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Research article

# Construction of an RNAi vector for knockdown of GM-ACS genes in the cotyledonary nodes of soybean

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ABSTRACT

*Background:* Ethylene plays an important role in the regulation of floral organ development in soybean, and 1-aminocyclopropane-1-carboxylate synthase (ACS) is a rate-limiting enzyme for ethylene biosynthesis. However, whether ACS also regulates floral organ differentiation in soybean remains unknown. To address this, we constructed an RNAi vector to inhibit *ACS* expression in cotyledonary nodes. Linear DNA cassettes of RNAi-ACS obtained by PCR were used to transform soybean cotyledonary nodes.
*Results:* In total, 131 of 139 transiently transformed plants acquired herbicide resistance and displayed GUS activities in the new buds. In comparison to untransformed seedling controls, a greater number of flower buds were differentiated at the cotyledonary node; *GM-ACS1* mRNA expression levels and ethylene emission in the transformed buds were reduced.
*Conclusion:* These results indicate that the cotyledonary node transient transformation system may be suitable for stable transformation and that the inhibition of *ACS* expression may be an effective strategy for promoting floral organ differentiation in soybean.



## 1. Introduction

Ethylene plays an important role in the growth and development of soybean [1]. Studies that included the spraying of inducers and inhibitors of ethylene biosynthesis on soybean seedlings showed that ethylene mainly regulates flowering and abscission in soybean flowers and pods [2]. The 1-aminocyclopropane-1-carboxylate synthase gene (*ACS*) encodes the rate-limiting enzyme in the biosynthesis of ethylene. Regulation of *ACS* expression can be used to modulate ethylene biosynthesis, and this has facilitated studies on the physiological functions of ethylene at different stages of growth and development in soybean [2,3]. However, whether ACS is involved in floral organ differentiation in soybean is unclear. The transient expression system is a simple yet powerful approach for studying gene function [4,5,6,7]. Linear DNA expression cassettes have been widely applied in transgenic plant studies because they reduce the risk of vector backbone sequences or genetic rearrangement that may introduce genetic modifications that compromise safety [8,9]. These linear cassettes can be transformed into the host cells of various plants without the aid of any hardware devices as they are small in size and easy to manipulate. In addition, the cassettes have a broader target host range as they can be directly transformed into both dicots and monocots [8]. Thus, the objective of the present study was to establish a cotyledonary node transient transformation system using a linear minimal DNA expression cassette and determine whether the *ACS* regulates floral organ development in soybean.

## 2. Materials and methods

### 2.1. Materials

Approximately 7 d after germination, 210 seedlings of the soybean cultivar Tie-Feng 31with two fully expanded cotyledons were used to study the transient expression system.

### 2.2. Construction of bivalent linear DNA cassettes of GUS and BAR

The structure of the bivalent linear transformation cassette for β-glucuronidase (*GUS*) and bialaphos resistance gene (*BAR*) is shown in Fig. 1. The cassette containing the *GUS* and *BAR* genes was generated by polymerase chain reaction (PCR) using pCAMBIA 3301

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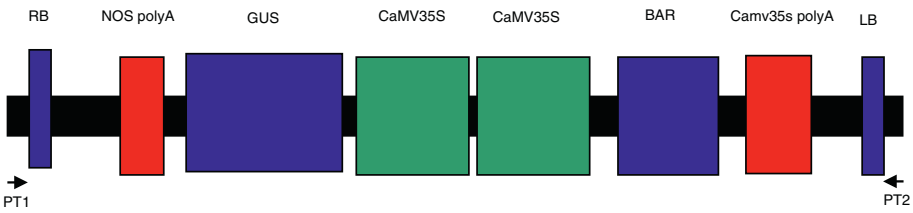


**Fig. 1.** Scheme of minimal linear *GUS* and *BAR* transformation cassette. The minimal linear *GUS* and transformation cassette contains eight basic elements: two *CaMV35S* promoters, one *GUS* reporter gene, one *BAR* gene, one *NOS* terminator, and two T-DNA border repeats (LB: left border; RB: right border).

as a template and the primers PT1 (5′-TGGCAGGATATATTGTGGTGT AAAC-3′) and PT2 (5′-GTTTACCCGCCAATATATCCTGTCA-3′) according to Liu et al. [9]. The relative positions of all primers on the minimal *GUS* reporter gene cassette are shown in Fig. 1.

