

Regulation of the Gravitropic Response and Ethylene Biosynthesis in Gravistimulated Snapdragon Spikes by Calcium Chelators and Ethylene Inhibitors¹

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The possible involvement of Ca^{2+} as a second messenger in snapdragon (*Antirrhinum majus* L.) shoot gravitropism, as well as the role of ethylene in this bending response, were analyzed in terms of stem curvature and gravity-induced asymmetric ethylene production rates, ethylene-related metabolites, and invertase activity across the stem. Application of Ca^{2+} chelators (ethylenediaminetetraacetic acid, *trans*-1,2-cyclohexane dinitro-*N,N,N',N'*-tetraacetic acid, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) or a Ca^{2+} antagonist (LaCl_3) to the spikes caused a significant loss of their gravitropic response following horizontal placement. Conversely, the Ca^{2+} ionophore A23187 or the agonist Bay K-8644 increased gravibending. Longitudinally halved stem sections had significantly higher amounts of ethylene, 1-aminocyclopropane-1-carboxylic acid, and 1-(malonylamino) cyclopropane-1-carboxylic acid compared with vertical controls, with the extra production arising exclusively from the lower half of the stem. *trans*-1,2-cyclohexane dinitro-*N,N,N',N'*-tetraacetic acid pretreatment completely abolished the gravity-induced ethylene gradient across the stem, thereby leading to a significant reduction of the curvature. Similarly, reduction of the ethylene produced in the gravistimulated stems with CoCl_2 or inhibition of its action by silver thiosulfate or 2,5-norbornadiene significantly inhibited the subsequent gravibending. Silver thiosulfate and CoCl_2 also abolished the gravity-induced gradient of invertase activity across the stem, which is associated with the asymmetric stem elongation. These results suggest that cytosolic Ca^{2+} may regulate auxin action in snapdragon spikes, manifested as increased ethylene production, which is, in turn, intimately correlated with stem bending. Therefore, both hormones seem to play significant roles in induction and progress of the gravibending of snapdragon spikes.

Gravitropism in shoots and roots of higher plants is the result of asymmetric growth. According to the prevailing model of Cholodny and Went (Li et al., 1991; Trewavas, 1992), the negative gravitropic response of grass seedling coleoptiles is brought about by gravity, which induces redistribution of auxin toward the lower side of this gra-

vireacting organ; this causes the growth asymmetry that leads to coleoptile reorientation. Originally devised for grass coleoptiles, this theory was soon generalized to explain the manifold gravitropic reactions of stems and roots as well. Evidence in favor of the Cholodny-Went hypothesis has emerged from various studies showing an asymmetric distribution of auxin, specifically IAA, in gravistimulated grass coleoptiles (McClure and Guilfoyle, 1989; Li et al., 1991). However, it appears that changes in sensitivity of the gravity receptor and time-dependent gravity-induced changes in sensitivity to auxin (Harrison and Pickard, 1989; Rorabaugh and Salisbury, 1989; Evans, 1991; Kim and Kaufman, 1995) or to other growth regulators (Firn and Digby, 1980; Mertens and Weiler, 1983; Kaufman et al., 1985), as well as other effectors such as calcium (Lee et al., 1983; Bjorkman and Leopold, 1987; Saunders, 1990), play important roles in the gravitropic response of these organs. Hence, in view of the immense volume of research accumulated so far, it seems that the Cholodny-Went hypothesis is fundamentally valid, although it represents a great oversimplification of a complex array of phenomena, and its scope should be extended (Trewavas, 1992).

It is becoming widely accepted that extracellular signals are mediated in plants through a transient increase in cytosolic calcium, which is normally maintained at submicromolar levels by membrane-borne Ca^{2+} transport systems (Hepler and Wayne, 1985; Saunders, 1990; Bush, 1995). In the last two decades, evidence has accumulated showing that the pathway involved in the hormone-induced gravitropic stimulus-response coupling may utilize components of the Ca^{2+} -based signal transduction pathways (Lee et al., 1983; Slocum and Roux, 1983; deGuzman and delaFuente, 1986; Gehring et al., 1990; Trewavas, 1992). The role of Ca^{2+} as a second messenger in mediating auxin redistribution and effects that lead to the gravitropic response has been suggested (Slocum and Roux, 1983); such a role was recently demonstrated by visualization of rapid changes in cytosolic calcium of oat coleoptiles that were directly correlated with increased gravity-stimulated cell

¹ Supported by grant No. IS-2434-94 from BARD, The United States-Israel Binational Agricultural Research and Development Fund. This paper is a contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 1640-E, 1995 series.

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Abbreviations: BAPTA, 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; CDTA, *trans*-1,2-cyclohexane dinitro-*N,N,N',N'*-tetraacetic acid; MACC, 1-(malonylamino)cyclopropane-1-carboxylic acid; NBD, 2,5-norbornadiene; STS, silver thiosulfate.

elongation (Gehring et al., 1990). In addition, Ca^{2+} has been found to be involved in all three steps of the signal transduction pathway leading to gravitropic bending in higher plants, namely signaling (Bjorkman and Leopold, 1987; Shigematsu et al., 1994), transduction by auxin (deGuzman and de la Fuente, 1986), and cell growth and elongation (Lee et al., 1983; Slocum and Roux, 1983; Brock et al., 1992). This last step may be mediated through the induced action of various enzymes such as invertase and β -glucanase (Gibeaut et al., 1990), which lead in turn to cell-wall loosening and extension (Morris and Arthur, 1984). Hence, it seems that, as in the case of roots (Lee et al., 1983; Bjorkman and Leopold, 1987), the process of stem gravitropic bending might also be mediated by Ca^{2+} as a second messenger, leading to auxin-induced differential stem elongation.

