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Planta (2005) 220: 403–413
DOI 10.1007/s00425-004-1359-6

ORIGINAL ARTICLE

Ernst J. Woltering · Peter A. Balk
Mariska A. Nijenhuis-deVries · Marilyne Faivre
Gerda Ruys · Dianne Somhorst
Sonia Philosoph-Hadas · Haya Friedman

An auxin-responsive 1-aminocyclopropane-1-carboxylate synthase is responsible for differential ethylene production in gravistimulated *Antirrhinum majus* L. flower stems

Received: 8 March 2004 / Accepted: 19 July 2004 / Published online: 2 September 2004
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Abstract The regulation of gravistimulation-induced ethylene production and its role in gravitropic bending was studied in *Antirrhinum majus* L. cut flower stems. Gravistimulation increased ethylene production in both lower and upper halves of the stems with much higher levels observed in the lower half. Expression patterns 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 of gravistimulated stems and in excised stem sections following treatment with different chemicals. One of the ACS genes (*Am-ACS3*) was abundantly expressed in the bending zone cortex at the lower side of the stems within 2 h of gravistimulation. *Am-ACS3* was not expressed in vertical stems or in other parts of (gravistimulated) stems, leaves or flowers. *Am-ACS3* was strongly induced by indole-3-acetic acid (IAA) but not responsive to ethylene. The *Am-ACS3* expression pattern strongly suggests that *Am-ACS3* is responsible for the observed differential ethylene production in gravistimulated stems; its responsiveness to IAA suggests that *Am-ACS3* expression reflects changes in auxin signalling. *Am-ACS1* also showed increased expression in gravistimulated and IAA-treated stems although to a much lesser extent than *Am-ACS3*. In contrast to *Am-ACS3*, *Am-ACS1* was also expressed in non-bending regions of vertical and gravistimulated stems and in leaves, and *Am-ACS1* expression was not confined to the lower side cortex but evenly distributed over the diameter of the stem. *Am-ACO* and *Am-ETR/ERS* expression was increased in both the lower and upper halves of gravistimulated stems. Expression of both *Am-ACO* and *Am-ETR/ERS* was responsive to ethylene, suggesting regulation by IAA-dependent differential ethylene production. *Am-ACO* expression and in vivo ACO activity, in addition, were induced by IAA, independent of the IAA-induced ethylene. IAA-induced growth of vertical stem sections and bending of gravistimulated flowering stems were little affected by ethylene or 1-methylcyclopropene treatments, indicating that the differential ethylene production plays no pivotal role in the kinetics of gravitropic bending.

Keywords 1-Aminocyclopropane-1-carboxylic acid · *Antirrhinum* (gravitropism) · Auxin · Ethylene · Flower stem · Gravitropism

Abbreviations ACC: 1-Aminocyclopropane-1-carboxylic acid · ACO: ACC oxidase · ACS: ACC synthase · AVG: L-α-(Aminoethoxyvinyl)glycine · IAA: Indole-3-acetic acid · 1-MCP: 1-Methylcyclopropene

E. J. Woltering (✉) · P. A. Balk · M. A. Nijenhuis-deVries
M. Faivre · G. Ruys · D. Somhorst
Agrotechnology & Food Innovations (A&F),
Wageningen University & Research Center,
PO BOX 17, 6700 AA Wageningen,
The Netherlands
E-mail: ernst.woltering@wur.nl
Fax: +31-317-475347

S. Philosoph-Hadas · H. Friedman
Department of Postharvest Science of Fresh Produce,
ARO, The Volcani Center,
PO BOX 6, 50250 Bet Dagan, Israel

Present address: G. Ruys
Laboratory of Plant Genetics,
Wageningen University & Research Center,
PO BOX 9101, 6700 HB Wageningen, The Netherlands

Introduction

Gravity is an important environmental cue that aids plants to optimally orient themselves with respect to life-supporting resources such as water and light. Plants respond to gravity by exerting differential growth be-

tween upper and lower sides in a variety of organs. Roots generally grow in the direction of the gravity vector whereas shoots grow in the opposite direction (negative gravitropism). 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 a few reports have been published on the gravitropic response of flower stalks (e.g. Halevy and Mayak 1981; Clifford and Oxlade 1989; Woltering 1991; Fukaki et al. 1996; Philosoph-Hadas et al. 1996; Friedman et al. 1998; Weise and Kiss 1999).

The gravitropic response in plants can be separated into three sequential steps: gravity perception, signal transduction and an asymmetric growth response. Asymmetric growth is thought to be controlled by changing hormone levels in different parts of graviresponding organs. Even now, asymmetric distribution of auxin, as already described by the Cholodny–Went theory (Went and Thimann 1937), is still considered to be the main causative factor for differential growth although the observed changes in free auxin levels are often rather small, non-existent or transient (Mertens and Weiler 1983; Clifford et al. 1985; Schwark and Bopp 1993; Philosoph-Hadas et al. 2001). Recent findings regarding the lateral relocation of the auxin efflux regulator PIN3 upon gravistimulation and the observed differential expression of a synthetic *DR5::GUS* auxin reporter element, however, strongly support the auxin-redistribution theory (Friml et al. 2002; Ottenschläger et al. 2003). Rapid cycling of PIN3 proteins between the plasma membrane and other compartments of the cell in gravisensing tissues (starch sheath layer, columella) provides a mechanism to rapidly respond to changes in orientation by redirecting auxin efflux and differential growth (Friml et al. 2002).

The role of ethylene in gravitropism has also been investigated. In a variety of gravi-responding systems, an increased ethylene production in the lower half of the gravistimulated stems is observed, and this is accompanied by increased levels of 1-aminocyclopropane-1-carboxylic acid (ACC) and internal ethylene (e.g. Woltering 1991; Philosoph-Hadas et al. 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 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 stems 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 gravistimulated stems (Woltering 1991; Woltering et al. 1991), indicating that differential ethylene production is regulated by ACS.