### *2.3. Construction of RNAi vector for the GM-ACS1 gene*

The DNA and protein sequence of *GM-ACS1* (GenBank accession number: DQ273841) are shown in Fig. 2. To produce RNAi expression clones that target the ACC synthase gene, fragments of the *GM-ACS1* were PCR amplified using gene-specific primers (attB1 + ACS primer: 5′-AAAAAGCAGGCT-(TAGGGGAGTGAGGTGACAT)-3′ and attB2 + ACS primer: 5′-AGAAAGCTGGGT-(TGGCACAAAGGCATAGAG)-3′) and cloned into pDONR/Zeo vector using the BP clonase reaction mix (Invitrogen, USA). After sequencing confirmation, the GM-ACS1 fragments were recombined into the RNAi vector pHGRV-ACS using the LR clonase reaction mix (Invitrogen, USA). The orientation of the inserted *GM-ACS1* fragments in vector pHGRV-ACS was confirmed using gene-specific forward primers (5′-TAGGGGAGTGAGGTGA CAT-3′) and vector-specific intron primers (5′-CTTTTATCTTCTTCGT CTTACAC-3′). The construction process and structure of the RNAi vector pHGRV-ACS is shown in Fig. 3. The recombinant vectors were transformed into *Escherichia coli* DH5α by the heat-shock method and used for the construction of linear elements as described above.

## DNA sequence:

>DQ273841.1 Glycine max 1-aminocyclopropane-1-carboxylate synthase (ACS) mRNA, complete cds

CAATTAAACATAGACTATATGGGGTTGATGGCTGCGAACCAAACTCAATTGTTGTCTAAGATGGCCATCGGAGAT
GGACATGGTGAAGCATCCCCATATTTTGATGGATGGAAGGCTTATGATGAAAACCCCTTTCATCCCAAAGAGAATC
CTAACGGGGTTATTCAAATGGGTCTTGCTGAGAATCAGCTTACTTCTGATTTGGTTGAAGATTGGATACTGAATAA
CCCAGAGGCCTCCATTTGCACTCCAGAAGGAATAAATGATTTCAGGGCAATAGCTAACTTTCAGGATTATCATGGT
CTACCCGAGTTCAGAAATGCTGTGGCTAAATTCATGGGTAGAACAAGAGGAAACAGAGTCACGTTTGATCCTGAT
CGTATTGTCATGAGCGGTGGAGCAACTGGAGCACACGAAGTCACTACCTTTTGTTTGGCAGACCCCGGTGACGCAT
TTTTGGTGCCAATTCCTTATTATCCAGGTTTTGACCGGGATTTGAGGTGGAGAACAGGAATTAAACTTGTTCCAGTT
ATGTGCGATAGCTCAAACAATTTCAAGTTGACAAAGCAAGCATTGGAAGATGCGTATGTGAAAGCCAAAGAGGAT
AACATTAGAGTGAAGGGCATGCTCATCACCAATCCTTCAAACCCATTAGGCACAGTCATGGACAGAAACACACTA
AGAACCGTGGTGAGCTTCATCAATGAGAAGCGTATCCATCTTGTATCTCATGAAATATACTCTGCAACAGTTTTTA
GCCGTCCCAGTTTCATAAGCATTGCTGAGATACTAGAGGAAGACACAGACATCGAATGTGACCGCAACCTCGTTC
ACATTGTTTATAGTCTTTCAAAGGATATGGGGTTCCCTGGCTTCAGAGTTGGCATCATATACTCTTACAATGATGCT
GTGGTCAATTGTGCACGCAAAATGTCAAGCTTTGGGTTGGTGTCAACACAGACTCAGCATCTTTTAGCATCAATGC
TAAATGATGATGAGTTTGTGGAAAGGTTTCTGGAAGAGAGTGCAAAAAGGTTGGCACAAAGGCATAGAGTTTTCA
CTTCGGGGTTGGCCAAAGTAGGCATAAAGTGCTTGCAAAGCAATGCTGGTCTCTTTGTGTGGATGGATTTAAGGCA
ACTTCTCAAGAAGCCAACGCTTGACTCTGAAATGGAGCTTTGGAGAGTGATCATTCATGAGGTTAAGATCAATGTT
TCACCTGGCTCTTCTTTCCATTGCACTGAGCCAGGGTGGTTTAGGGTGTGCTATGCCAACATGGATGATATGGCTGT
GCAAATTGCATTGCAAAGAATTCGAACCTTCGTGCTTCAAAACAAGGAGGTCATGGTTCCTAACAAGAAACATTG
CTGGCACAGTAACTTGAGGTTGAGCCTCAAAACCANAAGGTTTGATGATATCATGATGTCACCTCACTCCCCTATA
CCTCAGTCCCCTTTGGTTAAAGCCACAATTTGA

