

## Regulation and Role of Differential Ethylene Biosynthesis in Gravistimulated *Antirrhinum majus* L. Cut Flower Stems

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### Abstract

Gravistimulation induced differential ethylene production in *Antirrhinum majus* L. cut flower stems with highest levels in the lower halves of the gravistimulated stems. Expression levels of three different 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS) genes, an ACC oxidase (ACO) and an ethylene receptor (ETR/ERS homolog) gene were studied in the bending zone after 9 h of gravistimulation. One of the ACS genes (*Am-ACS3*) was abundantly expressed in the lower halves but not in the upper halves of gravistimulated stems. This strongly suggests that *Am-ACS3* is responsible for the observed differential ethylene production in gravistimulated stems. *Am-ACO* and *Am-ETR/ERS* gene expression was increased in both the lower and upper halves of gravistimulated stems, suggesting that they play no role in differential ethylene production.

When gravistimulation was performed in an environment enriched with either 20  $\mu\text{L/L}$  ethylene or 100  $\text{nL/L}$  1-methylcyclopropene (1-MCP), a slight stimulation of bending by 1-MCP and a slight inhibition of bending by ethylene were observed. The regulation and role of ethylene in gravitropism of cut snapdragon flowering stems is discussed.

### INTRODUCTION

Flowering stems of a variety of bulbous (e.g. *Gladiolus*, *Kniphofia* and *Tulipa*) and herbaceous (e.g. *Gerbera*, *Lupinus* and *Antirrhinum*) plants often show elongation growth during their postharvest life and may show severe bending when stored or transported horizontally (Halevy and Mayak, 1981). Despite the commercial importance of such commodities, most research on gravitropic responses has been done with seedlings, hypocotyls, vegetative stems and specific gravi-responsive organs such as coleoptiles, epicotyls and grass-shoot pulvini. Only few reports have been published on the gravitropic response of flower stalks including dandelion, *Kniphofia*, *Antirrhinum* and *Arabidopsis* (e.g. Halevy and Mayak, 1981; Clifford and Oxlade, 1989; Woltering, 1991; Philosoph-Hadas et al., 1995, 1996; Fukaki et al., 1996; Friedman et al., 1998; Weise and Kiss, 1999).

The gravitropic response is often accompanied by an increased ethylene production. In a variety of gravi-responding flower systems, an increased ethylene production in the lower half of the gravistimulated stems was observed, accompanied by increased levels of 1-aminocyclopropane-1-carboxylic acid (ACC) (e.g. Woltering, 1991; Philosoph-Hadas et al., 1995, 1996). In gravistimulated *Kniphofia* flower stems, the level of ACC was lowest in the peripheral cell layers in the upper half and highest in the lower half, suggesting a steep gradient of ACC and possibly of ethylene production over the entire diameter of the stem.

Based on the levels of ACC, malonyl-ACC and ethylene production in different parts of the stem it was estimated that ACC synthase (ACS) activity in the lower half of the stem increased over 100 fold during gravistimulation. ACC oxidase (ACO) activity was found to be approximately similar in upper and lower sides of the gravistimulated stems (Woltering, 1991; Woltering et al., 1991). This suggests that differential ethylene production in *Kniphofia* flower stems is regulated by ACS.

The role of the differentially produced ethylene in asymmetric growth is still

controversial (Madlung et al., 1999). In several reported cases, abolishment of the ethylene gradient by e.g. the use of ethylene production or perception inhibitors did not substantially alter the gravitropic response (e.g. Woltering, 1991; Madlung, et al. 1999), but in other cases such as in *A. majus* cut flowers, a clear effect of ethylene blockers was observed (Philosoph-Hadas et al., 1995, 1996, 1999).

In the present work we have further studied the regulation of gravistimulation-induced differential ethylene production and its role in gravitropic bending of *Antirrhinum majus* L. cut flower stems.