The role of the differentially produced ethylene in asymmetric growth is still controversial (discussed in Madlung et al. 1999). In several reported cases, abolishment of the ethylene gradient by, for example, the use of ethylene production or perception inhibitors did not substantially alter the gravitropic response (e.g. Woltering 1991; Madlung et al. 1999). In addition, mutant tomato seedlings defective in ethylene perception show only slight alteration in bending kinetics, indicating that ethylene is not an absolute requirement for the gravitropic response (Madlung et al. 1999).

We have investigated the regulation of differential ethylene biosynthesis in gravistimulated *Antirrhinum majus* flower stems by studying the expression patterns of ACS, ACO and ethylene receptor (ETR/ERS) genes. Based on the gene expression patterns in response to gravistimulation, and to treatments with IAA, ethylene and 1-methylcyclopropene (1-MCP) we conclude that localised expression of *Am-ACS3*, in response to changing auxin signalling, is responsible for differential ethylene production. The differential ethylene production, however, does not play a pivotal role in differential growth.

Materials and methods

Chemicals

Ethylene was from Praxair (Oevel, Belgium); a dilution of 1% in N_2 was used to obtain required concentrations in plant chambers. 1-Methylcyclopropene (1-MCP) was liberated from EthylBloc (a gift from Floralife, USA). All other chemicals were from Sigma.

Plant material and treatments

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 at 15 μmol photons m^{-2} s^{-1} at plant level). In initial experiments it was established that the kinetics of bending was similar in light and in dark. At different time points, 4-cm-long stem sections were excised from the bending zone and used for the analysis of ethylene production and gene expression. Sections excised from gravistimulated flower stems were longitudinally divided into 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. In some experiments, leaves, flowers and stem sections at non-bending locations were also sampled.

For desiccation treatment, isolated stem sections were left dry for 4 h at 20°C and 60% relative humidity. Wounding was done by making superficial cuts in the cortex of the isolated stem sections, after which they were placed for 4 h with their basal end in water.

To analyse the effects of ethylene and 1-MCP on indole-3-acetic acid (IAA)-induced growth and gene expression, isolated stem sections from the putative bending zone were placed with their basal end in small tubes containing a range (0.01–5 mM) of IAA concentrations and were subsequently placed in 70-l airtight stainless-steel chambers, to which either approximately 20 μl l^{-1} ethylene or approximately 100 nl l^{-1} 1-MCP was applied. Ethylene was applied from 1% dilution of ethylene gas; 1-MCP was applied from a 1,000 μl l^{-1} stock prepared from EthylBloc. Concentrations of both ethylene and 1-MCP were measured by GC. For RNA analysis, samples were taken after 6 h of treatment. For determination of growth, the length and weight increase of stem sections were determined after 24 h of treatment.

To study the effects of ethylene and 1-MCP on bending characteristics, gravistimulation was performed in 350-l transparent Plexiglas boxes, to which either 20 μl l^{-1} ethylene or 100 nl l^{-1} 1-MCP was applied.

Measurements of ethylene production and in vivo ACO activity

Stem sections excised at different times from the bending zone of vertically and gravistimulated stems were longitudinally halved and enclosed for 1 h in 30-ml tubes. Thereafter, a sample of the headspace was analysed for ethylene by GC (Chrompack model 437A). To study the effects of IAA and ethylene on in vitro ACO activity, stem sections were pre-treated for 16 h with either water or 0.1 mM L-α-(aminoethoxyvinyl)glycine (AVG) and thereafter supplied with a range of IAA concentrations for 4 h. Stem sections were then incubated in 30-ml closed glass vials containing either water or 1 mM ACC. After 2 h incubation, ethylene in the headspace was measured by GC.

Molecular cloning of ACS, ACO and ETR/ERS cDNA fragments

Total RNA from differently treated stem sections (non-treated, IAA-treated, 6 h gravitropic stimulation) was isolated according to Chang et al. (1993). All primers used were synthesised by Eurogentec, Belgium. To clone ACS cDNA fragments, RT–PCR, using degenerate primers 5′-GAGGATCCARATGGGIYTIGCIGAYAAYCA (forward) and 5′-GCAGATCTACICKRAACCAICCIGGYTC (reverse) was performed according to standard procedures. Reaction products were cloned using the pGEM-T Easy cloning system from Promega. Sequence analysis of the obtained products was done using sequencing equipment from Amersham Pharmacia. Cloning of ACO cDNA fragments was done using degenerate primers 5′-TGYGARAAYTGGGGHTTCTTTGAG (forward) and 5′-CATKGCYTCRAAYCTBGGCTCYTTDGC (reverse), and cloning of ETR/ERS cDNA fragments was done using degenerate primers 5′-TGGGTSCTHRTDCARTTYGGHGC (forward) and 3′-GCAGCATGWGARAGW GCSACWGC (reverse).

A full-length cDNA coding for *Am-ACS3* was isolated using the Marathon cDNA Amplification protocol from Clontech. 5′-RACE primer 5′-AGTTTCGTTGGCGGAGGTAGCG-3′ and 3′-RACE primer 5′-GATGCATTTACTCGAACGACCCG-3′ were used, being specific for the *Am-ACS3* cDNA fragment. PCR reactions were performed using the Advantage-2-PCR Enzyme System from Clontech. Reaction products were cloned using the pGEM-T Easy cloning system (Promega) and sequenced by Eurogentec (Belgium).

Expression analysis

Total RNA was isolated according to Chang et al. (1993) and 20 μg was separated on a 1.5% agarose gel. The RNA was blotted onto positively charged nylon membrane (Boehringer) and cross-linked by UV irradiation. Thereafter, the blot was deglyoxylated by boiling for 5 min in 20 mM Tris–HCl (pH 8.0), 1 mM EDTA. Blots were hybridised 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-hybridisation washes at high stringency, signal was detected using exposure to BioMax MS film (Kodak) or by using Phosphor Imager equipment (STORM 860; Molecular Dynamics). All experiments were repeated at least once; representative data are shown. Blots were re-probed with a tomato (*Lycopersicon esculentum*) DNA probe for ribosomal RNA (Nijenhuis-de Vries et al. 1994) to check for equal loading.