## protein sequence:

>ABB70230.1 1-aminocyclopropane-1-carboxylate synthase [Glycine max]

MGLMAANQTQLLSKMAIGDGHGEASPYFDGWKAYDENPFHPKENPNGVIQMGLAENQLTSDLVEDWILNNPEASICT
PEGINDFRAIANFQDYHGLPEFRNAVAKFMGRTRGNRVTFDPDRIVMSGGATGAHEVTTFCLADPGDAFLVPIPYYPGF
DRDLRWRTGIKLVPVMCDSSNNFKLTKQALEDAYVKAKEDNIRVKGMLITNPSNPLGTVMDRNTLRTVVSFINEKRIH
LVSHEIYSATVFSRPSFISIAEILEEDTDIECDRNLVHIVYSLSKDMGFPGFRVGIIYSYNDAVVNCARKMSSFGLVSTQTQ
HLLASMLNDDEFVERFLEESAKRLAQRHRVFTSGLAKVGIKCLQSNAGLFVWMDLRQLLKKPTLDSEMELWRVIIHEV
KINVSPGSSFHCTEPGWFRVCYANMDDMAVQIALQRIRTFVLQNKEVMVPNKKHCWHSNLRLSLKTXRFDDIMMSPH
SPIPQSPLVKATI

**Fig. 2.** The DNA and protein sequence of GM-ACS1.

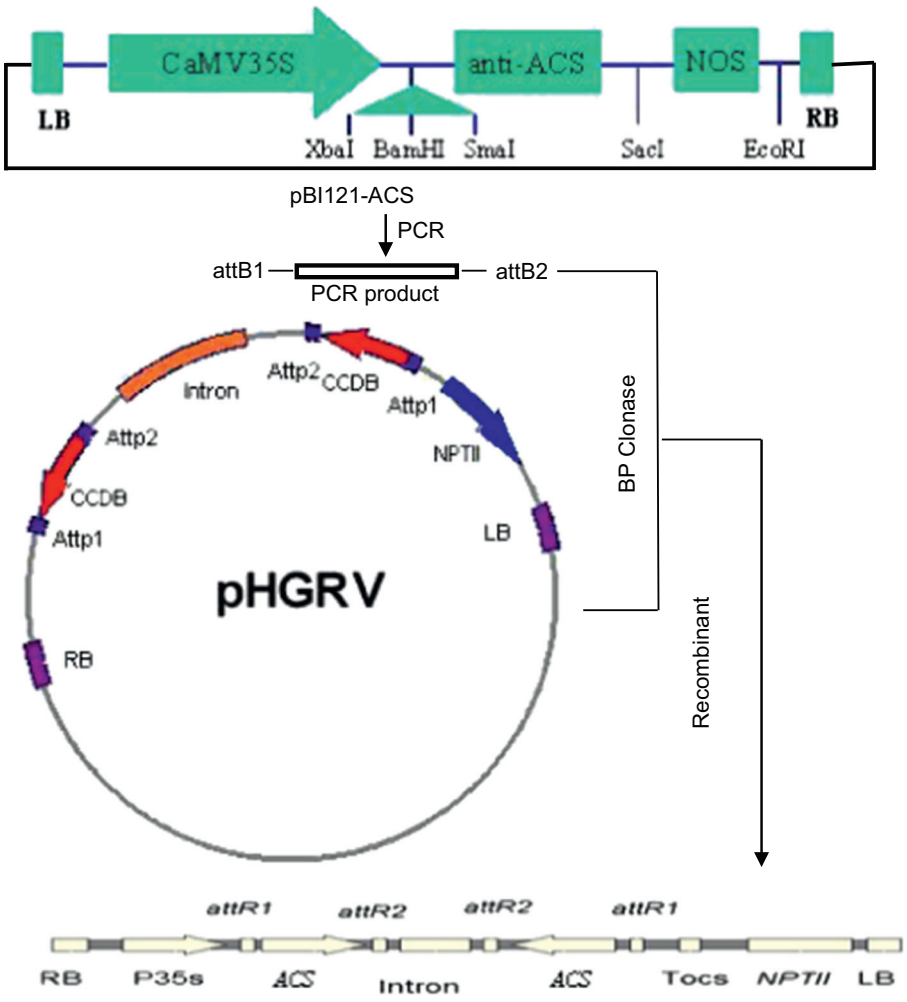


**Fig. 3.** Construction process and structure of the pHGRV-ACS RNAi vector.

*2.4. Transformation of the linear minimal DNA expression cassette into soybean cotyledonary nodes*

Murashige and Skoog medium supplemented with cofactors (0.1 mol/L $Ca^{2+}$, 0.01% Dow Corning Q2-5211, and a culture of *Agrobacterium* cells at an optical density of 1.0) was used as the basic transformation buffer. The buds and axillary buds of seedlings were carefully removed with a scalpel resulting in a wedge-shaped incision and lightly scratched 5–6 times parallel to the direction of the veins. Soybean cotyledonary nodes were then infected using the linear DNA expression cassette in transformation buffer solution. Following infection, the cotyledons and cotyledonary nodes were covered with black bags to maintain sufficient moisture and left to grow in the dark at 20–25°C for 5–8 d until buds appeared (Fig. 4). The black bags were then removed, and cotyledonary nodes were left to grow normally under exposure to light. After 10–20 d, bud differentiation at the cotyledonary node initiated, and parameters relevant to this study were subsequently measured.

*2.5. Selection of transformed plants*

Newly induced cotyledonary node buds were smeared with Basta solution (2%) for 72 h, and the amount of chlorosis that this induced was recorded. After the Basta solution screening, leaves from