## MATERIALS AND METHODS

Flower stalks (*Antirrhinum majus* L.) were obtained from a commercial grower. Flowers were harvested, held in an upright position and immediately transported to the laboratory. Flower stems were trimmed to a length of 60 cm and placed either vertically or horizontally in a 15 mL flower tube containing water, under controlled environmental conditions (relative humidity 60%, temperature 20°C and continuous light of 15  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at plant level). In initial experiments it was established that the kinetics of bending was similar in light and in the dark.

For the analysis of ethylene production and gene expression, 4 cm long stem sections were excised from the bending zone after 9 h of gravistimulation. Preliminary experiments revealed that maximum rates of ethylene production occurred at approximately 10 h of gravistimulation. Stem sections excised from gravistimulated flower stems were longitudinally divided in upper and lower halves and treated as separate samples; stem sections from vertically placed flowers were also longitudinally halved but were treated as one sample. For measurement of ethylene production stem sections were enclosed for 1 h in 30 mL tubes. Thereafter, a sample of the headspace was analysed for ethylene by gas chromatography.

For expression analysis total RNA was isolated and 20  $\mu\text{g}$  was separated on 1.5% agarose gel. The RNA was blotted onto positively charged nylon membrane and crosslinked by UV irradiation. Thereafter, the blot was deglyoxylated by boiling for 5 minutes in 20 mM Tris-HCl pH 8.0, 1 mM EDTA. Blots were hybridized in ULTRAhyb solution (Ambion) using antisense, radiolabeled RNA probes (Strip-EZ RNA, Ambion) transcribed from PCR fragments, covering the complete cloned cDNAs. Procedures were according to the description of the manufacturer (Ambion). After post-hybridization washes at high stringency, signal was detected and quantified using phosphor imaging equipment (STORM 860, Molecular Dynamics). To study the effects of 1-methylcyclopropene (1-MCP) and ethylene on bending characteristics, gravistimulation was performed in 350 L transparent plexiglass boxes, to which either 100 nL/L 1-MCP or 20  $\mu\text{L/L}$  ethylene were applied. 1-MCP was applied from a 1000  $\mu\text{L/L}$  stock prepared from EthylBloc<sup>TM</sup> (Rohm and Haas, USA); ethylene was applied from a 1% stock in nitrogen. Concentrations of 1-MCP and ethylene were measured by GC.

## RESULTS AND DISCUSSION

Gravistimulation causes an increase in ethylene production in the upper as well as the lower part of the bending zone whereas ethylene production in comparable stem sections from vertical stems was low (Fig. 1). The ethylene production in the lower part was much higher than that in the upper part of the of the gravistimulated stem.

Expression of ethylene genes was studied on Northern blots using labelled RNA probes representing three different ACC synthase genes (*Am-ACS1*, AF083814; *Am-ACS2*, AF038315 and *Am-ACS3*, AF083816), one ACO (*Am-ACO1*, AY333925) and one receptor (*Am-ETR1*, AY159363) gene. After 9 h of gravistimulation *Am-ACS1* expression was increased in both the lower and upper halves of the stem, with slightly higher level in upper halves (Fig. 2). Expression of *Am-ACS2* was not detectable under the conditions employed (data not shown). Expression of *Am-ACS3* was markedly increased in the lower halves but not in the upper halves (Fig. 2). *Am-ACO1* expression increased in both lower and upper halves at 9 h of gravistimulation, with slightly higher level in the lower halves

(Fig. 2). *Am-ETR1* expression level in gravistimulated stems was slightly higher than in vertical stems, with minimal difference between upper and lower halves (Fig. 2).

The data suggest that *Am-ACS3* expression is responsible for the increased ethylene production in the lower halves of gravistimulated stems and it determines the observed differential in ethylene production between upper and lower stem halves. In contrast to *Am-ACS3*, *Am-ACS1* gene expression, which increased in both the lower and upper halves, may be responsible for the increased ethylene production in the upper halves. Given the relatively small changes and lack of clear differential expression of *Am-ACO* and *Am-ETR1*, it is expected that these genes play no regulatory role in differential ethylene production.

