable to those for the wild-type indicating no impairment in the timing of the gravitropic response.

Gravitropic reversals. After a 90° reorientation of wild-type protonemata, an IR and MR, both temporarily downward, interrupt upward gravitropic curvature (Fig. 3; Young and Sack 1992). Nine out of ten divisions analyzed were accompanied by an MR.

Time-lapse analysis showed that *wwr-1* protonemata exhibited both types of reversal where curvature was temporarily upward. For example (Fig. 4), following reorientation, *wwr-1* first curved up (IR) at 30 min and then started to curve down (positive gravitropism). An

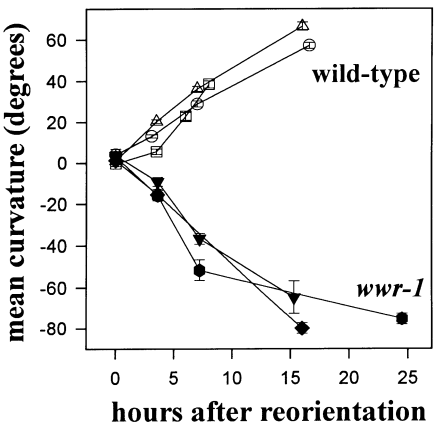

Fig. 2. Time course of gravitropic curvature for populations of *Ceratodon wwr-1* (*filled symbols*) and wild-type (*open symbols*) protonemata following reorientation to the horizontal for 0–25 h. Each curve indicates a separate experiment showing mean curvature for 8–25 protonemata ± SE (bars). Negative values reflect that curvature was downward (positive gravitropism) in *wwr-1*

IR occurred in 19 out of 25 series analyzed about 20 min after reorientation. The *wwr-1* IR was comparable in timing and magnitude to that of the wild-type (Fig. 3; Young and Sack 1992).

The mitotic reversal (MR) illustrated in Figs. 4 and 5 for a *wwr-1* protonema preceded cell division, and downward curvature resumed after the cell plate formed. In 18 out of 25 divisions the protonemata either grew upward (reversed direction) or stopped downward gravitropic curvature prior to cell division. Thus, the directions of gravitropism, the IR, and the MR in *wwr-1* were all opposite to those seen in wild-type protonemata.

Plastid zonation, sedimentation and protonemal appearance. Dark-grown wild-type protonemata have a characteristic plastid zonation (Fig. 3, zones 1–3 labeled at 1 h; Walker and Sack 1990). A plastid-free zone (zone 2) is present between non-sedimenting plastids present in the tip apex (zone 1) and plastids that sediment dramatically (zone 3).

For the most part, dark-grown *wwr-1* protonemata had a plastid zonation similar to that of the wild-type (Fig. 4, zones 1–3 labeled at 7 h), but the length of zone 2 and the number of plastids in zone 1 varied in *wwr-1*. The plastid-free zone was sometimes absent, but mostly it was very short. Thus, the sedimentation zone was closer to the tip in *wwr-1*.

Plastid sedimentation was striking in *wwr-1* protonemata and was first detected in zone 3 about 30 min after reorientation. Because of the high number of plastids in zone 3, especially adjacent to the plastid-free zone, as plastids sedimented, may accumulated on top of each other. This group of sedimented plastids appeared wedge-shaped in infrared video, and as the tip curved