**Fig. 4.** Explants of the transient expression system in soybean cotyledonary nodes. (a) Soybean seedling before excision; (b) soybean seedling after the excision of cotyledonary nodes; (c) new buds grown from the soybean seedling after excision (indicated by the arrow).

**Fig. 5.** Transformed plant selection using 2% Basta solution. Seedlings in the left and right pots are the control and transiently transformed treatments, respectively. Arrow shows the chlorosis symptoms induced by treatment with Basta solution.

chlorosis-positive plants were subjected to GUS activity analysis to confirm transformation [10].

### 2.6. Detection of GM-ACS1 gene expression and ethylene emission

Total RNA samples were extracted from 0.5 g of freshly detached new buds of the transformed plants to determine the relative expression level of *GM-ACS1* using the primers F: 5′- CACCTCAAA TCCCGGTCAA-3′ and R: 5′- AGCAACTGGAGCACACGAAG-3′ and using *ACT11* as an internal control (*ACT11* primers, F: 5′-ACCTCG ACATACTGGTGTTATGGTT-3′ and R: 5′- ATACCTCTTTTGGATTGGG CTTC-3′) [11]. RT-PCR for ACS gene expression and ethylene emission analysis were performed as described by Cheng et al. [12]. About 1 g of freshly detached organisms were placed in a 10-mL vial, and then the vial was sealed with a serum cap and parafilm. Ethylene production was quantified after 14 h of incubation at 30°C. Peak areas were determined using a Chromatopak C-R6A system (Shimadzu, Kyoto, Japan). The measurements were repeated thrice for each treatment. Each measurement data came from three individual reactions ($n = 3$), and the concentration was expressed as mean ± SD. Statistical analysis was performed by ANOVA using SAS version 8.01 (SAS Institute, Inc., Cary, NC, USA). Means were compared using the least significant difference t-test at 5% level of significance. Standard ethylene solution was purchased from the Institute of Organic Chemistry, Chinese Academy of Sciences.

## 3. Results

### 3.1. Transformed plant selection

New leaves began to form approximately 10 d after transformation. Following the application of Basta solution, the leaves on 41 of 180 plants showed typical chlorosis symptoms, whereas the color of the remaining 139 plants remained unchanged (Fig. 5). As further validation of transformation, we observed that BAR-dependent herbicide resistance developed in 77.22% of the plants. Furthermore, GUS activity was detected in the leaves of 131 plants of these 139 transiently transformed plants (Fig. 6). Overall, we observed transient transformation together with herbicide resistance in 72.78% of the plants.