The specific localization of *Am-ACS3* expression at the lower halves of gravistimulated stems correlates very well with earlier observations on differential ethylene production, ACC and malonyl-ACC levels in different parts of gravistimulated stems. In *A. majus*, both ACC and malonyl-ACC levels were higher in the lower half than in the upper half of gravistimulated flower stems (Philosoph-Hadas et al., 1996). Also in *Kniphofia* flower stems, ACC levels in the lower half of the bending zone increased within 1h of gravistimulation and, after 8h of gravistimulation the lower half contained approximately 25 times more ACC than the upper half (Woltering, 1991).

To investigate the effect of ethylene on differential growth, gravitropic bending was monitored of flowers that were placed in an ethylene or 1-MCP enriched environment. Gravistimulated stems consistently showed slightly increased bending as a result of 1-MCP and slightly decreased bending as a result of ethylene treatment (Fig. 3). This shows that despite the huge increase in ethylene production and its differential nature during gravitropism, ethylene may only play a minor role in differential growth.

Earlier studies on the role of ethylene in gravitropism in shoots and flowering stems have yielded conflicting results. In some studies a clear effect of ethylene on the kinetics of bending was observed; in others ethylene had no appreciable effect on bending (discussed in Madlung et al., 1999). In *Kniphofia* flower stems, abolishment of the ethylene gradient during gravitropism by treatment with ethylene, through inhibition of ethylene production by amino oxyacetic acid (AOA) or through blocking ethylene perception (and thereby the perception of a gradient) by e.g. silver thiosulphate (STS), 2,5-norbornadiene (NBD) or high CO<sub>2</sub> concentration, did not significantly alter the kinetics of bending (Woltering, 1991). *A. majus* flower stems, however, showed delayed gravitropic bending when treated with STS, NBD or CoCl<sub>2</sub> (Philosoph-Hadas et al., 1995, 1996, 1999). Together with the present results on the effects of ethylene and 1-MCP, this indicates that in this species, the differential ethylene production may slightly modify the bending response.

The physiological significance of ethylene in gravitropism under natural conditions (without added chemicals) has never convincingly been shown. At least some of the chemicals that inhibit gravitropic bending also to some extent modify straight growth of stems (Woltering, 1991). The slower bending rate may then result from overall slower growth rather than from a specific effect on differential growth. Indeed, we have found that concentrations of STS and AOA that slowed down gravitropic bending in *A. majus* also significantly slowed down elongation growth of the stems. However, concentrations of CoCl<sub>2</sub> that significantly inhibited gravitropic bending, did not inhibit straight growth (data not shown). CoCl<sub>2</sub> is a potent inhibitor of ACO activity and it abolished the ethylene gradient in *A. majus* stems (Philosoph-Hadas et al., 1996). The clear effect of CoCl<sub>2</sub> on bending indicates that indeed, in *A. majus*, endogenous ethylene may modify the gravitropic response. It seems therefore, that ethylene plays a certain role in the gravitropic response of *A. majus* flowering stems. However, the role of ethylene is not clearly manifested in most systems.

The results of the present study, which point to an important role of *Am-ACS3* in the gravitropism-related differential ethylene production, may provide a useful means to elucidate its role in gravitropism. Therefore, the ultimate experiment to determine the physiological significance of differential ethylene production in flower stem gravitropism

would be the use of ACS3-knock out plants.

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## Figures

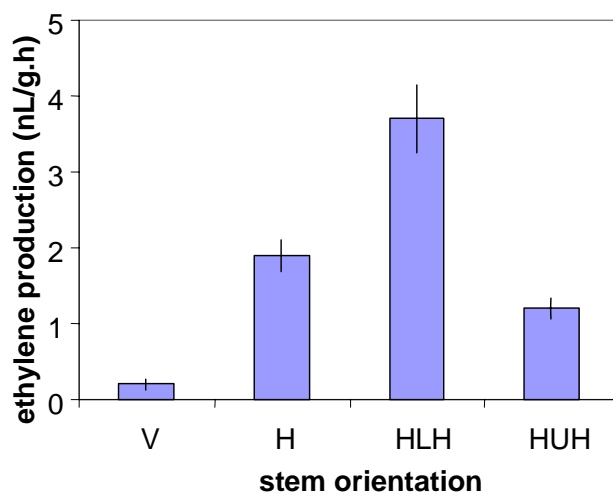


Fig. 1. Ethylene production rates of stem sections excised from the bending zone of *A. majus* stems after 9 h of gravistimulation. V = Vertical control, H = Horizontal stem, HLH = Horizontal Lower Half, HUH = Horizontal Upper Half. Vertical bars represent 2 x SE (n=6).