Results

Ethylene production and bending kinetics in gravistimulated flower stems

Gravistimulation induced a rapid increase in ethylene production in the upper as well as the lower halves of the bending zone, whereas ethylene production in comparable sections from vertical stems showed only minor variations (Fig. 1a,b). The ethylene production in the

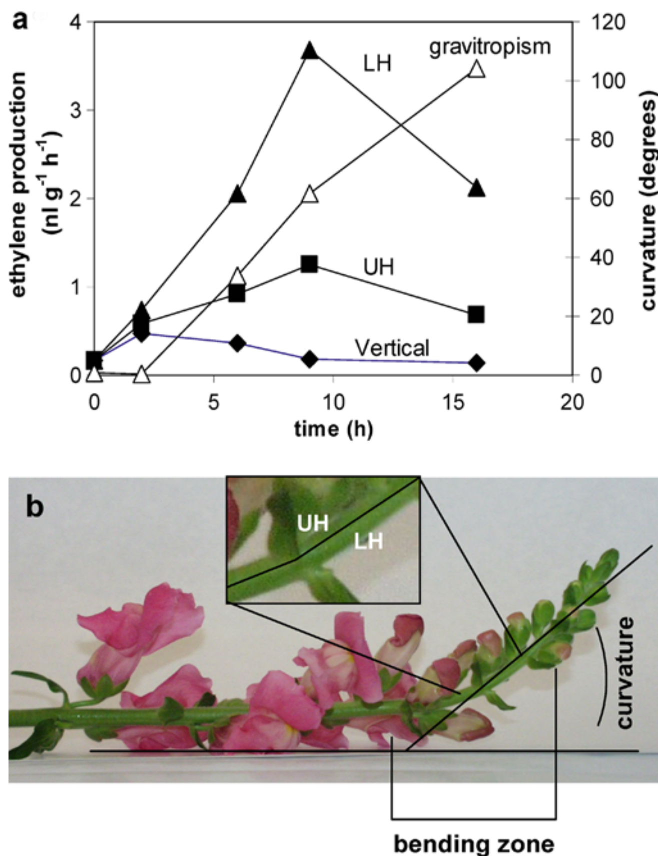

Fig. 1a,b Ethylene production rates and curvature during gravistimulation of *Antirrhinum majus* flower stems. **a** Ethylene production rates (*solid symbols*) of longitudinally halved 4-cm stem sections excised from the bending zone at different times in vertical controls (*V*) and in upper (*UH*) and lower (*LH*) halves of gravistimulated stems. SE values were less than 10% of the mean ($n=6$). Curvature (*open triangles*) was measured as the angle between spike orientation and the horizontal. **b** Photograph showing the bending zone and sample position in a gravistimulated stem

lower part was much higher than that in the upper part of the gravistimulated stem. Upward bending of the stem started within 2–3 h of gravistimulation. The differential increase in ethylene production closely coincided with the start of stem bending.

Cloning of cDNAs representing ACS, ACO and ETR/ERS genes

Using primers to conserved regions of *ACS*, *ACO* and *ETR*/*ERS*, partial cDNA clones of about 1,040, 825 and 730 base pairs, respectively, were obtained by PCR. Following cloning and sequencing of a number of independent putative *ACS* fragments, three different genes were identified and the complete coding sequence of *Am-ACS3* was obtained using RACE (*Am-ACS1*, AF083814; *Am-ACS2*, AF038315; *Am-ACS3*, AF083816). An alignment of the three deduced amino acid sequences is shown in Fig. 2. The three fragments were quite divergent with only 54 (ACS1:ACS2/3) to 57% (ACS2:ACS3) identity at amino acid level. The conserved active site and a number of additional amino acids conserved among amino transferases were identified in all three ACS deduced amino acid sequences (Fig. 2).

Cloning and sequencing of a number of independent putative *ACO* fragments yielded two different partial *ACO* cDNAs (*Am-ACO1*, AY333925; *Am-ACO2*, AY3333926) with 90% identity at amino acid level (data not shown). The primers for cloning *ETR*/*ERS* genes were chosen in such a way that the cloned fragments contained part of the putative membrane-embedded domain. Cloning and sequencing of a number of independent putative *ETR*/*ERS* fragments yielded two different partial *ETR*/*ERS* cDNAs (*Am-ETR1*, AY159363; *Am-ETR2*, AY159362) with 76% identity at amino acid level to each other (data not shown).

Expression patterns of ACS, ACO and ethylene receptor genes in gravistimulated stems

Patterns of gene expression were studied on Northern blots using labelled RNA probes representing the three different ACS gene fragments (*Am-ACS1*, *Am-ACS2*, *Am-ACS3*), one ACO gene fragment (*Am-ACO1*) and one ETR/ERS gene fragment (*Am-ETR1*) (Fig. 3). Under the conditions of high stringency, the *ACS* gene-specific probes did not cross-hybridise (data not shown).

The overall expression level of *ACS2* was very low. After prolonged exposure of the blot (24 h), *ACS2* expression levels became visible on the autoradiograph only in the 2-h samples of both vertical and gravistimulated stems (data not shown). The expression profiles of *ACS1* and *ACS3* could easily be determined on blots exposed for 6 h. *ACS1* expression in both vertical and horizontal stems showed huge variation over time with relatively high levels at 6 h and low levels at 9 h of gravistimulation. The consistency of this pattern in both vertical and horizontal stems implies an endogenous rhythm. Compared to *ACS1* expression levels in vertical stems, an increased expression in the upper halves and a decreased expression in the lower halves of gravistimulated stems were apparent during the first 6–9 h of gravistimulation. At 16 h of gravistimulation this differential was reversed. Expression of *ACS3* showed a very clear profile in all the experiments. Within 2 h of gravistimulation an appreciable increase in *ACS3* expression level was observed. This increase was restricted to the lower half of the stem. Between 9 and 16 h of gravistimulation the expression level decreased. In the upper half of the gravistimulated stem and in vertical stems, *ACS3* expression was not detectable.