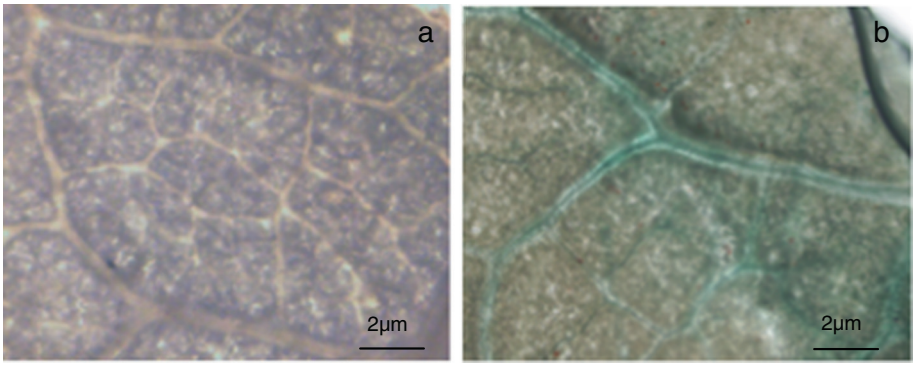


**Fig. 6.** GUS transient expression in a new bud following transformation at the cotyledonary node. (a) Control leaf; (b) transformed leaf.

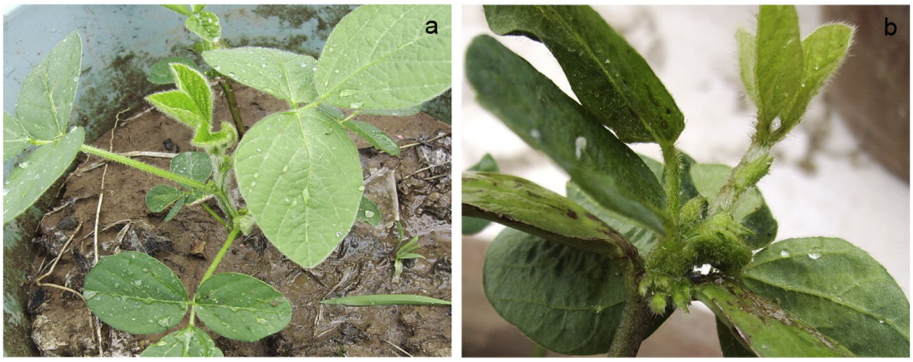


**Fig. 7.** Flower bud differentiation in transformed plants. (a) Control plant; (b) transformed plant.

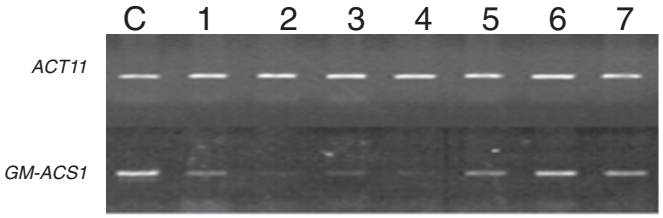


**Fig. 8.** RT-PCR detection of *GM-ACS1* transcript of transformed explants. C: Nontransformed control plant; 1–7: transformed plants. The bands brightness indicate the relative transcript level of *GM-ACS1* (primers F: 5′- CACCTCAAATCCCGGTCAA-3′ and R: 5′- AGCAACTGGAGCAC ACGAAG-3′) after normalization with *ACT11* (primers, F: 5′-ACCTCGACATACTGGTGTTAT GGTT-3′ and R: 5′- ATACCTCTTTTGGATTGGGCTTC-3′).

### *3.2. Phenotypic observation of transgenic acceptor materials of RNAi-GMACS*

As expected, new buds began to differentiate and develop at cotyledonary nodes between 1 and 3 weeks post transformation. Subsequently, some reproductive buds began to form on the RNAi-GMACS transgenic acceptors (Fig. 7). In plants transiently transformed with RNAi-GMACS, the number of reproductive buds ranged from 6–17, with an average of 8.4; in nontransformed plants, no reproductive buds were formed. These data indicate that transient transformation of soybean with RNAi-GMACS is an effective strategy for inducing flower differentiation.