Among other plant hormones, ethylene has been shown to be involved in various gravireacting systems (Wheeler and Salisbury, 1981; Mertens and Weiler, 1983; Kaufman et al., 1985; Wheeler et al., 1986). However, the role of ethylene in gravitropism is still controversial, since inhibitors of ethylene synthesis or action were not always effective in inhibiting the gravitropic response of various systems (Wheeler and Salisbury, 1981; Clifford et al., 1983; Kaufman et al., 1985; Balatti and Willemoes, 1989; Woltering, 1991). Also, the role of the increased ethylene production obtained in various plant organs upon their reorientation from the vertical to the horizontal is still obscure (Wheeler et al., 1986; Harrison and Pickard, 1989; Woltering, 1991).

The gravitropic response of flower stalks is important from a commercial point of view. However, despite the increasing volume of research on gravitropic bending, most research on the negative gravitropic response of shoots has been focused on seedlings (tomato, pea, bean, and sunflower) or oat coleoptiles. Consequently, there have been very few studies designed to examine the underlying complexities of the mechanism by which the gravity stimulus is perceived and transduced in intact, graviresponding flowering stems (see review by Halevy and Mayak, 1981; Woltering, 1991; Meichenheimer and Nackid, 1994). The major postharvest problems with such cut flowers is their upward bending as a response to gravity, mainly during transport when flowers are held horizontally. Since gravitropic bending is a growth process, it occurs only in flower shoot regions capable of linear growth after harvest, mainly during the first 24 hours in the vase (Halevy and Mayak, 1981). Hence, this problem is found only in cut flowers with actively growing long spikes (*Gladiolus*, snapdragon [*Antirrhinum majus* L.], *Eremurus*, *Ornithogalum*) or with growing peduncles (anemone, tulip).

Snapdragon inflorescences have 50- to 100-cm-long and 0.7- to 1-cm-thick growing flower stalks that show a rapid gravitropic response when placed horizontally, thereby causing a serious problem during their postharvest handling. Treatment of snapdragon stems with IAA, with various synthetic auxin derivatives, with anti-auxins and inhibitors of polar transport of auxin, or with ethylene inhibitors retarded their gravitropic bending to a certain extent (Joiner et al., 1977). However, treatments with Ca^{2+}

or calcium antagonists were not assayed with this species. Therefore, snapdragon spikes were taken as a suitable model for studying the possible involvement of calcium as a second messenger in shoot gravitropism and for evaluating the function of ethylene in the bending process. For this purpose, we have examined the changes in ethylene production rates (presumably induced by auxin) of longitudinally halved stem sections excised from gravistimulated spikes, through application of Ca^{2+} chelators or inhibitors of ethylene synthesis and action. In parallel, the curvature angle, invertase activity, and ACC and MACC levels were determined. Invertase is a cell-wall-loosening enzyme associated with elongation of graviresponding organs (Gibeaut et al., 1990; Wu et al., 1993), whereas ACC and MACC contents represent a manifestation of auxin action, which induces ACC synthase activity (Yang and Hoffman, 1984).

MATERIALS AND METHODS

Plan Material and Treatments

Freshly cut flower spikes of snapdragon (*Antirrhinum majus* cv Axium) at the prescribed harvesting stage were obtained from a local commercial grower and held vertically with the stem bases in water. At the beginning of the experiments, the stems were trimmed to a length of 55 or 70 cm and groups of 10 flowers were placed vertically in 0.5- or 2-L plastic cylinders, each containing 100 or 500 mL, respectively, of one of the following calcium-related solutions: Ca^{2+} -chelating agents, EGTA (20 mM), CDTA (20 mM), and BAPTA (6.5 mM) (all from Sigma); CaCl_2 (20 mM); Ca^{2+} ionophore A23187 (0.18 mM) (Sigma); Ca^{2+} -channel agonist Bay K-8644 (0.29 mM) (Calbiochem); and a Ca^{2+} -channel blocker, LaCl_3 (10 mM) (Sigma). Spikes were also similarly pulsed with solutions of the ethylene inhibitors STS (1.5 mM) or CoCl_2 (6.7 mM). STS solution, composed of AgNO_3 and $\text{Na}_2\text{S}_2\text{O}_3$, was prepared according to Reid et al. (1980). The concentrations of the chemicals used were determined according to preliminary tests with each chemical, in which a wide range of concentrations was assayed. All treatments were performed in a conditioned room maintained at 22°C with 60 to 70% RH and illumination of 12 h per day at a light intensity of $14 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool-white fluorescent tubes. After 20 h of pulsing, the flowers were divided into bunches consisting of five stalks each and transferred to 1-L plastic cylinders filled with 100 mL of preservative solution (TOG-6, Assia Reizel Ltd., Ramat-Gan, Israel), which contained $50 \mu\text{g mL}^{-1}$ of chlorine as the active ingredient, complexed in sodium dichloroisocyanurate. Gravitropic stimulation was provided by orienting the cylinders horizontally for an additional 24 h. Control cylinders were maintained in a vertical position during this period. The kinetics of stem bending was estimated by monitoring the angle of curvature of 10 replicates at hourly intervals, using a protractor. An angle of 180° represents a noncurved stem. In one experiment the 24-h-gravistimulated spikes, which reached an angle of 90°, were reoriented to the vertical and allowed to return to their upright position during the 4 subsequent

d. Consequently, the stems bent upwards at a new bending zone, forming an S-like shape. The deviation from vertical orientation, defined as the distance between two parallel perpendicular lines drawn at the two bending zones, was recorded at the end of this period. Experiments were repeated with similar results each time. The results presented are from individual experiments.

NBD Treatment

The gaseous ethylene inhibitor NBD was applied as described previously (Woltering, 1991). Groups of 10 snapdragon spikes were placed horizontally in 30-L plastic barrels kept at 22°C in darkness. A known amount (500 μL) of liquid NBD (Aldrich) was injected into each barrel to yield a concentration of 4000 $\mu\text{L L}^{-1}$ NBD. Control inflorescences were placed horizontally under identical conditions, except that endogenously produced ethylene and CO_2 were removed by absorption with Ethisorb (Stayfresh Ltd., Manchester, UK) and a 0.2 M KOH solution, respectively. After 20 h of treatment, the barrels were opened and the bending angle was recorded.