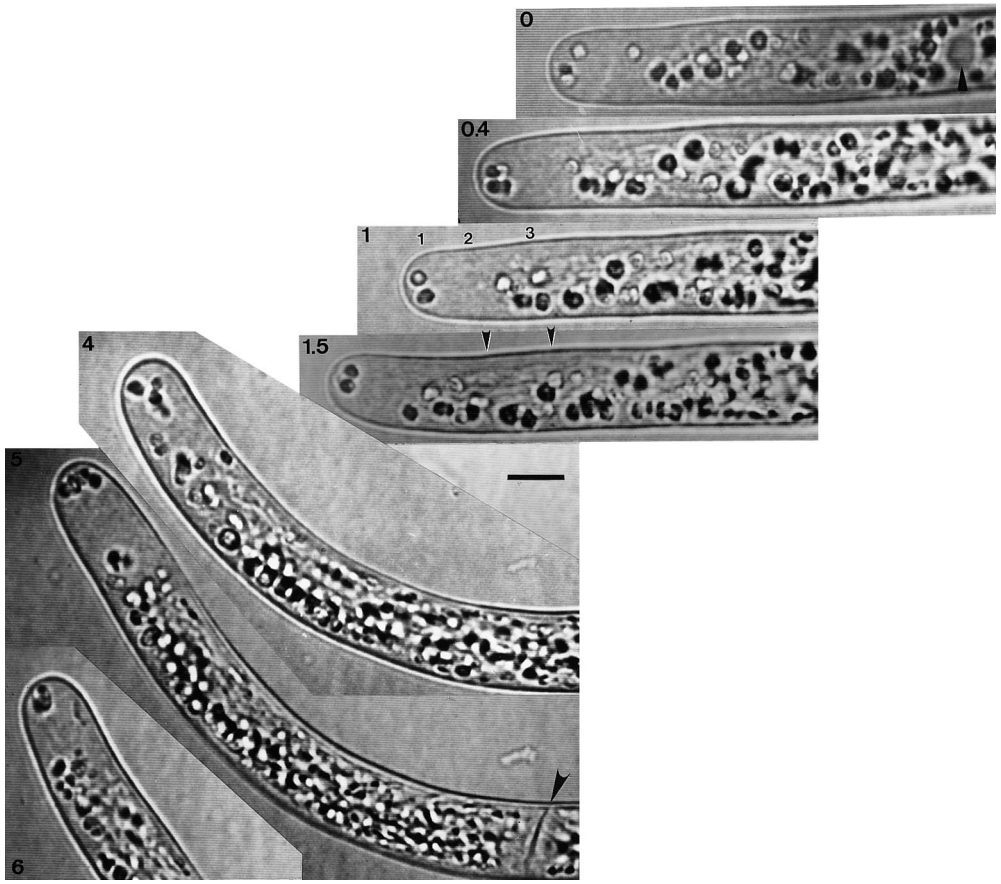

Fig. 3. Time-lapse series of upward gravitropism in wild-type *Ceratodon* protonema following horizontal placement. Sets of micrographs 0–1.5 h and 4–6 h are aligned to show growth. A slight initial reversal (IR) of the tip can be detected by the downward slope of the upper side of the tip (0.4 h). The downward curvature is retained at that position (between arrow heads at 1.5 h) even after upward growth. A mitotic reversal (MR) started between 4 and 5 h after reorientation. Note that the new cell wall (*arrowhead*) was complete at 5 h, and upward curvature had resumed by 6 h. Plastid zones 1–3 (see text) are labeled at 1 h. (Modified from Young and Sack 1992.); 150; bar = 50 μm

