Accessory Publication

Control of gravitropic orientation. I. Non-vertical orientation by primary roots of maize results from decay of competence for orthogravitropic induction

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Expanded materials and methods

Seedling growth

Seeds of *Zea mays* L. cv. Silver Queen were obtained from Hummert Seed Co. (Earth City, MO) and Park Seed Co. (Greenwood, SC). Selection of this cultivar was based on its conspicuous plagiogravitropic responsiveness (Millet and Pickard 1988), on the lore of its diagravitropic behaviour in the dark, and on side-by-side comparisons with one seed lot of the variety Merit (data not shown). Under our conditions, Silver Queen seedlings were less susceptible to water stress, produced straighter primary roots, and, in long-term experiments, showed fewer erratic curvatures than Merit seedlings. Seeds were germinated and grown in a room maintained at 26 ± 1°C according to a protocol modified in four ways, as follows, from that of Millet and Pickard (1988). (1) After seeds were surface-sterilised in 10% fresh commercial [NaOCl] hypochlorite bleach for 5 min and rinsed for 1 min, residual bleach was neutralised with one or two 1-min rinses in 0.01 N HCl. (2) Imbibed seeds were set with embryo up on wetted pads consisting of seven layers of paper towels, and covered with one layer. The brand of paper toweling used was Scott® Surpass® Scottex® 2-ply Roll Towels (Kimberly-Clark, Roswell, GA; if reproduction of the experiment is attempted, it should be noted that the properties of this brand have been modified and no longer support rapid, uniform, healthy seedling growth. High-grade laboratory tissues or commercial germination papers may perhaps be substituted). (3) Plants were maintained in darkness except for exposures to IR or dim green work-lights as described below. (4) For experiments of Figs 4–7, beginning a little more than 24 h after imbibition was initiated the seedlings were grown upright on milled 1.9-cm-wide plastic racks to each of which was pressed a 1.2 × 23 cm strip of Scotch Double Stick Tape (3M Corp., St Paul, MN). To fix the newly germinated seeds to a rack, their coats were gently dried by blotting with laboratory tissue and they were pressed against the tape with the axis-side out. Two such racks were mounted between apposed 33 × 25 cm plastic trays supporting wetted wicks cut from three-layer pads of 2-ply paper toweling. Using large rubber bands as spacers, the wicks were lightly pressed together above, beside, and below each rack to form an air-filled cocoon around it. The separation of the wicks was approximately 1 cm in the immediate vicinity of the seedlings; thus the distance from roots to wicks was just enough to assure that the roots would remain suspended freely during growth. The wicks, which were cut longer than the trays, extended at top and bottom through a 1-cm space maintained between the rims of the trays. The assembly was mounted with its base in a reservoir of distilled water with tops and edges of the wicks exposed to room air of roughly 70% RH.

Experimental protocol

Gravitropism test dishes were prepared as follows. A set of 10-cm square plastic Petri dishes (Becton Dickinson Labware, Lincoln Park, NJ) were deepened by gluing 2-cm-wide, 0.05-mm-thick strips of acrylic plastic to the sides of the dishes. An 8-cm-long strip of clay-like Mortite® Weatherstrip and Caulking Cord (Mortite Inc., Kankakee, IL) was sealed inside each dish, centered 3 cm from the side which would be uppermost during vertical seedling orientation; because the strip did not reach the walls of the dish, water could drain around it. Three pads each consisting of four layers of the 2-ply toweling specified above stapled to one sheet of black, cotton rag paper (Arches Cover, Arjo Wiggins, distributed by Legion Paper Corp, New York, NY) were cut to fit inside the dish. After wetting with distilled water, two pads were placed in the bottom of the dish — one below and one above the Mortite® strip — and one pad was placed in the lid, all with black side facing inward. (Although the black paper was used only to provide a contrasting photographic background, it was included in each pad to assure symmetry of dish environment. Before use in pads, it was soaked in 95% ethanol and rinsed repeatedly in distilled water to remove any contaminants.) Each dish was held vertical by a special stand.

Approximately 46 h after initiation of imbibition, seedlings selected for straight roots approximately 10 mm long (see statistics below) were mounted in the gravitropism test dishes by gently blotting moisture from the seeds and pressing them, still in the vertical orientation, into the Mortite® strip. For most experiments seven or eight seedlings were fitted onto each strip with axis side out, but in specially identified cases up to 13 seedlings were fitted with the axis facing sideways. Seedlings for most experiments were manipulated under dim ‘green’ fluorescent light filtered through combined 5-mm sheets of green and dark-blue Plexiglas (Rohm and Haas, Philadelphia, PA). Intensity was less than 0.05 μmol m^{-2} s^{-1}, as determined with a LI-COR radiometer (model LI-185B with sensor Q 3855, LI-COR, Inc., Lincoln NE). In a few experiments, and as controls in some other experiments, seedlings were manipulated using a Find-R-Scope® and its IR source (FJW Optical Systems, Palatine, IL); this source illuminates with wavelengths above 800 nm. Immediately after seeds were mounted in a dish, the dish was set vertical in near-darkness with seedlings upright and they were equilibrated for at least 1 h. Because this period of near-darkness varied between test dishes, the durations were recorded, and in large experiments any effects were compensated for as described below.