Ethylene Measurements

For measurements of ethylene production rates during gravitropic bending of snapdragon spikes, 5-cm stem segments were excised from the bending zone (just above the last open floret, 5–10 cm beneath the apex) of treated and untreated spikes at various intervals following gravistimulation. In one experiment, 5-cm stem sections were excised from the apex along 25 cm of the flowering stem following 4 h of gravistimulation. After leaves and florets had been detached, the stem sections, either whole or longitudinally halved, were weighed and individually placed in 25-mL Erlenmeyer flasks sealed with rubber serum caps for 1 h at room temperature to allow ethylene accumulation. The upper and lower halves of the horizontally placed stems were maintained in their original positions during the ethylene measurements. Ethylene concentration in the various flasks was analyzed by withdrawing a 2-mL gas sample with a hypodermic syringe and injecting it into a gas chromatograph (Varian, Palo Alto, CA) equipped with an activated-alumina column and a flame-ionization detector. The longitudinally halved stem sections used for ethylene measurements (0.2–0.3 g fresh weight) were frozen in liquid nitrogen and stored at -80°C until used for ACC, MACC, and invertase analyses.

Extraction and Analysis of ACC and MACC

Extraction and determination of ACC and MACC were performed basically as described previously (Jiao et al., 1986; Woltering, 1991), except that each frozen segment was homogenized in 5 mL of 95% ethanol by means of a Polytron (Kinematica, Luzern, Switzerland) homogenizer. The homogenate was centrifuged at 10,000g for 10 min, the supernatant was evaporated to dryness at 55°C, residues were dissolved in 2 mL of distilled water, and pigments were removed by addition of 0.5 mL of chloroform. All subsequent steps of ACC and MACC analysis were per-

formed as detailed previously (Jiao et al., 1986). Three replicates were employed for each determination.

Extraction and Assay of Invertase

Soluble invertase was extracted as described by Gibeaut et al. (1990). Stem segment halves (0.2 g fresh weight) were homogenized in 3 mL of 10 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT, 1 mM EDTA, and 0.1 mM PMSF, using a Polytron homogenizer. The homogenate was spun at 30,000g for 20 min and the supernatant obtained was assayed for invertase activity as detailed by Wu et al. (1993). Briefly, the reaction mixture contained, in 1 mL, 0.1 mM sodium acetate buffer (pH 5.5), 21 mM Suc as a substrate, and 100 μL of stem extract (20 μg of protein). The mixture was incubated for 30 min at 37°C, and the reaction was terminated by addition of 1 mL of 3,5-dinitrosalicylic reagent and subsequent heating for 10 min at 100°C. The resulting reducing sugars obtained (D-Glu and D-Fru) were quantitated by measurements of A_{540} using a Kontron (Zurich, Switzerland) Uvikon-820 spectrophotometer. Protein content of the extract was assayed according to Bradford (1976), using γ -globulin as a standard. Units of invertase activity were expressed in $\text{mol Suc kg}^{-1} \text{protein min}^{-1}$. Three replicates were employed for each assay.

RESULTS

Results depicted in Figure 1 show that the gravitropic bending response of cut snapdragon flowering stalks was significantly inhibited by application of various Ca^{2+} -chelating agents (EGTA, CDTA, BAPTA). Consequently, the chelator-treated flowers had an average bending angle of 140° following 8 h of gravistimulation, whereas in the untreated flowers an angle of 80° was obtained. However,

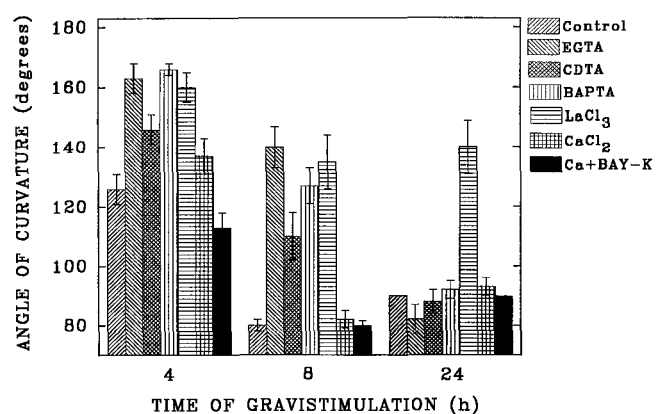


Figure 1. Effect of pulsing snapdragon cut flowers with Ca^{2+} chelators or a Ca^{2+} antagonist or agonist on their angle of curvature following 24 h of gravistimulation. Spikes were pulsed for 20 h with one of the following solutions: 20 mM EGTA, 20 mM CDTA, 6.5 mM BAPTA, 10 mM LaCl_3 , 20 mM Ca^{2+} , or 20 mM CaCl_2 + 0.29 mM Bay K-8644 and placed horizontally in cylinders containing preservative solution (TOG-6) for an additional 24 h at 22°C. Control spikes were similarly pulsed with preservative solution. The angles of 10 flowers per treatment were monitored following 24 h of gravistimulation and each bar represents the mean \pm SE.

although after 24 h of gravistimulation the angle of the chelator-treated flowers was similar to that of control flowers, the lanthanum-treated flowers still retained an average angle of 140° , suggesting that this Ca^{2+} antagonist was the most effective (Fig. 1). The effect of CaCl_2 was not pronounced during this period of gravistimulation, and the Ca^{2+} agonist Bay K-8644 enhanced slightly the angle of curvature only after 4 h of gravistimulation (Fig. 1). The Ca^{2+} ionophore A23187 had no effect during the 24 h of gravistimulation (data not shown).