More-precise localisation of the expression of the three *ACS* genes was studied in a similar experiment where, following 6 h of gravistimulation, the lower half of the stem was divided into the inner vascular cylinder and the outer cortical layers. These outer layers contained the starch sheath as evidenced by staining with KI

Fig. 2 Comparison of the deduced amino acid sequences of *Antirrhinum majus* ACC synthases. Conserved amino acids in the three *A. majus* ACC synthases and in the *Lycopersicum esculentum* (*Le-ACS3*, U17972) ACC synthase are marked by *asterisks*. Invariable amino acids, conserved among aminotransferases and ACC synthases from different sources, are highlighted in *grey*. The *boxed* peptide sequence is part of the active site of ACC synthases. *Underlined* sequences show the regions used for primer design

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Am-ACS1     ---------- ---------- ---------- ---------- ---------L
Am-ACS2     ---------- ---------- ---------- ---------- ---------V
Am-ACS3     MA-LSRKATC NSHGQDSSYF LGWEEYEKNP YDAVLNPKGI IQMGLAENQL
Le-ACS3     MKLLSEKATC NSHGQDSSYF LGWQEYEKNP YDEIQNPKGI IQMGLAENQL
            *  ** **** ********** *** ****** **   ***** *********

SFDLVEAWIR DNPKASICTP EGAYNFKNIA NFQDYHGLPV FRNAVARFME KVRGNRVRFD
SFDLLEEYLE KHSEATNCDS RNSG-FRENA LFQDYHGLLS FRKAMASFME QIRGGRATFD
SFDLLESWLS ENPDAAGFKR NGESIFRELA LFQDYHGLPA FKNAMVNFMS EIRGNKVNFD
SFDLLESWLA QNPDAAGFKR NGESIFRELA LFQDYHGLPA FKNAMTKFMS EIRGNRVSFD
**** *         *           *   *  *******   *  *   **     *    **

PDKIVMSGGA TGAQETLAFC LADPGDALLV PTPYYPGNDR DITWRTGIQL LPVVCESSNE
PERVVITAGA PPANELLTFI LANPGDALLI PTPYYPGFDR DLRWRTGVNI VPIHCDSSNN
PNKLVLTAGA TSANETLMFC LAEPGDAFLL PTPYYPGFDR DLKWRTGVEI VPIQCTSSNG
SNNLVLTAGA TSANETLMFC LANQGDAFLL PTPYYPGFDR DLKWRTGAEI VPIHCSSSNG
    *   **   * * * *  **  *** *  ******* ** ** ****     *    ***

FKLTHEALES AYKKAQDSNI KVKGLLLNNP SNPLGIVLDK QTLTDSLNFT NDKNIHLICD
FEITPQALEA AYNEAESKNI KVKGVLITNP SNPLGATIKR AILEQILEFV TRKNIHLISD
FRITAPALED AYELAQKRNL KVKGVLVTNP SNPLGITLTR QELNLLVKFI DKKGIHLISD
FRITESALEE AYLDAKKRNL KVKGVLVTNP SNPLGTTLNR NELELLLTFI DEKGIHLISD
*  *  ***  **  *   *  **** *  ** *****  *     *     *    * **** *

EIYSATVFSQ PS------FT SIAEIVQENE NYNR----DL IHIVYSLSKD LGFPGFRVGI
EIYSGSSFSP E------EFV SIAEILEAQD -YKNS---ER VHIVYSLSKD LGLPGFRVGT
EIYSGTVFNS RILSASWKFS RTENMLMIKS -Y-----WNR VHIEYSLSKD LGLPGFRVGC
EIYSGTVFNS PGL-VSVMEV LIEKNYM-KT R-----VWER VHIVYSLSKD LGLPGFRIGA
****   *                                     ** ****** ** **** *

IYSYNQT-VM NCARKMSSFG LVSTQTQHLI ATMLSDSDFV DRFIDESAKR LGKRHGVLSR
IYSYNDK-VV TTARRMSSFT LISSQTQHLL ASMLSDKEFT KEYININRSR LRKRYEMIVD
IYS-NDPVVV AAATKMSSFG LVSSQTQYLL SAMLSNKKFA RSYIVENQKR LRTVHAMLVR
IYS-NDEMVV SAATKMSSFG LVSSQTQYLL SCMLSDKKFT KKYISENQKR LKRRHAMLVK
*** *   *    *  ****  * * *** **   ***         *     * *

GLAQVGIGSL KSNAGLYCWM DLRRLLKEPT FEAELDLWRV ILNEVKLNVS PGASFHCS--
GLKEAGIECL KGNAGLFCWM NLKPLLEKPT KEHEVELWKL IMYEVKLNIS PGSSCHCS--
GLKSTGISCL ESNAGLFCWV DMRHLLSSNT FEAEMDLWKK IVYEVGLNIS PGSSCHCDEP
GLKSAGINCL ESNAGLFCWV DMRHLLSSNN FDAEMDLWKK IVYDVGLNIS PGSSCHCTEP
**   **  *   **** **      **           **   *   * ** * ** * ** **

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---------- ---------- ---------- ---------- ---------- ----------
GWFRVCFANM SEETLELAIQ RIKLFVNRHH CN-------- ---------- ----------
GWFRVCFANM SEDTLDLAMR RIKDFVESTA PNATNHQNQQ QSNANSKKKS FSKWVFRLSF
********** ** ** **   *** **      *

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NDRQRER
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(data not shown). *Am-ACS2* expression was very low and no relation with stem orientation could be established (Fig. 4). *Am-ACS1* showed slightly higher expression in the lower half of gravistimulated stems but there was no difference in expression levels between vascular cylinder and cortical layers. *ACS3* was highly expressed in the lower half of gravistimulated stems and expression was exclusively restricted to the cortical cell layers (Fig. 4).

Am-ACO1 expression in vertical stems showed considerable variation throughout the day, indicative of an endogenous rhythm. Compared to expression levels in vertical stems, an increase was observed in both lower and upper halves at 9 and 16 h of gravistimulation (Fig. 3). In vertical stems, expression levels of *Am-ETR1* were low and did not show appreciable changes over time (Fig. 3). During the first 9 h of gravistimulation, *ETR1* expression levels gradually increased in both the lower and upper halves of the gravistimulated stem. At 16 h, expression levels had decreased again (Fig. 3).