### *3.3. GM-ACS1 gene expression and ethylene emission*

Buds were regenerated from the cotyledonary section after the soybean cotyledonary nodes were excised, transformed, and left to grow for approximately a week. Following RT-PCR (see Materials and methods), we observed that in vivo expression of the interference vectors in the transformed plants was normal, whereas levels of *GM-ACS1* mRNA in the transformed buds were reduced (Fig. 8). On the basis of a significant reduction in *GM-ACS1* expression, cotyledonary nodes of five transformed plants were examined for ethylene emission (Fig. 9). The reduction in ethylene production varied between samples, with an average of 51.91% reduction compared to the control. Sample ACS-1 yielded the lowest reduction in ethylene production (15.39% compared to the control), whereas ACS-2 and ACS-5 reduced ethylene production by less than 73%. The greatest decline in ethylene production was 86.67%. Taken together, these data show that RNAi-mediated reduction of *GM-ACS1* expression is associated with a drop in ethylene emission.

## 4. Discussion

Genetic transformation of soybean has generally been associated with low efficiency and poor reproducibility. Analysis of the effects of genetic modifications on soybean is also challengingas tissue culture and regeneration following transformation is difficult in this plant [13]. In the present study, we used the cotyledonary node transformation system, which does not rely on tissue culture and can directly transform soybean seedlings in the field. This method has facilitated the generation of transformed buds at the cotyledonary nodes in the background of an otherwise unmodified plant that contains nontransformed parts such as rhizomes. This method has successfully overcome the limitations of genetic transformation and regeneration in the soybean; thus, it can be directly applied to the functional analysis of genes that regulate soybean differentiation. In addition, the cotyledonary node transient transformation system can be directly applied for stable transformation and genetic improvement of the soybean if suitable positive progeny can be selected.

Ethylene mainly affects the abscission of pods by affecting the process of flower differentiation. Reproductive bud differentiation is challenging in the presence of high levels of ethylene [2,14]. In contrast, reducing ethylene production through RNA interference elicited early flowering, and a T-DNA insertion mutant of the *etr*2 gene resulted in increased sensitivity to ethylene and also induced early flowering [15]. Here, we found that RNAi leads to the degradation of the mRNA encoding the rate-limiting enzyme in ethylene biosynthesis, *GM-ACS1*, thereby regulating the target gene at the post-transcriptional level. Consistent with these earlier reports, we found that the inhibition of ACC synthase expression is another strategy that can be used to modulate floral organ differentiation in soybean.

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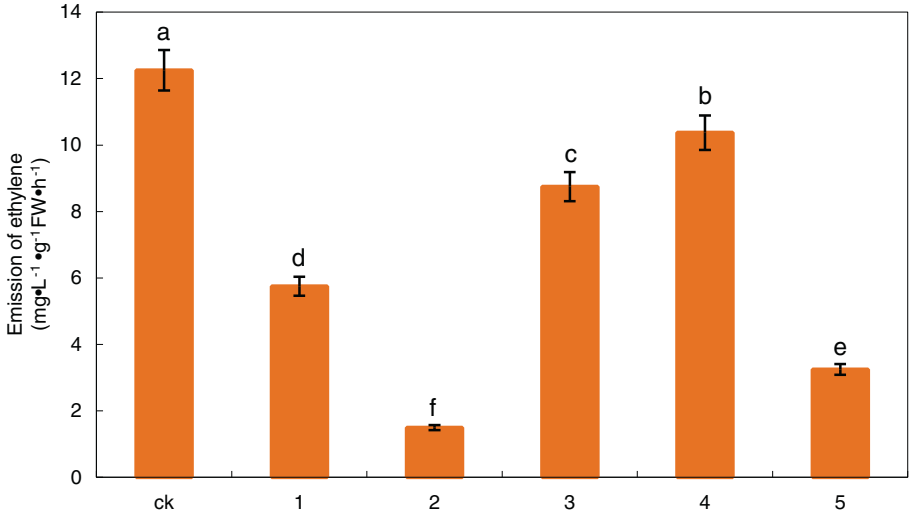


**Fig. 9.** Ethylene production in the cotyledonary nodes of transformed plants. ck: Control plants; 1–5: Five transformed explants. Different lower-case letters above each column indicate significant difference at $P = 0.05$.

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## Conflict of interest

The authors declare that they have no conflict of interest.

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