To further understand the role of Ca^{2+} in the gravitropic response, we examined the effect of lanthanum, Ca^{2+} , and Ca^{2+} plus A23187 on deviation from vertical position following 24 h of horizontal incubation and subsequent 4 d of vertical reorientation. In the lanthanum-treated spikes, the deviation from vertical was doubled compared with untreated spikes (Fig. 2A), since this Ca^{2+} antagonist probably slowed down the return of the stems to their upright orientation. Hence, the deviation from vertical reflects the time required for readjusting growth following stem reorientation, and it seems that lanthanum significantly alters the kinetics of this response. On the other hand, in Ca^{2+} -

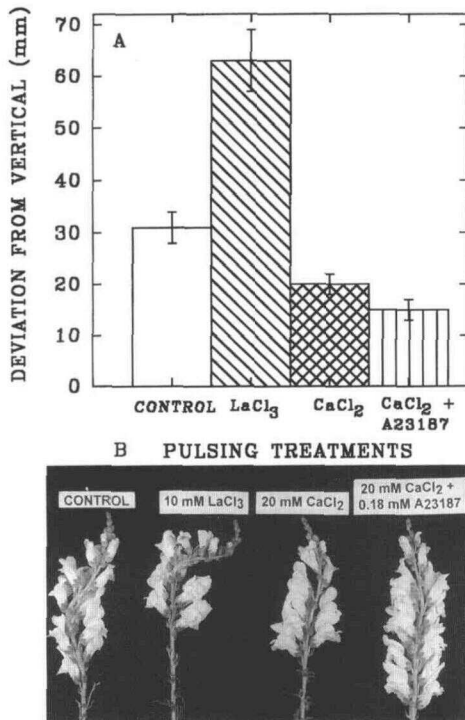


Figure 2. Effect of pulsing snapdragon cut flowers with a Ca^{2+} antagonist or with CaCl_2 in the presence or absence of a Ca^{2+} ionophore on the deviation from vertical (A) and on stem shape (B) following 24 h of gravistimulation and subsequent 4 d at upright position. Spikes were pulsed for 20 h with one of the following solutions: 10 mM LaCl_3 , 20 mM CaCl_2 , or 20 mM CaCl_2 + 0.18 mM A23187, placed horizontally in cylinders containing preservative solution (TOG-6) for an additional 24 h, and then reoriented to the vertical for the subsequent 4 d at 22°C . Control spikes were similarly pulsed with preservative solution. The deviation distances of 10 flowers per treatment were monitored at the end of this period, and each bar represents the mean \pm SE.

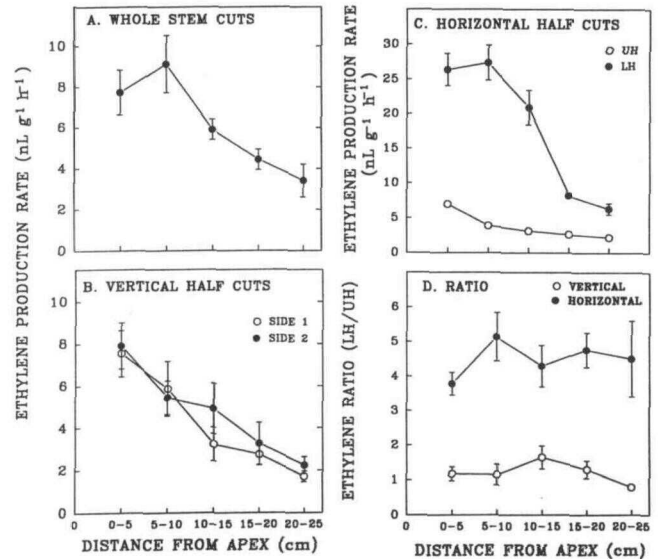


Figure 3. Patterns of ethylene evolution rates from whole (A) or longitudinally halved stem sections cut along vertical (B) or gravistimulated (C) snapdragon spikes and their ethylene production ratios (D). Stem sections 5 cm long were excised along the flowering stem following 4 h of gravistimulation and incubated for 1 h in sealed glass vials for ethylene accumulation, either as a whole cut or as a longitudinal half cut. Each point is the average \pm SE of 10 replicates. UH, Upper halves; LH, lower halves.

treated spikes, the deviation was reduced compared with the control and was even further decreased in the presence of the Ca^{2+} ionophore A23187 (Fig. 2A), which facilitates Ca^{2+} entry into the cytosol. The shapes of the spikes that resulted from these treatments are illustrated in Figure 2B.

To determine the exact bending zone in snapdragon flowering stems that start to bend, whole cuts or longitudinally halved stem sections were excised at various distances from the apex along the flowering spike and their ethylene production rates were monitored following 4 h of gravistimulation. Whole-stem cuts (Fig. 3A) and longitudinally halved segments cut along the stem of vertically oriented snapdragon spikes (Fig. 3B) showed similar ethylene production rates. A wounding-induced ethylene response could be detected only within 8 h of incubation of the longitudinally halved cuts (data not shown). This implies that longitudinal cutting of the stem sections into halves elicited no measurable wound effect during the 1 h of ethylene incubation. Therefore, all subsequent ethylene measurements were performed with longitudinally halved cuts. Ethylene production rates of both whole (Fig. 3A) and longitudinally halved stem sections (Fig. 3B) decreased with increasing distance from the apex. Whereas upper and lower segments cut along the stem of vertically oriented snapdragon spikes showed similar ethylene production rates (Fig. 3B), a significant gravity-induced differential ethylene production was obtained in stem halves excised along the stem of horizontally oriented spikes (Fig. 3C). Thus, after 4 h of gravistimulation, the ratio of asymmetric ethylene production found between upper and lower halves excised along the stem remained around 4:1 in favor

of the lower half (Fig. 3D). Nevertheless, whole cuts (Fig. 3A) or lower-half sections (Fig. 3C) excised from the stem region located 5 to 10 cm below the apex showed the highest ethylene production rates following 4 h of gravistimulation. This high ethylene production was indicative of the location of the bending zone, and, therefore, stem sections located 5 to 10 cm below the apex were employed in all subsequent experiments. A similar asymmetric distribution between ACC and MACC contents of lower and upper sections cut from different regions along the stem was found in horizontally oriented snapdragon spikes following 4 h of gravistimulation (Table I).

