Optimizing Environmental Conditions for Mass Application of Mechano-dwarfing Stimuli to *Arabidopsis*

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ABSTRACT. Obtaining uniform mechano-dwarfing of *Arabidopsis thaliana* (L.) Heynh. seedlings within dense plantings is problematic. Alternative forms of mechano-stimulation were applied to seedlings in effort to obtain uniform growth reduction compared with undisturbed controls in both greenhouse and controlled growth environments. *Arabidopsis* grown under low photosynthetic photon flux (PPF) artificial light grew upright with limited leaf expansion, which enhanced mechano-responsiveness compared to that of rosette-growing plants under filtered sunlight or high PPF artificial light. Hypocotyls of seedlings grown at PPFs $>60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ elongated less and had 6% less sensitivity to mechanical stress than seedlings grown at PPFs $<60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. Fluorescent lamps alone (F) or fluorescent plus incandescent (F+I) lamps were compared for seedling responses to mechanical stress. Under F lighting, hypocotyl elongation was reduced 25% to 40% by twice-daily brush or plate treatments, and brushed seedlings exhibited more growth reduction than did plate treatments. Seedlings grown under F+I lamps exhibited similar stress-induced growth reduction compared to seedlings grown under F only, but stressed F+I seedlings lodged to a greater extent due to excessive hypocotyl elongation. Temperature-response studies using standardized F-only lighting indicated increased hypocotyl elongation but decreased leaf expansion, and decreased mechano-responsivity to brushing over the temperature range from 20 to 28 °C. Daylength studies indicated similar degrees of mechano-inhibition of hypocotyl elongation over the daylength range of 12, 16, 20, and 24 hours, whereas fresh weight of stressed seedling shoots declined compared to controls. A combination of environmental growth parameters that give repeatable, visual mechanical dwarfing of *Arabidopsis* include low-PPF fluorescent lighting from 55 to 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, ambient temperatures from 22 to 25 °C, and twice-daily brush treatments.

Mechanical stresses are common among plants growing in nature. Mechanical stress causes physiological responses that can reduce height, leaf area, and biomass compared to undisturbed controls (Jaffe, 1973; Mitchell et al., 1975). The resulting growth-inhibitory effects have inspired the intentional application of stress to seedlings growing in greenhouses to adapt them for outdoor transplant (Latimer, 1991). Various forms of mechanical stress have been tested for their hardening effects on horticultural crops. Brushing (Pontinen and Voipio, 1992) or static impedance (Garner and Bjorkman, 1997) lead to growth reduction with no visible damage. Few studies have been done to optimize environmental growth parameters for mechano-sensitivity (Jaffe, 1973; Heuchert and Mitchell, 1983; Jones and Mitchell, 1992).

This project identifies a set of conditions that permit uniform response of *Arabidopsis* seedlings *en masse* to mechanical stress and is part of a broader effort to identify T-DNA insertional mutant phenotypes unresponsive to mechano-stimuli and genes mediating the process.

Individual *Arabidopsis* plants receiving daily, noninjurious contact stress exhibit growth reductions during bolting (Braam and Davis, 1990). However, to develop an effective mass mechano-dwarfing screen, plants must be small and vegetative to avoid competing physical interactions that occur among closely spaced, bolting plants. The present article describes the systematic approach used to enhance and validate the uniform mechano-dwarfing re-

sponse of wild-type *Arabidopsis* that could be developed into a mutant screen.

Methods and Materials

Initial experiments were conducted in greenhouse (GH) and growth-room (GR) environments, where brush and static-impedance stresses were applied and plant responses compared. Twenty-four flats each were planted with 400 *Arabidopsis thaliana* (L.) Heynh. seeds of the C-24 ecotype in commercial potting mix (Scotts Metromix 360) preleached with water. The flats were incubated for 4 d at 4 °C and then moved under intermittent mist (8-s spray every 10 min) in a greenhouse. Seedlings emerged after 2 d under mist, and, after 4 d, 12 flats were moved into a GH under 70% shade; the remaining 12 flats were transferred to the GR. Stress treatments were assigned randomly to flats, and each treatment (brush, plate, control) was replicated four times. Treatment was started after emergence of the first set of true leaves, ≈ 4 d after removal from the mist. Brush treatments were applied using a tri-layered, fringed, burlap brush. Each treatment consisted of 10 back-and-forth strokes applied twice daily 7 h apart beginning at ≈ 0900 HR. Static-impedance treatments involved placing a 0.05-cm-thick perforated polycarbonate transparent plate on the flats for 12 h overnight. Photosynthetic photon flux (PPF) from high-intensity discharge lamps was measured using a cosine-corrected quantum meter (LI-1776; LI-COR, Lincoln, Neb.) at bench level in the GR at 130 to 150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, whereas PPF in the GH was uncontrolled. Temperatures were set at 22 °C days/20 °C nights in the GR. Temperatures in the GH were controllable to within 10 °C of ambient outside air during the summer. Flats were subirrigated to avoid mechano-effects from overhead watering. Commercial

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Table 1. Effects of 6 d of mechanical stress treatments in two different growth environments on shoot dry weight and leaf area of *Arabidopsis* seedlings.

