Research article

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

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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.

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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 31 with 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...
as a template and the primers PT1 (5′-TGGCAGGATATTTGTGGTGTAAAC-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′-AGAAGCTGGGT-(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 pHRGRV-ACS using the LR clonase reaction mix (Invitrogen, USA). The orientation of the inserted GM-ACS1 fragments in vector pHRGRV-ACS was confirmed using gene-specific forward primers (5′-TAGGGGAGTGAGGTGACAT-3′) and vector-specific intron primers (5′-CTTTTATCTTCTTCGCTTACAC-3′). The construction process and structure of the RNAi vector pHRGRV-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
CAATTAAACATAGACTATATGGGGTTTGGCTGCGAACCAAACTCAATTGTTGTCTAAGATGGCCATCGGAGATGGACATGGTAAAGCATTTTGATGGATGGAAGGCTTATGATGAAAACCCCTTTCATCCCAAAGAGAATCCTAACGGGGTTATTCAAATGGGTCTTGCTGAGAATCAGCTTACTTCTGATTTGGTTGAAGATTGGATACTGAATAACCCAGAGGCCTCCATTTGCACTCCAGAAGGAATAAATGATTTCAGGGCAATAGCTAACTTTCAGGATTATCATGGTCTACCCGAGTTCAGAAATGCTGTGGCTAAATTCATGGGTAGAACAAGAGGAAACAGAGTCACGTTTGATCCTGATCGATTGTTCATGAGCGGTGGAGCAACTGGAGCACACGAAGTCACTACCTTTTGTTTGGCAGACCCCGGTGACGCATTGTTTTGGTGCCAATTCCTATTATCCAGGTTTTGACCGGGATTTGAGGTGGAGAACAGGAATTAAACTTGTTCCAGTTATGTGCGATAGCTCAAACAATTTCAAGTTGACAAAGCAAGCATTGGGAAGATGCGTATGTGAAAGCCAAAGAGGATAACATTAGAGTGAAGGGCATGCTCATCACCAATCCTTAAACCCATTAGCACTGACATGACGAAACACATCAAGACCTGGTGAGCTCTACTCCTCTGTGCTTACATGAAATACCTCTGCCACACGTTTTTA

protein sequence:
> ABB70230.1 1-aminocyclopropane-1-carboxylate synthase [Glycine max]
MGLMAANQTQLSLKAIGDGHGEASPYPFDGWKADVENPHFHPKENPVGQIMGLAENQLTSDLVEDWILNPEASICTPEGIDFRAIAFQPYHHIGPEFRANAVAKFGRGGTRFTGVRFTDIVMSGGAATGHEVITFCLADPGDLAVPYPYPQGDRLRWRKLVKLVPMDCSSSNFKQLTKQALEYAVKAEKDNIRVKGMLTNPSNPLGTVMDRNLTRLTVSFIPEREIHLVWSHEISATVFRSPFISAEILEEDTIEIDCNRLHVHYLSSKMLDSMFPGFRVGYIYSDYNVANCARKMSSFLGVSSTQHLLASMLNDDEFVRLESEASKRLAQRIRVFSLAGKVGKICLSANAFGLFWMDRLQLQKPLTDSEMELWRRVIIHYEKNIVSPGSFHCETEPGWFRVCYANMDMAVQIALQRIETFVLQNEKVMVPNNKHCHWSNLRLSIXRFDIMMSHPSPIQPSLVKATI

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

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).
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′-CACCTCAAATCCGGTCAA-3′ and R: 5′-AGCAACTGGAGCACACGAAG-3′ and using ACT11 as an internal control (ACT11 primers, F: 5′-ACCTCGACATCTGGGTATATGT-3′ and R: 5′-ATAACTCTTTTGGATTCGCTC-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.
ACS-1 yielded the lowest reduction in ethylene production, an average of 51.91% reduction compared to the control. Sample reduction in ethylene production varied between samples, with plants examined for ethylene emission (Fig. 9). The 3.3. GM-ACS1 gene expression and ethylene emission reduced (Fig. 8). On the basis of a significant difference at \( P = 0.05 \). 

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′-CACCCTATCCGCTAA-3′ and R: 5′-AGGACTGAGGACACACGGAG-3′) after normalization with ACT1 (primers, F: 5′-ACCTCGAGATCAGGTGTATGGTT-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 challenging as 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 etr2 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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Conflict of interest

The authors declare that they have no conflict of interest.

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