ACS gene expression is tissue- and stimulus-specific

To investigate the tissue specificity of the three *Am-ACS* genes, mRNA levels were analysed in different parts of the flower stem, in leaves and in different flower parts. Samples were taken from both vertical and 6-h-gravistimulated flowering stems. None of the *ACS* genes was expressed in flower parts of either vertical or gravisti-

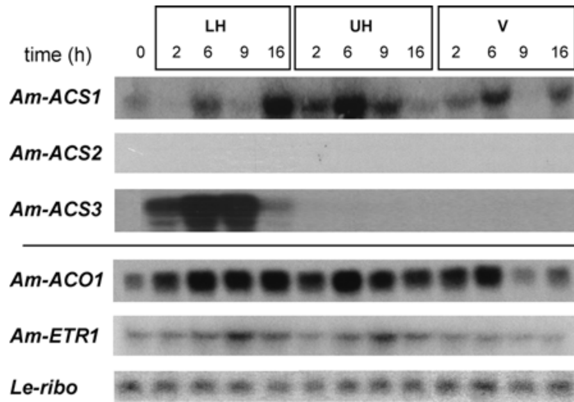

Fig. 3 Expression profiles of ethylene biosynthetic and receptor genes during gravistimulation. RNA was isolated from lower (*LH*) and upper halves (*UH*) of *A. majus* stems at different times following gravistimulation and from control vertical (*V*) stems. Blots were hybridised with labelled antisense RNA probes representing three different *Am-ACS* genes, one *Am-ACO* and one *Am-ETR*/*ERS* gene. Blots were exposed for 6 h. RNA loading was analysed using a tomato (*Lycopersicon esculentum*) DNA probe for ribosomal RNA (*Le-ribo*)

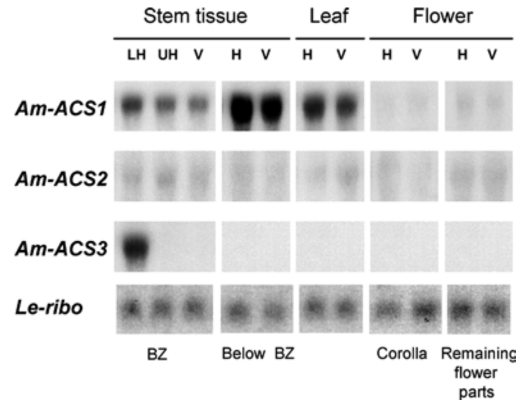

Fig. 5 Tissue-specific expression of *Am-ACS* genes in *A. majus*. RNA was isolated from 4-cm-long bending-zone (*BZ*) stem segments of lower (*LH*) and upper halves (*UH*) of 6-h-gravistimulated stems and of vertical (*V*) stems. RNA was also isolated from a position just below the stem BZ, and from leaves and flowers of 6-h-gravistimulated (*H*) and of vertical (*V*) flowering stems. The flower was divided into corolla and remaining flower parts including reproductive organs. Blots were hybridised with labelled antisense RNA probes representing three different *Am-ACS* genes and exposed for 6 h. RNA loading was analysed using a tomato DNA probe for ribosomal RNA (*Le-ribo*)

mulated stems (Fig. 5). *ACS1* expression was clearly detectable in stem parts, both at the position of the bending zone and in the non-bending region of the stem. In both vertical and gravistimulated stems, *ACS1* expression was much more pronounced in the non-bending than in the (putative) bending part of the stem. *ACS1* was also abundantly expressed in leaf tissue. Gravistimulation caused a slight increase in *ACS1* expression in both stem parts and in leaves (Fig. 5). Expression of *ACS3* was exclusively restricted to the lower side of the bending zone of gravistimulated stems, showing its tight relation to the observed ethylene production during gravistimulation (Fig. 5). Expression levels of *ACS2* were very low in all investigated plant parts and no information about possible tissue specificity was obtained.

Stem sections excised from the putative bending zone were exposed to different treatments known to affect ACS gene expression in other systems (moderate desiccation, wounding, ethylene, IAA) and expression levels of the three ACS genes were investigated (Fig. 6). Desiccation strongly decreased *ACS1* and *ACS2* expression compared to the level on control stems. Wounding increased the expression level of *ACS1* but did not affect

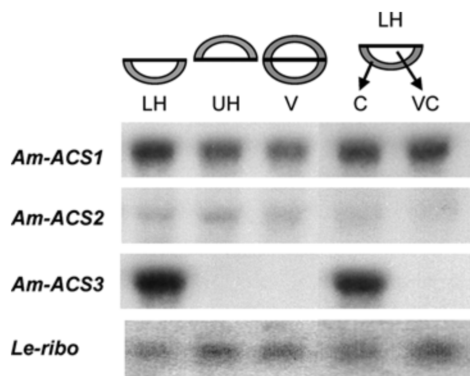

Fig. 4 *Am-ACS3* expression is restricted to the cortical layers of the gravistimulated *A. majus* flower stem. RNA was isolated from lower (*LH*) and upper halves (*UH*) of gravistimulated stems, from vertical stems (*V*), and from cortex (*C*) and vascular cylinder (*VC*) of the lower stem halves following 6 h of gravistimulation. Blots were hybridised with labelled antisense RNA probes representing three different *Am-ACS* genes and exposed for 6 h. RNA loading was analysed using a tomato DNA probe for ribosomal RNA (*Le-ribo*)

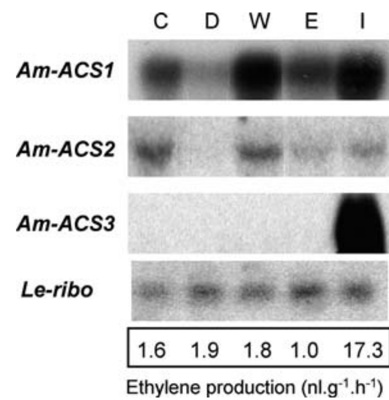

Fig. 6 Effect of different treatments on ethylene production rates and the expression of ACC synthase genes in excised stem sections of *A. majus*. Stem sections were treated as follows: placed in water and left untreated (*C*); left dry (*D*); placed in water and wounded (*W*) or treated with ethylene (*E*); placed with their basal end in a 1 mM IAA solution (*I*). After 4 h, ethylene production rates were determined and samples for RNA analysis were taken. Blots were hybridised with labelled antisense RNA probes representing three different *Am-ACS* genes and exposed for 6 h. RNA loading was analysed using a tomato DNA probe for ribosomal RNA (*Le-ribo*)