Treatment	Greenhouse		Growth room	
	Dry wt (mg/plant)	Leaf area (cm ² /plant)	Dry wt (mg/plant)	Leaf area (cm ² /plant)
Control	3.39 a ²	0.273 a	8.31 a	0.419 a
Brush	3.74 a	0.293 a	9.40 a	0.429 a
Plate	3.26 a	0.276 a	11.2 a	0.433 a

²Data in columns followed by the same letter are not significantly different at $P = 0.05$ according to Tukey's ω -procedure.

micronutrient and NPK-enriched water was given to each flat every third watering. Plants received 14 consecutive days of mechanical-stress treatments and were 28-d-old from seeding at harvest.

Total leaf area per flat was determined from overhead digital photographs. The images were imported into Photoshop and converted into grayscale. Black pixel counts of the leaves were compared to those for a standard area. Average leaf area per plant was calculated based on pixel counts and number of plants per flat. Growth also was expressed as average shoot fresh and dry weights per plant. Shoot fresh weight of total seedlings per flat was taken immediately after harvest, and dry weights were measured after the biomass was dried for 24 h in a 65 °C forced-air drying oven.

Subsequent experiments conducted in the laboratory used a growth medium that improved control of nutritional and water-status parameters. Rockwool (Grodan) slabs (28 × 20 × 4.5 cm) were placed in aluminum trays and each slab was hydrated with 1500 mL of 0.25× Hoagland's no. 1 nutrient solution (pH 5.5). Because high calcium concentrations have been related to decreased mechano-sensitivity (Myers and Mitchell, 1993), calcium concentration in the nutrient solution was reduced to 0.5 mM. Seed weights equivalent to 400 seeds were mixed with 3 g acid-washed sand and sown onto each wetted slab using a salt shaker. Trays were covered with saran wrap and stratified for 4 d at 4 °C. They were then moved under light banks, where the saran wrap was replaced with a propped transparent plastic dome. Lamp height was adjusted so that PPF was 55 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ under the domes. Treatments were started 3 d after removal from the cooler. Brush treatments consisted of twice-daily applications (10 back-and-forth strokes), and static impedance was applied for 1 hour twice daily (0900 to 1000 HR, 1600 to 1700 HR) by placing one plate (0.33 g·cm⁻²) on each flat. A 16-h daylength was used unless otherwise indicated. Treatments were given for 6 d before harvest. Harvested seedlings were 13-d-old from planting.

An experiment was conducted to identify the PPF needed for adequate hypocotyl elongation as well as for best mechano-sensitivity. Four light banks were used, each containing two 32-W fluorescent lamps. Four flats were placed under each bank at varying distances from the lamps to obtain four different PPF levels (40, 50, 70, and 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$).

An experiment was conducted to compare plant response to mechanical stress under either fluorescent plus incandescent lamps (F+I) or fluorescent lamps alone (F). Each lamp bank was equipped with two 32-W fluorescent lamps, plus three 15-W incandescent bulbs spaced 30.5 cm apart along the 122-cm bank (1.44:1 ratio of fluorescent-to-incandescent wattage). Both lighting regimes were adjusted to maintain a total PPF of 55 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

A study was conducted to find the temperature range optimal for mechano-sensitivity. Four growth chambers (Conviron models E8 and E15) were set at different day/night temperature regimes (22/20, 24/22, 26/24, 28/26 °C). Each chamber contained brush and control treatment trays replicated four times each. Daylength was

set at 16 h. Average daily temperatures (ADTs) of the treatments were 21.3, 23.3, 25.3, and 27.3 °C, respectively.

A laboratory experiment was conducted to distinguish effects of varying daylength on mechano-stress responsiveness. Fluorescent lamp banks were maintained at daylengths of 12, 16, 20, or 24 h.

Growth was measured as hypocotyl length and leaf area. Hypocotyl length was measured for a random subset of 25 seedlings from each flat. Leaf area was determined using the photographic protocol described. Statistical analysis of data included analysis of variance (ANOVA) and mean-separation tests (Tukey's ω -procedure) (Steele and Torrie, 1960) at the 95% confidence level.

Fig. 1. Effects of 6 d of brushing treatment on leaf area (top) and hypocotyl length (bottom) under differing PPF levels. Different letters indicate significant differences at $P = 0.05$, Tukey's ω -procedure.

Table 2. Effects of transparent domes on photosynthetic photon flux (PPF), phytochrome photostationary state (PPS) and hypocotyl length of *Arabidopsis* seedlings after 6 d of brush treatment.