Gravitropism from the horizontal position was typically initiated by rotating the dishes 90° within the vertical plane; initiation at alternative angles is described where applicable. When ‘white’ light was provided during the experimental period, the source was a pair of Cool White fluorescent tubes (Sylvania, Danvers, MA), and the intensity at the top of each dish was approximately 20 μmol m^{-2} s^{-1} as measured with the LI-COR radiometer. Sets of seedlings for experiments shown in Figs 2–4 were imaged under the standard green light (0.1 μmol m^{-2} s^{-1}, total exposure ≤ 1 min) with CCD camera (Sony model XC-77, New York, NY) or, for experiments shown in Fig. 7, under the standard white light (approximately 20 μmol m^{-2} s^{-1}) with CCD camera (Nikon CoolPix 990, Tokyo, Japan). To permit photography the lid of each dish

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was removed. In an experiment parallel to that of Fig. 2, but without a paper pad on the lid and with the lid permanently in place, it was established that transient lowering of the humidity around the plants during lid removal did not influence the outcome of the experiments. For experiments shown in Figs 2–6, each dish was set flat on the camera stand. For the experiment shown in Fig. 7, dishes were set upright with seedling axes horizontal during photography. It was carefully tested to ensure that these brief positional changes did not result in measurable differences in subsequent curvature.

Mounting seedlings sometimes required as much as 2–3 h. As expected, based on extensive studies of roots illuminated with red or white light then held for various durations of darkness in the vertical orientation, and finally laid horizontal in darkness for curvature development (Scott and Wilkins 1969; Wilkins 1977; Feldman 1981, 1984; Feldman and Briggs 1986; Leopold and LaFavre 1989), the dim green work-light did enhance subsequent root gravitropism, an effect documented in Fig. 3*A*. In any case, the competence for enhanced response dwindles when plants wait in darkness or near-darkness before gravitropic stimulation (i.e. for an 8-h stimulus, 34 ± 2° after a 0-h wait, 27 ± 1° after a 1-h wait, and 19 ± 2° after a 5-h wait; $n = 13$). Therefore, when seedling-filled dishes were allocated to replicate treatments within an experiment, they were selected so that those prepared relatively early were matched with those prepared relatively late. The effectiveness of this matching procedure was statistically evaluated, and its use justified, as described at the end of the following section.

Measurements and statistics

Root orientations and lengths were assessed from the digital images using the graphics program NIH Image. To measure an orientation angle (angle with respect to the horizontal), a line was drawn from the root tip to a midpoint 1 mm basal to the tip, and the angle between this line and a fiducial line representing the horizontal was recorded. Root curvature was calculated as orientation angle at a specified time minus the orientation angle at zero time, after correcting for any dish reorientations between the two times. For root lengths, a line was marked across the root base using as morphological indicator the tip-most remnant of the coleorhiza; side-by-side comparisons of consecutive images ensured that the same reference feature was consistently marked. Successive line segments were fitted to the mid-line of the root from its basal mark to the tip of its root cap, and these were summed to determine root length in pixels. Lengths were calibrated on each image by referring to the pixel count for the 94-mm dish width. The gravitropism test dish was deep enough that the growing, nutating roots seldom made contact with the wetted pads that humidified the dish air. When a root did touch, its cap left a visible mucilage deposit, and on this basis it was excluded from the dataset.

ANOVAs with initial root length as covariant and curvature as dependent variable showed that, within the range of root lengths utilised, starting length did not affect outcome. For long-term experiments of the type depicted in Fig. 3, the question of whether gravitropic responsiveness is affected by length and age of roots becomes particularly relevant. To address this, a series of experiments using dark-grown seedlings of the standard starting root length, approximately 10 mm, was carried out with seedlings set up in green light as usual, held vertical in complete darkness for periods varying from 8 to 30 h, and then turned horizontal for a further 8 h in darkness. Their curvature was independent of time in darkness and their lumped average was 13.7 ± a s.e. of 0.6°, with $n = 109$. This was the same as the average curvature (13.7 ± 0.7°, $n = 69$) of roots manipulated in IR light, then immediately turned to the horizontal for 8 h. Root lengths at the beginning of horizontal orientation in the first case varied from 20–60 mm, whereas those in the second case averaged approximately 10 mm. From these findings and the ANOVAs on data for roots of approximately 10 mm length summarised above, it appears that responsiveness of dark-adapted roots does not change with length under the conditions used here.

Initial angles of roots averaged 1° with a standard deviation of 5°; the variability resulted largely from nutation after selection and before zero-time photography. ANOVAs with initial angle as covariant and curvature as dependent variable were performed on most of the experiments represented here. They showed that starting angle influenced response by a measurable, often surprising, amount, but almost all means were changed less than 2°, and so the uncorrected means are presented throughout. Deviant initial angle has a trivial influence on the component of gravity initially acting perpendicular to the root when it is placed horizontal but influences the time a root remains in a range where that perpendicular vector is close to 1 g.

The data of an early repetition of the experiment shown in Fig. 4 were used to confirm the effectiveness of the procedure, described in 'Experimental protocol', for balancing the post-selection periods of waiting in near darkness. That was done by using the waiting period for each dish ($n = 7$ dishes per treatment with two treatments) as a covariant in an ANOVA with dish curvature mean as dependent variable. This analysis corrects values for each dish and each treatment mean while leaving the experiment mean unchanged. If the matching procedure has been successful, the treatment means should be little affected by this correction even if the dish corrections are large. Comparisons of corrected and uncorrected dish curvature values showed a maximum difference of 3° (15%), but treatment means differed at most by 0.5° (2%), which was well within the standard errors of the uncorrected means. We concluded that our matching procedure is adequate and, therefore, we did not statistically adjust data for the waiting-period effect.

Fitting a Boltzmann sigmoidal equation to the time-course data (Fig. 2) was performed with Origin graphics software, Version 3, Microcal Software, Inc., Northampton, MA.

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