The kinetics of ethylene production rates during gravistimulation of snapdragon flowering stems was examined by monitoring ethylene production rates in longitudinally halved stem sections excised from their bending zone. In flower stalks maintained in a vertical position, both upper and lower segments showed the same pattern of ethylene evolution, peaking 8 h after vertical orientation, and no significant differences in the ethylene production rates of the two stem halves could be detected within 24 h (Fig. 4A). However, significantly higher amounts of ethylene evolved from the gravistimulated stem sections (Fig. 4B) than from the vertical controls (Fig. 4A) when the extra ethylene production arose exclusively from the lower half of the stem and peaked 8 h following gravistimulation. Consequently, during 4 h of gravistimulation, the asymmetry in ethylene production between the upper and lower halves reached a ratio of 3.5:1 in favor of the lower half (Fig. 4C). A ratio of 2:1 between the ethylene production rates of lower and upper stem halves was maintained during the remainder of the incubation. The increased ethylene production rates found in the gravistimulated lower stem halves (Fig. 4B) were very closely correlated with the sharp angles of curvature (80–90°) (Fig. 4C), whereas the non-curved (180°) vertical controls (Fig. 4C) exhibited much lower ethylene rates (Fig. 4A). The peak of ethylene production obtained during the initial 8 h of gravistimulation (Fig. 3B) coincided well with the bending process leading to formation of the sharp angles of curvature (80°) (Fig. 2C). However, once an angle of 80 to 90° was obtained following 10 h of gravistimulation, no further bending could be detected (Fig. 4C), and ethylene production rates were markedly reduced (Fig. 4B). This suggests that high levels of ethylene may be required for the elongation response of the lower stem half, leading to bending.

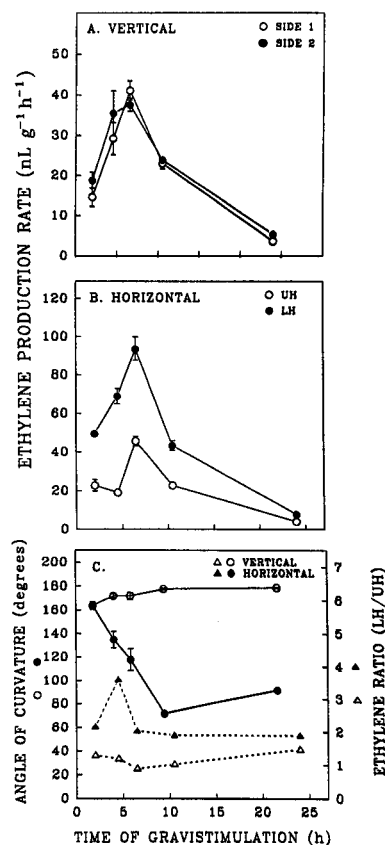


Figure 4. Time course of ethylene evolution from longitudinally halved stem sections cut from the bending zone of vertical (A) and gravistimulated (B) snapdragon spikes and their ethylene production ratio and kinetics of curvature (C). Stem sections 5 cm long were excised from the bending zone at the indicated intervals after start of gravistimulation, cut longitudinally into halves, and incubated for 1 h in sealed glass vials for ethylene accumulation. Each point is the average \pm SE of 10 replicates. UH, Upper halves; LH, lower halves.

The ethylene gradient obtained between the lower and upper halves of the stem during gravistimulation (Fig. 5A) was abolished completely by pretreatment of the stems with 20 mM CDTA (Fig. 5B), which significantly inhibited the gravity-induced extra ethylene production observed in the lower stem half (Fig. 5A). Hence, CDTA reduced the ethylene production rates of lower-half sections to the basal ethylene production rates monitored in upper stem

Table I. Effect of distance from the apex of snapdragon spikes on levels of ACC and MACC measured in longitudinally halved stem sections following 4 h of gravistimulation

Stem sections 5 cm long were excised along the flowering stem following 4 h of gravistimulation and cut longitudinally into halves, and their levels of ACC and MACC were analyzed. UH, Upper halves; LH, lower halves.

| Distance from Apex cm | ACC Level | | MACC Level | | ACC Ratio | MACC Ratio |
|--------------------------|-------------------------------------|-------|------------|------|--------------|------------|
| | UH | LH | UH | LH | | |
| | <i>nmol g⁻¹ fresh wt</i> | | | | <i>LH/UH</i> | |
| 0–5 | 0.363 | 0.773 | 8.35 | 13.2 | 2.13 | 1.58 |
| 5–10 | 0.444 | 0.705 | 8.40 | 11.5 | 1.58 | 1.36 |
| 10–15 | 0.145 | 0.733 | 5.65 | 9.16 | 5.05 | 1.62 |
| 15–20 | 0.175 | 1.385 | 6.48 | 8.43 | 7.91 | 1.30 |
| 20–25 | 0.485 | 0.452 | 5.19 | 14.9 | 0.93 | 2.87 |

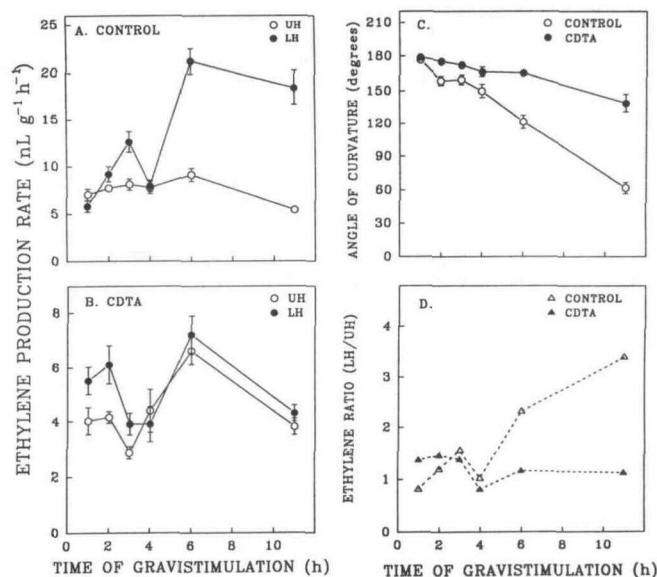


Figure 5. Effect of pulsing snapdragon spikes with CDTA on the time course of ethylene evolution (A and B) from longitudinally halved stem sections cut from their bending zone on the kinetics of curvature (C) and on their ethylene production ratio (D) during 12 h of gravistimulation. Flowering stems were pulsed with 20 mM CDTA for 20 h and placed horizontally. Ethylene production was monitored in longitudinally halved stem sections as detailed in Figure 4. Each point is the average \pm SE of 10 replicates. UH, Upper halves, LH, lower halves.