Treatment	PPF	PPS	Hypocotyl length	
	($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$)		Control (mm)	Stress (mm)
Domes	54.0	0.8387	6.77 ± 0.186	4.10 ± 0.177
No Domes	58.5	0.8366	3.56 ± 0.057	2.57 ± 0.180

Results

Stressed seedlings exhibited no consistent growth reductions compared to controls after 10 d of twice-daily treatments when grown in the GH or GR (Table 1). Plants in the GR grew twice as large as those in the GH, but dry weight of stressed plants did not differ significantly from that of controls in either environment. Leaf area of brushed plants also was not reduced compared to controls, but the leaf area of plated seedlings undergoing static impedance treatments actually increased. Static-impedance plates prevented upward growth of seedlings during treatment, but stimulated lateral leaf expansion, even though plates were in place for only 2 h·d⁻¹.

Seedlings grown using an inert growth medium and artificial low PPF assumed an upright, caulescent growth habit compared to the acaulescent, rosette growth habit of seedlings grown under higher PPF in the GH or GR environments. Brush treatments were applied to trays of seedlings grown under different PPFs (40, 50, 70, or 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Leaf area of brushed seedling was not significantly affected by stress, but there was a strong trend toward reduced leaf expansion compared to controls (Fig. 1). Hypocotyl length of brushed seedlings was significantly shorter than that of controls after 6 d of treatment. Low PPF (40 or 50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) *per se* increased hypocotyl growth 42% compared to that under the higher PPFs tested (70 or 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Fig. 1).

Seedling growth under low-PPF artificial light was compared with and without plastic, transparent domes covering the flats. Use of domes reduced total PPF only 7% but maintained a constant phytochrome photostationary state (PPS). PPS is the equilibrium achieved between the red-light-absorbing phytochrome (Pr) and the far-red-absorbing form (Pfr), and was measured with a spectroradiometer (LI-C1800). Hypocotyl elongation of stressed seedlings was reduced 39% compared to controls when domes were used. When domes were not used, only a 28% growth reduction occurred (Table 2).

Two mechanical-stress treatments (brush or plates) were compared under two light-quality regimes (F vs. F+I) (Figs. 2 and 3). No significant differences in leaf area to mechano-response occurred for brushed vs. control seedlings (Fig. 2). The most obvious, visual response to stress was reduced hypocotyl elongation. Hypocotyl elongation was reduced 39.4% by brushing plants grown under F lights. Under F+I lights, hypocotyl elongation was reduced 34.5% by stress compared to controls. Overall, F+I seedlings grew taller but lodged more easily when stressed. The hypocotyls of F-lighted seedlings grew thicker and were less prone to lodging

Fig. 2. Effects of 6 d of brushing treatment on leaf area (top) and hypocotyl length (bottom) under fluorescent (F) or fluorescent plus incandescent (F + I) lighting regimes. Different letters indicate significant differences at $P = 0.05$, Tukey's ω -procedure.

Fig. 3. Effects of twice-daily 1-h impedance plate treatments on leaf area (top) and hypocotyl length (bottom) under fluorescent (F) or fluorescent plus incandescent (F + I) lighting regimes. Different letters indicate differences at $P = 0.05$, Tukey's ω -procedure.

Fig. 4. Effects of different day/night temperature regimes and the resultant average daily temperature (ADT) on average plant hypocotyl length (**top**) and average leaf area per plant (**bottom**), as well as on plant responsiveness to daily brush treatments. Second-order regression equations are as follows:

Length/control: $y = 0.054x^2 - 0.098x + 0.45$

Length/stress: $y = 0.044x^2 - 0.079x + 0.24$

Leaf area/control: $y = -0.016x^2 + 0.033x + 0.30$

Leaf area/stress: $y = -0.013x^2 + 0.086x + 0.0094$

when stressed. Leaf area of plated seedlings also was not reduced significantly (Fig. 3). A 31.5% reduction in hypocotyl elongation was measured under F lights, and a 26.0% reduction was measured for F+I plated plants. The seedlings of plate treatments required a longer reorientation period once the plates were removed compared to brush treatments.

Hypocotyl elongation increased concomitant with increasing average daily temperature (ADT), but sensitivity to brush treatments declined (Fig. 4). On the other hand, leaf area expansion declined with increasing ADT.

Overall, stress sensitivity was constant over the range of day-lengths tested. As both daylength and daily PPF ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$) increased at constant, instantaneous PPF ($\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), shoot fresh weight also increased, but sensitivity to brushing declined from a 37.6% reduction in fresh weight with a 12-h daylength to a 9.2% reduction with a 24-h daylength (Fig. 5).