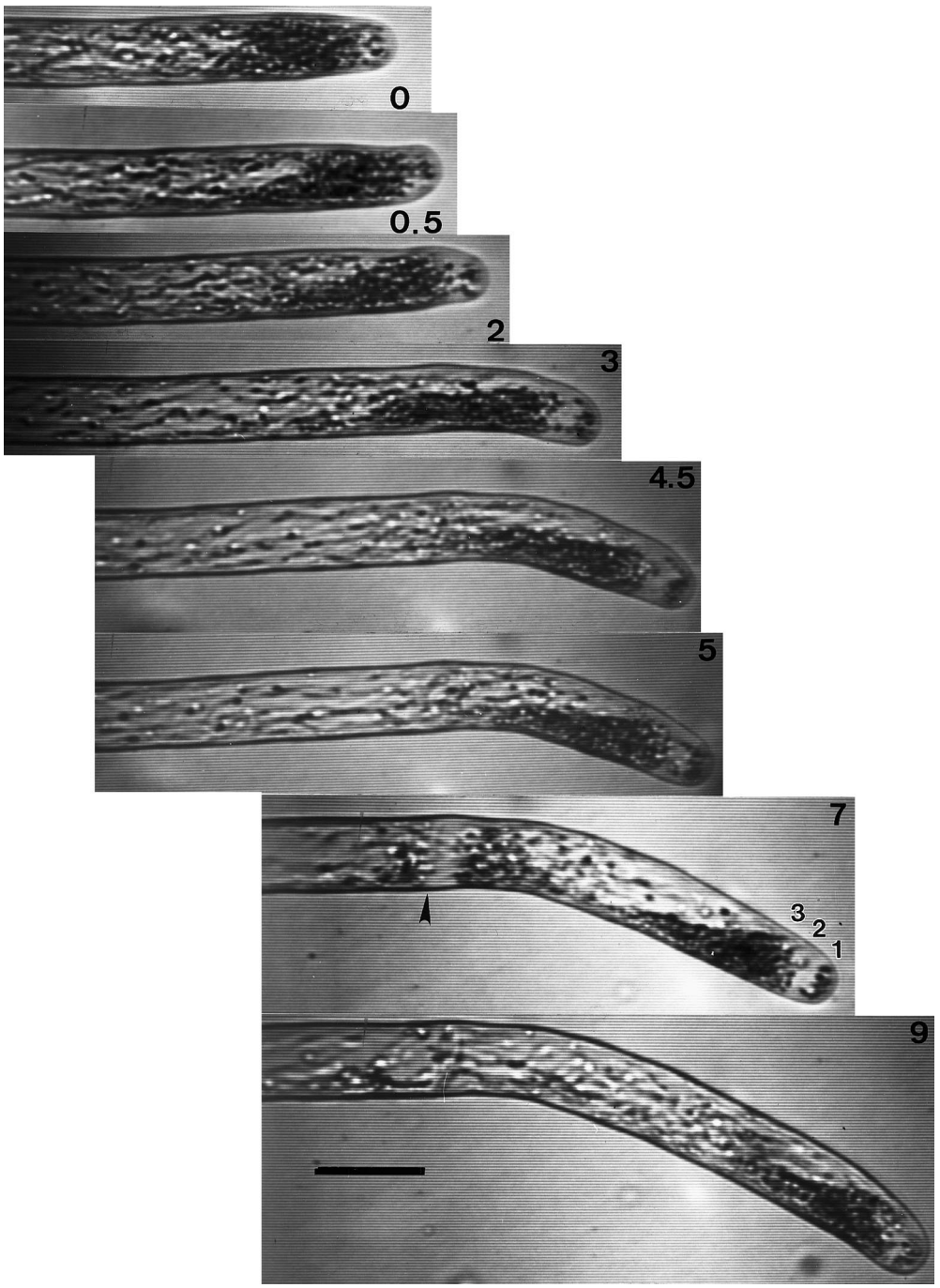

Fig. 4. Time-lapse series showing downward curvature of *wwr-1* protonema following reorientation. A clear upward IR can be detected 30 min after reorientation. An MR is seen at 5 h compared to 4.5 h (see Fig. 5). The *arrowhead* points to the cell plate first visible at 7 h. Downward curvature resumed by 9 h. Plastid zone 1–3 are labeled at 7 h. 310; bar = 50 μm

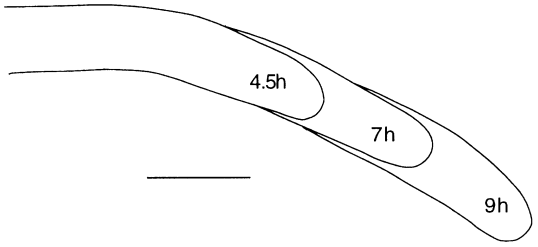

Fig. 5. Tracings of micrographs from Fig. 4 superimposed to show tip curvature and growth of the protonema during mitosis. The MR, though subtle, is visible at 7 h. Bar = 50 μm

downward, the upper surface of the wedge became horizontal and later inclined downward (Fig. 4, 7 h).

Dark-grown *wwr-1* protonemata had many more plastids than the wild-type, and the protonemal tips appeared greener (Fig. 1B,C). Protonemata of *wwr-1* were about 10 μm wider than those of the wild-type (Fig. 1) and showed more branching (data not shown). Even though the tips appeared green, 7-d-old cultures of dark-grown *wwr-1* appeared brown overall (Fig. 1). Brown pigments were also seen in dark-grown wild-type cultures but usually not before two weeks of growth.

Discussions

The directions of gravitropism, the MR, and the IR are coordinated. The most important finding of this study is that *Ceratodon wwr-1* protonemata mirror the wild-type in the directions of gravitropic curvature and of the IR and MR. Whereas wild-type cells grow up with downward reversals, *wwr-1* protonemata grow down overall but the IR and MR are temporarily upward. This argues against the hypothesis that all downward curvatures, including the IR and the MR in the wild-type, as well as positive gravitropism in *wwr-1*, result from the same default program. If all upward growth required a single gene, then the probable loss of this gene's function would eliminate the IR and MR in *wwr-1* protonemata.