halves (Fig. 5, A and B). Consequently, the CDTA-treated flowers had an average bending angle of 150° following 11 h of gravistimulation, compared with an angle of 80° obtained in control flowers (Fig. 5C). Therefore, the ethylene production ratio of 3:1 found between control upper and lower halves at the bending zone was reduced to 1:1 following CDTA treatment (Fig. 5D). It should be noted that during the initial 4 h after gravistimulation, no significant bending was obtained (Fig. 5C), and, therefore, neither significantly increased ethylene production rates (Fig. 5A) nor ethylene ratios larger than unity (Fig. 5D) could be detected during this period. The remarkable effect of CDTA on the bending of snapdragon floral spikes following 11 h of gravistimulation is illustrated in Figure 6. It should be noted that the chelator treatments did not reduce flower longevity or quality or appearance within the time frame of the experiments.

To further establish the possible physiological role of the gravity-induced changes in ethylene production across the stem, the effects of various ethylene inhibitors on the angle of curvature and invertase activity were studied in relation to their effects on the ethylene biosynthesis pathway in upper and lower stem halves. Results depicted in Figure 7A demonstrate that reduction of ethylene production or action in the gravistimulated snapdragon stems by pre-treatment of the stems with inhibitors of ethylene synthesis (CoCl_2) or action (STS, NBD) significantly inhibited the subsequent gravitropic bending. The ethylene synthesis inhibitor CoCl_2 significantly reduced ethylene production rates in both stem halves and completely abolished the



Figure 6. Effect of pulsing snapdragon flowering stems with 20 mM CDTA on their angle of curvature and appearance following 11 h of gravistimulation.

gravity-induced ethylene gradient across the stem (Fig. 7B). On the other hand, the effect of STS, which inhibits ethylene action but has a stimulatory effect on ethylene synthesis, was in the opposite direction, showing a remarkable stimulation of ethylene production in both stem halves while maintaining the original gradient between them (Fig.

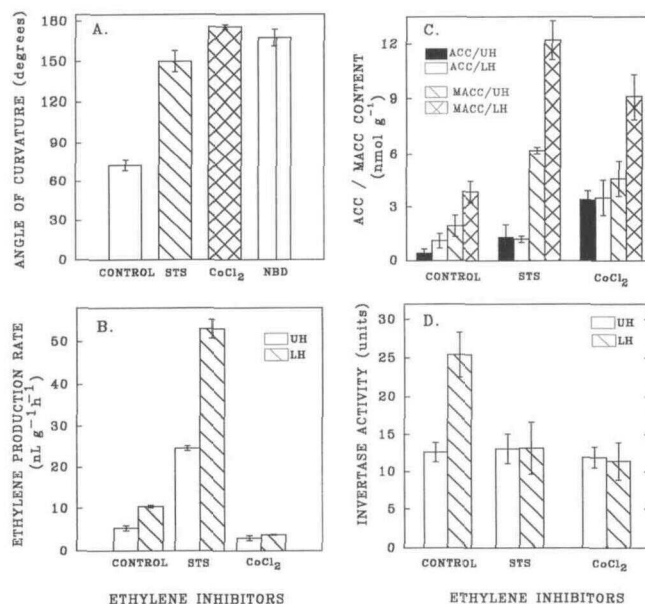


Figure 7. Effect of inhibitors of ethylene action or synthesis on angle of curvature of snapdragon spikes (A), on ethylene evolution rates (B), ACC and MACC levels (C), and invertase activities (D) in longitudinally halved stem sections cut from the bending zone following 24 h of gravistimulation. Flowering stems were pulsed for 20 h with 1.5 mM STS or 6.7 mM CoCl_2 or exposed to $4000 \mu\text{L L}^{-1}$ NBD, and their angle of curvature was monitored following 24 h of incubation in a horizontal position. Ethylene production was monitored in longitudinally halved stem sections as detailed in Figure 4. The same sections were subsequently analyzed for their ACC and MACC levels and for invertase activity. Each point is the average of \pm SE of 10 replicates (A and B) or of 3 replicates (C and D). UH, Upper halves; LH, lower halves.

7B). Nevertheless, STS was still effective in inhibiting snapdragon bending (Fig. 7A). STS increased the ACC level only in the upper segment, whereas CoCl_2 significantly increased ACC levels to a similar extent in both upper and lower stem halves (Fig. 7C). Consequently, the gravity-induced ACC gradient obtained in control lower and upper stem segments was not pronounced in either STS- or CoCl_2 -treated stems (Fig. 7C). MACC levels in upper and lower stem halves increased significantly in the presence of both inhibitors and, unlike the ACC gradient, the MACC gradient across the stem was maintained (Fig. 7C). As a result, the sum total of ACC plus MACC levels accumulated in the lower stem half following gravistimulation was about 3 times higher than the corresponding levels in the upper stem half. Additionally, STS and CoCl_2 , which significantly inhibited bending (Fig. 7A), reduced the gravity-induced extra invertase activity found in the control lower stem halves to the basal level of the upper halves (Fig. 7D). Consequently, the gravity-induced gradient of invertase activity across the stem was abolished in the noncurved STS- and CoCl_2 -treated spikes (Fig. 7D).

DISCUSSION

Several new ideas expanding the basic Cholodny-Went hypothesis postulating that gravitropism is caused by an asymmetric auxin distribution were examined in gravire-sponding snapdragon (*A. majus* L.) spikes. Accordingly, the postulate that increases in cytosolic concentration of Ca^{2+} may be regarded as one of the primary events in induction of auxin redistribution and sensitivity to auxin in the organ, and/or may be involved in the action of the hormones (auxin, ethylene) and enzymes (invertase) associated with the elongation response, was examined. Experiments were designed to modulate endogenous cytosolic Ca^{2+} by means of Ca^{2+} chelators or Ca^{2+} antagonist or agonists in order to affect auxin action, as well as to modulate ethylene level and action by ethylene inhibitors. These two hormones were found to play a significant role in induction and progress of the gravitropic response in snapdragon flowering stems.