Discussion

A successful mass mechano-dwarfing screen for *Arabidopsis*

must be reliable, repeatable, and, most important, visual. *Arabidopsis* seedlings grown in the GH/GR, while showing high sensitivity to mechanical stress at the bolting stage (Fig. 6), were far too large at that stage of development, growing together and causing many physical interactions among plants. From the interactions between seedlings and the need to select variant individuals from the flats, screened plants would have to remain more widely spaced or be smaller at the time of screening. In the GH/GR environments, PPF was found to be supraoptimal at levels around $150 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and higher. Unfavorable root-zone environments (variable water status, high pH, and salt accumulation) along with uncontrolled temperatures also reduced uniformity of response.

Plate treatments increased lateral leaf expansion, possibly due to localized humidification and improved water status, although those conditions lasted only 2 out of every 24 h.

The typical rosette growth habit of *Arabidopsis* protects the recessed shoot apex from receiving mechanical stimulation. It was concluded that upright stem growth is a practical necessity for mechano-dwarfing of vegetative *Arabidopsis*.

Under laboratory conditions, the reasons for upright hypocotyl growth and mechano-responsivity were suspected to be related to low total irradiance, dosage of blue light, and/or red/far-red ratio of the light source. Use of low PPF (40 to $50 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) permitted significant mechano-dwarfing because of the

drastic increase in hypocotyl elongation of controls that does not occur at higher PPF (70 to $80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) (Fig. 1). While low PPF under either F+I (Fig. 7) or F-only (Fig. 8) lighting treatments both promoted hypocotyl elongation, it was found that F lighting alone at low PPF was the most effective lighting regimen. In Experiments where total PPF was varied from 40 to $80 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ (Fig. 1), the changes in hypocotyl elongation could be attributed to blue-light dosages sensed by cryptochrome (Ahmad and Cashmore 1993), but in experiments testing mechano-sensitivity between F and F+I lighting the blue light dosage did not change, indicating that the far-red output of incandescent lamps also had a significant, opposite effect on hypocotyl elongation.

Preliminary studies using transparent plastic domes loosely covering seeded rockwool slabs induced superior growth double that of seedlings grown without domes. The dome effect also promoted sensitivity to mechanical stress (Table 2). The domes retarded water loss from the growth system presumably by creating a localized microclimate with increased humidification. Domes also protect seedlings from uncontrolled room air currents that otherwise might harden off seedlings to mechano-treatments. Phytochrome photostationary state was measured under fluorescent light with and without domes, and the ratios of $[P_{fr}]$ to total $[P]$ at photo-equilibrium were not different (Sager et al. 1988). Spectroradiometer scans across the entire visible range of wavelengths suggested that domes caused no selective wavelength filtering, and decreased total PPF only 7%.

In previous studies, Jaffe (1976) and Heuchert and Mitchell (1983) found that mechanical-stress sensitivity is reduced if plants

Fig. 5. Effects of different daylengths at a constant PPF of $55 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on hypocotyl length (**top**) and shoot fresh weight per plant (**bottom**), as well as on responsiveness to daily brush treatments. Bar pairs with different letters were significantly different from each other at $P = 0.05$, Tukey's ω -procedure.

Fig. 6. *Arabidopsis* at the bolting stage grown in the greenhouse. Control (**left**) and stressed (**right**) seedlings after 7 d of plate treatment.

Fig. 7. Control (**left**) and stressed (**right**) *Arabidopsis* seedlings grown under fluorescent-plus-incandescent lamps after 8 d of twice daily-brush treatments.

Fig. 8. Control (**left**) and stressed (**right**) *Arabidopsis* seedlings grown under fluorescent lamps only after 6 d of twice-daily brush treatments.

are grown outside their optimal temperature range. Plants grown at the low end of the temperature range ($22/20^\circ\text{C}$) in this study had the most sensitivity to mechanical stress but also grew the slowest (Fig. 4). Since one goal is to screen thousands of mutant lines as quickly as possible, a $24/22^\circ\text{C}$ day/night temperature regime was selected as an effective compromise temperature regime for enhancement of *Arabidopsis* seedling growth while still giving adequate stress responses.

Since similar mechano-induced hypocotyl growth reductions occurred at all daylengths tested, a 16-h daylength was selected because it gave reasonable growth within 6 d of growth and treatment. Longer daylengths were associated with higher daily dosages of photosynthetically active radiation. Growth measurements based on shoot fresh and dry weight indicated less sensitivity to mechanical stress at higher daily PPF, even though plants were grown at an instantaneous PPF that was conducive to good mechanical response. Use of a 16-h daylength was an effective compromise leading to uniform mechano-dwarfing.

Conditions selected for an optimum visual screen of *Arabidopsis* mechanical-stress response include a combination of fluorescent lights at low PPF (50 to $60 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 16 h, ambient temperatures at $24/22^\circ\text{C}$, twice-daily brushing treatments, use of protective transparent domes, and a uniform and controlled nutrient solution and growth medium.

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