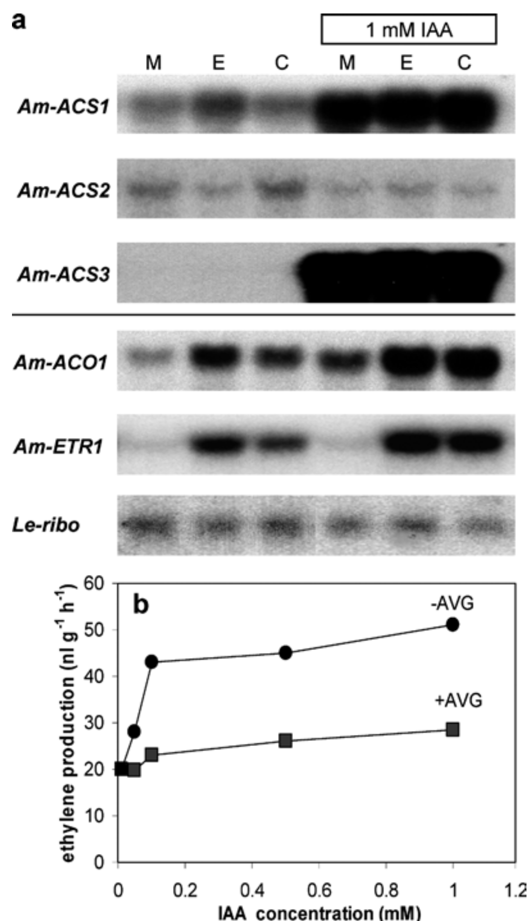

Fig. 7a,b Effect of IAA and ethylene on expression of ethylene-related genes and in vivo ACC oxidase activity in stem sections of *A. majus*. **a** Stem sections were placed with their basal end in either water or a 1 mM IAA solution and were simultaneously treated with either 1-MCP (*M*), ethylene (*E*) or left untreated (*C*). Samples for RNA were collected after 4 h of treatment. Blots were hybridised with labelled antisense RNA probes representing three different *Am-ACS* genes, one *Am-ACO* and one *Am-ETR/ERS* gene. Blots were exposed for 6 h. RNA loading was analysed using a tomato DNA probe for ribosomal RNA (*Le-ribo*). **b** Stem sections were pre-treated with either water or 0.1 mM AVG for 16 h and thereafter treated for 4 h with a range of IAA concentrations. Subsequently, 1 mM-ACC-stimulated ethylene production (in vivo ACO activity) was determined. SE values were less than 10% of the mean ($n=6$)

expression of the other *ACS* genes. Ethylene increased expression of *ACS1*, decreased expression of *ACS2* and had no effect on expression of *ACS3*. IAA treatment strongly increased expression of *ACS1* and *ACS3* and decreased expression of *ACS2*. IAA caused a substantial increase in ethylene production (Fig. 6) and the observed changes in ACS gene expression may have resulted from interaction with the simultaneously produced ethylene.

The role of ethylene in IAA-induced gene expression

To further investigate the possible role of ethylene in IAA-induced up-regulation of *ACS1* and *ACS3* and down-regulation of *ACS2* expression, stem sections were treated with IAA in the presence of ethylene or the gaseous inhibitor of ethylene perception, 1-MCP. This experiment confirmed that IAA increases expression levels of *ACS1* and *ACS3* but decreases expression of *ACS2* (Fig. 7a), and showed that IAA-induced changes in ACS gene expression are independent of (IAA-induced) ethylene (Fig. 7a).

The effects of IAA and ethylene on *ACO1* and *ETR1* expression were also studied. *ACO1* expression was increased in ethylene and decreased in 1-MCP-treated stem sections, indicating that *ACO1* expression is regulated by ethylene (Fig. 7a). *ACO1* expression levels were also increased in IAA-treated samples. *ACO1* expression in samples from the combined IAA + ethylene and IAA + 1-MCP treatments were clearly higher than in their respective non-IAA-treated counterparts, indicating that IAA-induced *ACO1* expression is under control of both IAA and (IAA-induced) ethylene. *ETR1* expression was regulated by ethylene; IAA-induced expression of *ETR1* may solely result from IAA-induced ethylene as 1-MCP treatment completely abolished IAA-induced expression (Fig. 7a).

The effect of IAA on ACC-induced ethylene production, being a measure of in vivo ACO activity, was studied in stem sections pre-treated with the inhibitor of ACS, L-α-(aminoethoxyvinyl)glycine (AVG). AVG-treated stem sections did not produce any increased ethylene following IAA treatment, whereas non-AVG-treated stem sections showed a marked increase in ethylene production in response to IAA (data not shown). In vivo ACC oxidase activity was stimulated by addition of IAA. Over a wide range of IAA concentrations, in vivo ACC oxidase activity was considerably lower in AVG-treated samples than in water-treated samples (Fig. 7b), confirming the view that maximum IAA-induced ACO gene expression and activity requires IAA, as well as ethylene-related signalling pathways.