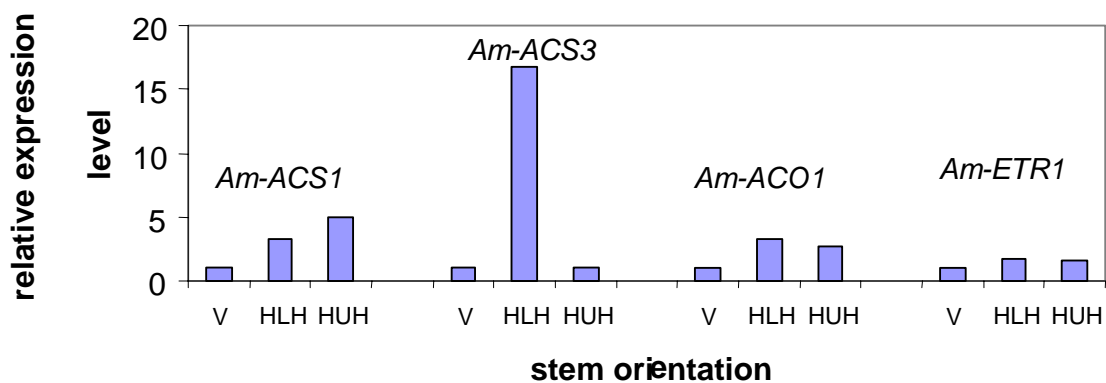


Fig. 2. Relative expression levels (Vertical control = 1) of *Am-ACS1*, *Am-ACS3*, *Am-ACO1* and *Am-ETR1* in stem sections excised from the bending zone of *A. majus* stems after 9 h of gravistimulation. V = Vertical control, HLH = Horizontal Lower Half, HUH = Horizontal Upper Half.

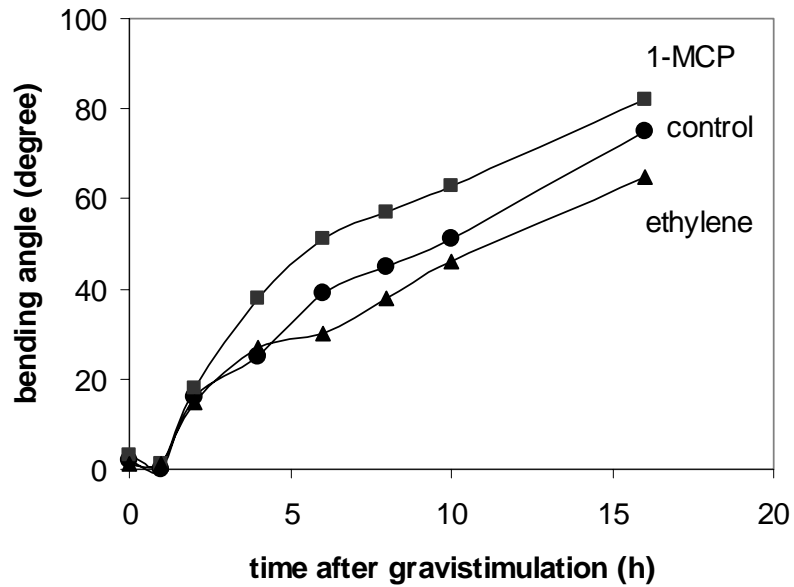


Fig. 3. Effect of 1-MCP and ethylene on the kinetics of *A. majus* stem bending. The gases were continuously present during gravistimulation. At most time points differences in bending between 1-MCP or ethylene and control were not statistically different (n=8). In repeat experiments a similar pattern was observed. In a 6h gravistimulation experiment with larger number of flowers (25), differences between 1-MCP or ethylene and control differences in bending were statistically significant.