The Ca^{2+} chelators BAPTA and CDTA have the highest affinity and specificity for Ca^{2+} , whereas EGTA has high affinity for Ca^{2+} in the presence of Mg^{2+} . Nevertheless, all three chelating agents inhibited the gravitropic bending equally well (Fig. 1) but had no effect after flowers were vertically reoriented (data not shown). On the other hand, the curvature-inhibitory effect of the Ca^{2+} channel antagonist LaCl_3 was much more significant, since it lasted longer (Fig. 1) and could be observed also in the second bending after spikes were vertically reoriented (Fig. 2). Indeed, La^{3+} is known as a very strong competitor for biological Ca^{2+} binding and transport sites (Bush, 1995), and Figure 2 shows that its effect is irreversible. It seems, therefore, that La^{3+} affects cytosolic Ca^{2+} concentration in a manner different than that of the Ca^{2+} chelators. Stimulation of Ca^{2+} influx into cells by either the Ca^{2+} ionophore A23187 or by the Ca^{2+} -channel agonist Bay K-8644, which causes opening of the so-called "L-type" Ca^{2+} channels (Fleckenstein, 1985), stimulated the bending process

slightly (Figs. 1 and 2). The effect of Bay K-8644 was significant only after 4 h of gravistimulation (Fig. 1), indicating that the changes in calcium level leading to bending may occur at initial stages of the process. These results suggest that the signal transduction pathway leading to shoot gravitropic bending may involve calcium mediation, as previously suggested and demonstrated for roots (Lee et al., 1983; Bjorkman and Leopold, 1987).

Although the effect of calcium antagonists on stalk elongation rate was not examined, there is indirect evidence indicating that calcium antagonists did not exert their inhibitory effect on snapdragon bending through inhibition of overall stem growth. Accordingly, the results shown in Figures 1 and 2 demonstrate clearly that bending, which is basically a growth process that could not happen if overall growth were inhibited, did occur with time in spikes treated with calcium antagonists. Also, no visible difference in stem length or impaired flower quality, which could result from overall growth inhibition, were observed in either LaCl_3 -treated (Fig. 2B) or CDTA-treated stalks (Fig. 6). Hence, it may be suggested that CDTA significantly reduced the curvature of snapdragon spikes (Figs. 1 and 5C) through inhibition of their differential ethylene production rates (Fig. 5), which are presumably induced by differential auxin distribution. This was partially confirmed in stem sections of gravisensitive *Ornithogalum* stalks incubated with exogenous IAA, which showed remarkable IAA-induced ethylene production rates that were significantly inhibited by CDTA or EGTA (S. Philosoph-Hadas, S. Meir, A.H. Halevy, unpublished results). Therefore, the results shown in Figure 5 may indicate that cytosolic Ca^{2+} might regulate auxin action, manifested as increased ethylene production.

The auxin-induced ethylene production was found to promote coleoptile elongation in concert with auxin, probably by prolonging the possible duration of the auxin-stimulated elongation (Burg and Burg, 1967; Ishizawa and Esashi, 1983). Hence, these two plant hormones are linked closely together to regulate normal elongation of various systems (Yang and Hoffman, 1984; Stange and Osborne, 1988). Accordingly, it follows that ethylene should mediate the gravitropic response induced by auxin (Burg and Burg, 1967). Increased ethylene production rates in the lower stem half apparently result from increased ACC-synthase activity, which is presumably induced by auxin (Yang and Hoffman, 1984). The resulting auxin-induced higher ACC levels can be metabolized in turn to MACC, which is supposed to dissipate high ACC levels in the tissue (Yang and Hoffman, 1984; Jiao et al., 1986). Hence, differential auxin redistribution and action across the stem will be expressed in differential total levels of ACC and MACC. The data obtained in this study clearly demonstrate that gravistimulation induced differential ACC-plus-MACC levels along the stem (Table I) and at the bending zone (Fig. 7C), and this gradient was accompanied by differential ethylene production in those regions (Figs. 3 and 7B). This differential production was shown to be induced by gravistimulation and not by wounding (Fig. 3, A and B). Based on the direct and indirect evidence presented, it may be

concluded that the ethylene asymmetry might merely reflect the asymmetric distribution of auxin across the gravistimulated stem, which is in accordance with the Cholodny-Went hypothesis (Trewavas, 1992). Similar gravity-induced ethylene gradients across the stem, with extra ethylene in the lower half, were reported previously in dandelion peduncles (Clifford et al., 1983), in tomato and cocklebur seedlings (Wheeler et al., 1986), and in *Kniphofia* flowering stems (Woltering, 1991). The gravity-induced ethylene gradient of *Kniphofia* was also accompanied by the corresponding ACC and MACC gradients (Woltering, 1991).

Besides being a possible marker for auxin, ethylene may be directly involved in the gravitropic response of snapdragon spikes. The data of the present study indicate that manifestation of curvature was associated with the existence of an ethylene gradient across the graviresponding stem, as well as with elevated ethylene production rates in a distinct stem region. Ethylene production rates decreased with increasing distance from the stem apex (Fig. 3, A and B), and the lower halves of longitudinally cut sections of gravistimulated spikes showed the highest ethylene production rates in the regions close to the stem apex (Fig. 3C), which is the location of the bending zone (Figs. 4, B and C, and 5A). Hence, although a constant ethylene gradient (Fig. 3D), which presumably reflects an auxin gradient across the stem (Table I; Fig. 7C), seems to be formed along the whole spike as a response to gravistimulation, only the region showing increased ethylene production rates in the lower-half sections is capable of bending. This indicates that the snapdragon spike can bend in various regions, and the location of the bending zone, determined according to the last open floret, is dependent on the stage of maturity of the spike at harvest. Further evidence of the intimate correlation between bending and ethylene production was provided by comparing the kinetics of the two processes: (a) Ethylene production rates of lower halves of gravistimulated stems increased (Fig. 4B) in parallel to the decrease in the curvature angle of the stem (Fig. 4C). (b) Once the stems reached an angle of 80 or 90°, indicating that the bending process was terminated (Fig. 4C), ethylene production rates of the lower-half sections were significantly reduced (Fig. 4B). (c) The bending process of stems depicted in Figure 5 was much slower than that demonstrated in Figure 4. Accordingly, during the initial 4 h of gravistimulation, no significant differences could be detected in the ethylene production rates of lower and upper halves (Fig. 5A), in the angle of curvature (Fig. 5C), or in the ethylene ratios (Fig. 5D). Only when the ethylene production of the lower halves increased significantly from the basal level, between 4 and 11 h of gravistimulation (Fig. 5A), was a significantly sharper bending angle detected (Fig. 5C).