Ethylene is not required for differential growth

To investigate if ethylene produced during gravistimulation affects stem growth, stem sections excised from the bending zone were treated with a range of IAA concentrations in the presence of ethylene or 1-MCP. IAA was ineffective at 0.001 mM (data not shown); at higher concentrations, IAA induced linear growth of stem sections with maximum growth between 0.05 and 1 mM IAA (Fig. 8a). The response of the stem sections to IAA was affected neither by ethylene nor 1-MCP, indicating that stem growth is regulated by IAA independent of (IAA-induced) ethylene (Fig. 8a). Similar results were obtained when growth was measured by weighing the stem sections (data not shown). To investigate the effect of ethylene on gravitropic bending, flowers were placed in either an ethylene- or 1-MCP-enriched environment. Gravistimulated stems consistently showed slightly increased bending in the presence

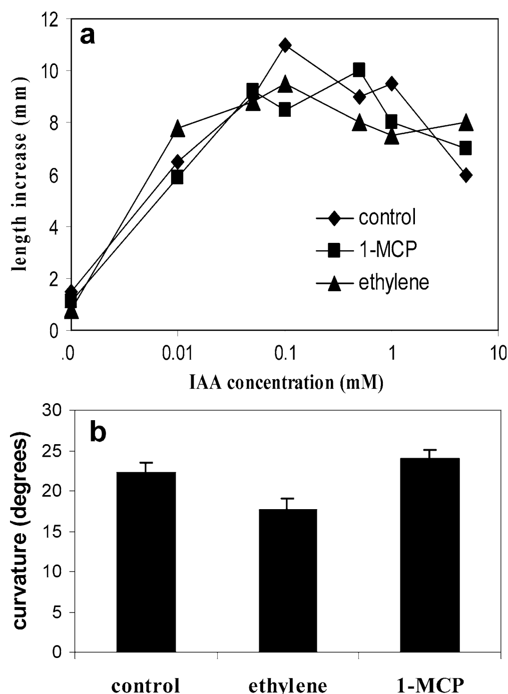

Fig. 8a,b Effect of ethylene and 1-MCP on IAA-induced elongation and gravitropic bending of *A. majus* stems. **a** Stem sections (4 cm length) were placed with their basal end in solutions of different IAA concentrations and either left untreated (*control*) or simultaneously treated with either 1-MCP or ethylene. After 24 h the increase in length was measured. SE values were less than 12% of the mean ($n=12$). **b** Flowering stems were gravistimulated in air (*control*) and under ethylene- or 1-MCP-enriched conditions. Bending angle was measured after 6 h of gravistimulation. Vertical bars represent SE ($n=25$). The difference between the control and ethylene treatment was statistically significant ($P<0.005$); the difference between the control and 1-MCP treatment was not significant ($P=0.11$)

of 1-MCP and slightly decreased bending in the presence of ethylene, indicating a minor inhibitory effect of ethylene on differential growth (Fig. 8b).

Discussion

Plants generally contain small families of the ethylene biosynthetic and perception genes and several examples of temporal, tissue- and stimulus-specific expression of *ACS* family members have been described (e.g. Liang et al. 1992; Yip et al. 1992; Ten Have and Woltering 1997; Arteca and Arteca 1999). Although to a lesser extent, such variability in tissue- and stimulus-specificity of ACO (e.g. Kende 1993; Barry et al. 1996) and ETR/ERS (e.g. Tieman and Klee 1999; Muller et al. 2000) genes is also apparent. This implicates the existence of a differentiated molecular machinery to enable plants to respond in a specific way to a diversity of developmental and environmental signals.

To investigate the regulation of ethylene production during gravitropic bending of above-ground plant parts, we isolated three different *ACS*, two *ACO* and two *ETR/ERS* cDNAs from *A. majus* flower stems and studied their expression in the bending zone of gravistimulated stems. The isolated ACS genes showed very divergent expression patterns and tissue- and stimulus-specificity. One of the genes, *ACS3*, was exclusively expressed in the cortex of the bending zone at the lower side of gravistimulated stems and no expression was observed either in vertical stems or in other parts of (gravistimulated) stems, leaves or flowers. *ACS3* was not responsive to ethylene but was strongly induced by IAA (Figs. 3, 4, 5, 6). The expression pattern of this gene in gravistimulated stems strongly suggests that *ACS3* is responsible for the pronounced increase in ethylene production in the lower side of gravistimulated stems (Fig. 1). The strict localisation of *ACS3* expression to the lower-side peripheral layers of gravistimulated stems correlates very well with earlier observations on ethylene production, ACC and malonyl-ACC levels in different parts of gravistimulated stems. In *Kniphofia* flower stems, ACC levels in the lower half of the bending zone increased within 1 h of gravistimulation, and after 8 h of gravistimulation the lower half contained approximately 25 times more ACC than the upper half. Over 80% of the ACC in the lower half was confined to the peripheral layers (Woltering 1991). Also 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).

The strong inducibility of *ACS3* by IAA (Figs. 6, 7a) and the higher amounts of free IAA observed in the lower half of gravistimulated stems during the first hour of gravistimulation (Philosoph-Hadas et al. 2001) suggest that *ACS3* expression may be related to auxin signalling and may therefore be an indirect effect of gravistimulation-induced changes in auxin activity. The pattern of *ACS3* expression shows similarity to expression of the early auxin-responsive genes *Am-SAUR1* and *Am-AUX/IAA* that increase in the lower half of gravistimulated *A. majus* stems (Philosoph-Hadas et al. 2001), being another indication that *ACS3* expression may reflect changes in auxin activity.

Gravity sensing is thought to occur through sedimentation of starch-containing plastids (amyloplasts) in specialised cells known as statocytes. In gravistimulated roots, sedimentation of amyloplasts is found in the central columella cells of the root cap whereas in gravistimulated shoots this has been observed in the innermost layer of the cortex, designated "starch sheath" or "endodermis" (Sack 1997). The absence of a normal endodermis in *A. thaliana* mutants lacking a gravitropic response in shoots and inflorescence stems confirmed these earlier observations, and shows that indeed this layer is important for gravisensing (Tasaka et al. 1999). In *A. majus* flowering stems, gravity-induced sedimentation of starch-containing chloroplasts in the inner cortex and around the vascular system in the stele was observed (Friedman et al. 1998, 2003), which shows that a similar process is involved in gravisensing in *A. majus*. Recently, an *A. thaliana* auxin efflux regulator (PIN3), involved in asymmetric auxin

distribution and differential growth, was identified (Friml et al. 2002). In the root columella cells, PIN3 rapidly relocalises laterally upon gravistimulation. *PIN3* expression and protein levels were high in the shoot endodermis and around the vasculature, indicating that these are the locations where auxin redistribution occurs following gravistimulation. Our observation that *ACS3* expression is restricted to the cortical layers (including the starch sheath) of the *A. majus* flower stems (Fig. 4) strongly suggests that its expression is regulated by changes in auxin levels in this tissue.