The results show that the *WWR-1* gene product most likely functions between gravitropic sensing and the response. The *wwr-1* protonemata are clearly able to align their axis with respect to gravity with kinetics comparable to those of the wild-type. Thus, the *WWR-1* gene product is not necessary for the establishment of gravitropic polarity or for gravitropism but is needed to process information derived from sensing so that protonemata normally grow upward. It is possible that *WWR-1* may act to establish or interpret the polarity of a signal gradient to counteract or suppress default positive gravitropism.

Also, a functional *WWR-1* gene product is unnecessary for a coordinated response since the directions of the IR and MR in *wwr-1* protonemata are opposite to those of the wild-type. The simplest explanation could be that the directions of the IR and MR are coordinated to be opposite to the direction of gravitropism in the wild-type and *wwr-1*.

Implications for models of protonemal gravitropism. The positive gravitropism of *wwr-1* protonemata argues against a model (Sack 1993) of gravitropism in *Ceratodon* protonemata where plastid sedimentation leads to a downward displacement of Golgi stacks and exocytic vesicles to produce faster growth of the lower flank. Since plastids sediment in both wild-type and *wwr-1* protonemata, yet the directions of gravitropism are opposite, sedimentation is unlikely to be passively coupled to vesicle fusion. Stereological analysis also fails to indicate a redistribution of Golgi stacks or vesicles (Walker and Sack 1997). Thus, new models of protonemal gravitropism are required (Sack et al., in press).

Possible pleiotropy. In *Physcomitrella*, positive gravitropism was shown by somatic cell genetics to be due to a single recessive locus (*gtrC*; Knight et al. 1991). By extrapolation, positive gravitropism in *wwr-1* may also result from a single defective gene. The *wwr-1* mutant has been subcultured for over a year at weekly intervals, and the phenotype has remained stable. Unfortunately, *Ceratodon* has not been developed as a genetic system, and it is not possible at this time to determine the genetic basis for the *wwr-1* phenotype. Auxotrophic strains that make somatic hybrid genetics possible in *Physcomitrella* (Grimsley et al. 1977) are not yet available in *Ceratodon*. However, it seems unlikely that *wwr-1* is a composite of three different mutations, one reversing the MR, one reversing the IR, and one reversing net gravitropism.

In addition to the change in gravitropic polarity, *wwr-1* protonemata are wider, branch more, have more plastids, and turn brown earlier than wild-type protonemata. It is not known whether these morphological changes are related to each other and result from the same mutation that causes positive gravitropism, or whether they are due to a second mutation. Despite these morphological differences, *wwr-1* cells closely resemble wild-type caulonemata in plastid zonation and morphology and in the presence of oblique cross walls. Wild-type *Ceratodon* rhizoids are positively gravitropic, but they occur only rarely in our protonemal cultures and are not readily confused with caulonemata which are wider and contain more plastids. Therefore, it is unlikely that the *wwr-1* mutation has somehow caused the appearance of a new cell type or the overproduction of rhizoids.

Other gravitropic mutants with reversed polarity. There are some cases where the polarity of gravitropism reverses during the development of organs of wild-type plants, e.g. the peanut gynophore which switches from negative to positive gravitropism following fertilization (Shushu and Cutter 1990). Interestingly, several mutations reverse the normal polarity of gravitropism, e.g. *gtrC* in *Physcomitrella, lazy-2* in tomato, and *lazy* in maize (Jenkins et al. 1986; Gaiser and Lomax 1993). The mechanisms of gravitropism in multicellular shoots and in protonemal apical cells are likely to be fundamentally different. Nevertheless, it is critical to identify and sequence both types of gene since they provide entry into the pathway of gravitropism both upstream to sensing and downstream to differential growth.

Supported by a grant from the NASA Graduate Student Researchers Program (NGT-51281) to T.A.W., by NASA Space Biology grants NAGW-3594 and NAG10-0179 to F.D.S., and by a grant from the Leverhume Trust to D.J.C. Thanks to Tamara Frasure, Kathy Howard, and Connie Lee for technical assistance and to Dr. Jeff Young for the micrographs in Fig. 3.

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