Additional support for the role of ethylene in gravitropism of snapdragon spikes is provided by studies employing inhibitors of ethylene synthesis or action. Unlike previous studies with *Kniphofia* flowering stems (Woltering, 1991), all ethylene inhibitors tested significantly decreased the curvature of snapdragon stems following 24 h of gra-

vistimulation (Fig. 7A). These results indicate that the growth response of snapdragon stems following gravistimulation is ethylene dependent. A similar remarkable delay in the gravitropic response of cocklebur, tomato, and castor bean stems was obtained by application of various ethylene inhibitors (Wheeler and Salisbury, 1981; Wheeler et al., 1986). Hence, it seems that ethylene involvement in the gravitropic response of various systems is tissue dependent.

Similar to the effect obtained in CDTA-treated stems (Fig. 5B), the ethylene synthesis inhibitor CoCl_2 , which inhibits the conversion of ACC to ethylene (Yang and Hoffman, 1984), completely abolished the gravity-induced ethylene gradient across the stem (Fig. 7B), thereby leading to inhibition of bending. However, although both agents had a similar final effect on stem curvature, their modes of action seem to be different. CoCl_2 abolished the ethylene gradient by inhibiting ethylene synthesis in both upper and lower stem halves (Fig. 7B), whereas CDTA did so by moderating the extra ethylene production of the lower section to the basal level of ethylene production of the upper half of the stem (Fig. 5B). Hence, it seems that CDTA prevents gravibending by inhibiting auxin action, and thus indirectly reducing ethylene production, whereas CoCl_2 , being an ethylene synthesis inhibitor, prevents the curvature through direct inhibition of ethylene production. Alternatively, since the ethylene asymmetry may presumably reflect an asymmetric distribution of auxin across the stem, it is still possible that CDTA might also inhibit the differential auxin distribution (Lee et al., 1983).

The effect of STS, which significantly inhibited ethylene action (Fig. 7A) even though it had a remarkable stimulatory effect on its synthesis (Fig. 7B), further indicates that ethylene plays a significant role in induction of the gravitropic response of snapdragon stems. The remarkable stimulatory effect of silver ions (which are the active ingredient of STS) on both ACC and ethylene biosynthesis was previously demonstrated in vegetative tissues (Philosoph-Hadas et al., 1985, 1994) and was also suggested to result from increased conversion of ACC to ethylene (Mattoo and Lieberman, 1982). In this respect, it may be suggested that the STS-induced elevated ethylene production rates observed in lower halves of snapdragon stalks compared to upper halves (Fig. 7B) may be due to increased activity of ACC oxidase, since ACC content of lower half and upper half stem sections was identical in the presence of STS (Fig. 7C). Hence, it seems in this case that silver ions induced remarkable ethylene production rates mainly through stimulating the conversion of ACC to ethylene, as suggested previously by Mattoo and Lieberman (1982).

STS exhibited a rather efficient inhibition of the bending process, since although the differential ethylene distribution across the stem was maintained and even elevated in its presence (Fig. 7B), it still significantly inhibited ethylene action (Fig. 7A). However, it should be noted that the differential ACC-plus-MACC gradient across the stem was still maintained in the presence of both ethylene inhibitors (Fig. 7C), suggesting that these agents did not abolish the auxin gradient across the stem. Hence, in view of these

results, it may be concluded that formation of an ethylene gradient in the bending zone is essential for manifestation of the gravibending in snapdragon stems. This conclusion is reinforced by the data depicted in Fig. 7D, showing that the invertase gradient across a snapdragon stem, which may be associated with cell growth and elongation (Gibeaut et al., 1990), was ethylene dependent. Invertase is responsible for the hydrolysis of Suc to D-Glc and D-Fru, thereby providing substrate for starch synthesis in the gravisensors and for cell-wall biosynthesis or maintenance of turgor pressure in elongating cells of graviresponding organs (Wu et al., 1993). It was previously demonstrated that the activity of invertase in oat-leaf sheath pulvini is differentially up-regulated in top compared with bottom halves of the graviresponding organ (Gibeaut et al., 1990; Wu et al., 1993).

Based on the accumulated evidence, a putative sequence of events in shoot gravitropism may be suggested as follows: graviperception initiates, through Ca^{2+} as a second messenger, auxin redistribution and/or modulation of auxin sensitivity, which in turn results in differential auxin action, manifested as asymmetric auxin-induced ethylene production and ethylene-mediated asymmetric invertase activity across the shoot. This will lead to asymmetric cell extension (through activation of cell-wall-loosening enzymes) and gravicurvature. The acidification effect of auxin, as well as ethylene action, may also be Ca^{2+} mediated (Gehring et al., 1990; Saunders, 1990).

According to the outlined hypothesis, controlling cytosolic Ca^{2+} by the use of Ca^{2+} chelators or antagonists would abolish the signal for auxin lateral movement but would not interfere with the polar movement of auxin, as do the traditionally used auxin-transport inhibitors (Halevy and Mayak, 1981). Consequently, the gravitropic bending would be prevented without affecting cell elongation or other auxin-dependent functions. This would enable a better strategy for postharvest handling of graviresponding cut flowers to be designed.

ACKNOWLEDGMENTS

The excellent technical assistance of Ms. Hili Mann and Ms. Lilian Sonego is gratefully acknowledged.

Received August 7, 1995; accepted October 19, 1995.
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