One of the other ACS genes (*ACS1*) also showed increased expression in gravistimulated and IAA-treated stems, although to a lesser extent than *ACS3*. In contrast to *ACS3*, *ACS1* was responsive to ethylene (Fig. 7a) and was also expressed in non-growing regions of vertical and gravistimulated stems and in leaves (Fig. 5). *ACS1* expression was not confined to the cortical cell layers but evenly distributed over the diameter of the stem (Fig. 4). The lack of tissue specificity and observed minor changes in response to gravistimulation show that *ACS1* is not a main regulator of differential ethylene production in gravistimulated stems. *ACS1* may be responsible for wound ethylene production as its expression levels were strongly induced by wounding (Fig. 6). Expression of *ACS2* in flower stems was generally very low and no pattern related to gravitropism could be detected.

The RNA probes used to study expression of *ACO* and *ETR*/*ERS* were not gene-specific and may have detected more than one member of the respective gene families. Nevertheless, it was shown that expression of these gene family members was increased in both the lower and upper sides of the gravistimulated stem with slightly higher levels of *ACO* in the lower side (Fig. 3). As expression of both genes was responsive to ethylene, their increased expression following gravistimulation may be induced by the increased ACS-dependent (differential) ethylene production (Fig. 7a). Apart from regulation by ethylene, *ACO* expression and ACO activity were also induced by IAA independent of ethylene (Fig. 7a,b).

Together a picture emerges in which the locally changing auxin activity in specific layers of the stem bending zone induced by gravistimulation triggers expression of an auxin-inducible ACC synthase gene (*ACS3*), resulting in local increases in ACC and ethylene production. The produced ethylene presumably functions as a secondary trigger for expression of ACC oxidase and ethylene-receptor genes. The latter events may, however, not be of significance for the overall regulation of ethylene production following gravistimulation. In vivo ACO activity in stem sections was over 10 times higher than actual ethylene production rates during gravistimulation, indicating that ACO is most probably not a regulatory step. *ACS3* expression in response to IAA was independent of ethylene. This indicates a lack of regulation by either positive or negative feedback mechanisms, ruling out a regulatory role for ETR/ERS.

The physiological role of gravitropism-related differential ethylene production remains unclear. Generally, ethylene inhibits growth of roots and shoots, although there are numerous exceptions. Depending on the species, plant organ, developmental and environmental conditions, ethylene may stimulate, inhibit or have no effect on growth. Ethylene is known to affect both polar and lateral auxin transport (Schwark and Schierle 1992), and recently a direct interaction between ethylene and auxin transport has been established. *A. thaliana PIN* mutants, defective in polar auxin transport (see discussion above), showed a decreased sensitivity to ethylene, indicating that ethylene may, at least partly, mediate growth through its effect on PIN function (Chen et al. 1998).

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). *A. majus* flower stems showed delayed gravitropic bending when treated with inhibitors of ethylene action (silver thiosulphate, 2,5-norbornadiene) or production ($CoCl_2$), indicating that, in this species, the differential ethylene production may modify the bending response (Philosoph-Hadas et al. 1996).

Assuming that differential growth is primarily caused by locally increased auxin levels, we studied the effect of ethylene and 1-MCP (inhibitor of ethylene action) on IAA-induced growth of stem sections. On the basis of both length and weight increase, IAA-induced growth was affected neither by ethylene nor 1-MCP, which indicates that ethylene plays no pivotal role in auxin-mediated growth in *A. majus* flower stems (Fig. 8a). When gravistimulation was performed in an environment enriched with either 20 $\mu l\ l^{-1}$ ethylene or 100 $nl\ l^{-1}$ 1-MCP, we consistently observed a slight stimulation by 1-MCP and a slight inhibition of bending by ethylene (Fig. 8b). In addition, 1-MCP-treated stems frequently showed bending beyond the vertical before regaining their vertical orientation whereas no such effect was observed in ethylene-treated stems (data not shown). These observations indicate that ethylene may indeed modify the gravitropic response. Given the marginal effects of these quite severe treatments on the kinetics of gravitropic bending it is expected that the more gradual and moderate changes in ethylene production during gravistimulation in a natural environment do not appreciably affect bending kinetics. This conclusion is supported by recent studies in mutant tomato seedlings. In seedlings of the never ripe (NR) mutant, the kinetics of gravistimulation-induced bending only slightly differed from the bending kinetics observed in wild-type tomato, indicating that the differential ethylene production in wild-type seedlings does not play a pivotal role in gravitropic bending (Madlung et al. 1999).

If ethylene does not play an important role during gravitropic bending, the question remains of why the

ethylene biosynthetic pathway in many plants responds so strongly to gravistimulation. We hypothesise that ethylene may play a role in maintaining the re-established vertical position by affecting, for example, stiffness of the stem. Earlier, Cosgrove (1997) speculated on a role of peroxidase-dependent reactive oxygen species (ROS), in particular H_2O_2, in cell wall stiffening during gravitropism. Only recently it was shown that gravistimulation indeed induces an oxidative burst and that asymmetric application of H_2O_2 induces bending in maize primary roots (Joo et al. 2001; Moseyko et al. 2002). Ethylene has been shown to facilitate the production of H_2O_2 during, for example, defensive responses and cell death (De Jong et al. 2002; Moeder et al. 2002), and ethylene has been shown to induce peroxidase activity (Argandona et al. 2001). Although there is currently no experimental evidence, ethylene may similarly stimulate H_2O_2 production during gravitropic bending and thereby facilitate processes involved in cell wall and stem stiffening and maintenance of the re-established vertical orientation. This hypothesis will be the subject of future research.

Acknowledgements This work was supported by grants from the Ministry of Agriculture, Fisheries and Nature Management Netherlands (LNV), DIARP (to M.A. Nijenhuis-deVries), and ATTEA (to M. Faivre). 1-MCP was a gift from Floralife, USA.

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